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CHARACTERISATION OF AGARWOOD HYDRODISTILLATION BY-PRODUCTS: CHEMICAL, PHYSICAL AND MICROBIOLOGICAL PROPERTIES

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CHARACTERISATION OF AGARWOOD HYDRODISTILLATION BY-PRODUCTS: CHEMICAL, PHYSICAL AND MICROBIOLOGICAL PROPERTIES

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189309

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ABSTRACT

Resinous agarwood produced from *Aquilaria* trees can be extracted for its essential oils. Agarwood and its essential oil are broadly employed in perfumes, food, and beverages. Hydrodistillation of agarwood has yield two liquid by-products namely residual water and hydrosol. These by-products might have some economic values since they are believed to contain small fraction of agarwood oil and other water-soluble bioactive compound which can be further utilized in manufacturing of cosmetics products. The present study is carried out in order to characterise the agarwood hydrodistillation by-products based on chemical, physical and microbiological properties. The residual water that contains tiny wood debris, dirt, suspended particles and microorganisms has to be clarified before further analysis and application. Three recovery processes such as centrifugation, microfiltration and redistillation were proposed in this study and their effectiveness were studied based on the properties. Chemical properties such as chemical profile by GC-MS analysis (for by-products), pH value and total dissolved solids; physical properties such as visual observation, turbidity, particle size analysis; and for microbiological properties such as microbial load were studied for all water samples. The findings suggested that hydrosol is more suitable to be applied for further usage as compared to residual water since hydrosol shows better results in the analysis of chemical, physical and microbiological properties. The presence of major aromatic compound in hydrosol namely benzylacetone has proved that it is suitable for manufacturing of cosmetics products for example soap and other aesthetic products providing that it must be collected aseptically and stored in good conditions to prevent any contamination that will reduce its shelf-life. However, the properties of recovered agarwood residual

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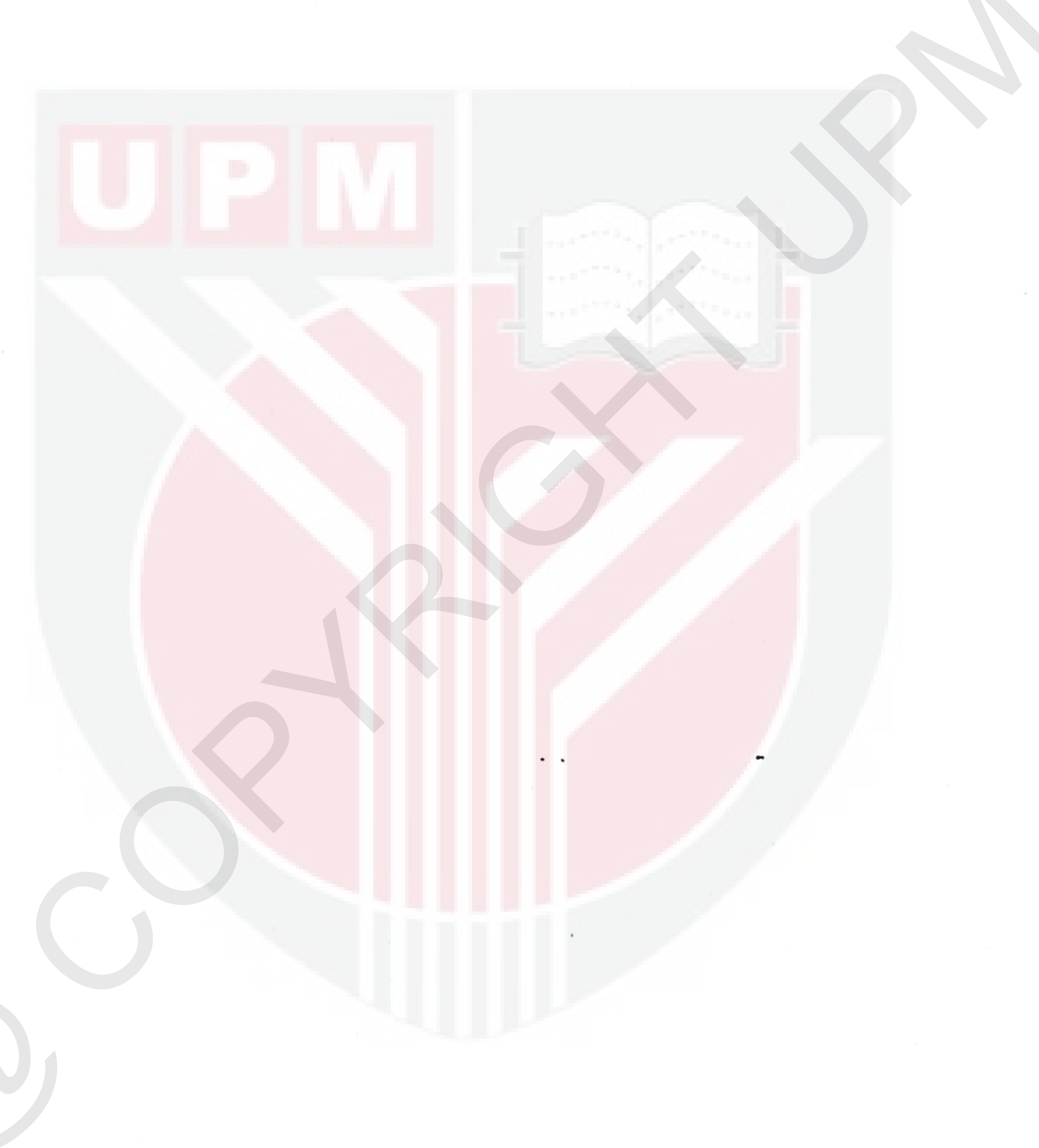
water have to be studied in more detail before commercialising it as a product. Among all proposed recovery processes, microfiltration has given an optimum performance in removing all the contaminants while retaining the quality and appearance of the residual water.



ABSTRAK

Kayu gaharu yang dihasilkan daripada pokok *Aquilaria* dapat diekstrak untuk minyak patinya. Gaharu dan minyak patinya banyak digunakan dalam minyak wangi, makanan, dan minuman. Hidrodistilasi gaharu telah menghasilkan dua produk sampingan cair iaitu sisa air dan hidrosol. Produk sampingan ini mungkin mempunyai beberapa nilai ekonomi kerana diyakini mengandungi sebilangan kecil minyak gaharu dan sebatian bioaktif larut air lain yang dapat digunakan lebih lanjut dalam pembuatan kosmetik. Kajian ini dijalankan untuk mencirikan produk sampingan hidrodistilasi gaharu berdasarkan sifat kimia, fizikal dan mikrobiologi. Air sisa yang mengandungi serpihan kayu kecil, kotoran, zarah terampai dan mikroorganisma perlu dijelaskan sebelum analisis dan penggunaan selanjutnya. Tiga proses pemulihan seperti sentrifugasi, mikrofiltrasi dan redistilasi dicadangkan dalam kajian ini dan keberkesanannya dikaji berdasarkan sifatnya. Sifat kimia seperti profil kimia oleh analisis GC-MS (untuk produk sampingan), nilai pH dan jumlah pepejal terlarut; sifat fizikal seperti pemerhatian visual, kekeruhan, analisis ukuran zarah; dan untuk sifat mikrobiologi seperti beban mikrob telah dikaji untuk semua sampel air. Hasil kajian menunjukkan bahawa hidrosol lebih sesuai digunakan untuk penggunaan lebih lanjut dibandingkan dengan air sisa kerana hidrosol menunjukkan hasil yang lebih baik dalam analisis sifat kimia, fizikal dan mikrobiologi. Kehadiran sebatian aromatik utama dalam hidrosol iaitu benzylacetone telah membuktikan bahawa ia sesuai untuk pembuatan produk kosmetik contohnya sabun dan produk estetik lain dengan syarat ia mesti dikumpulkan secara aseptik dan disimpan dalam keadaan baik untuk mengelakkan pencemaran yang akan mengurangkan jangka hayatnya. Walau bagaimanapun, sifat-sifat air sisa gaharu yang diproses harus dikaji dengan lebih

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terperinci sebelum mengkomersialkannya sebagai produk. Di antara semua proses pemulihan yang dicadangkan, penyaringan mikro telah memberikan prestasi optimum dalam membuang semua bahan cemar yang tidak diingini sambil mengekalkan kualiti dan penampilan sisa air tersebut.

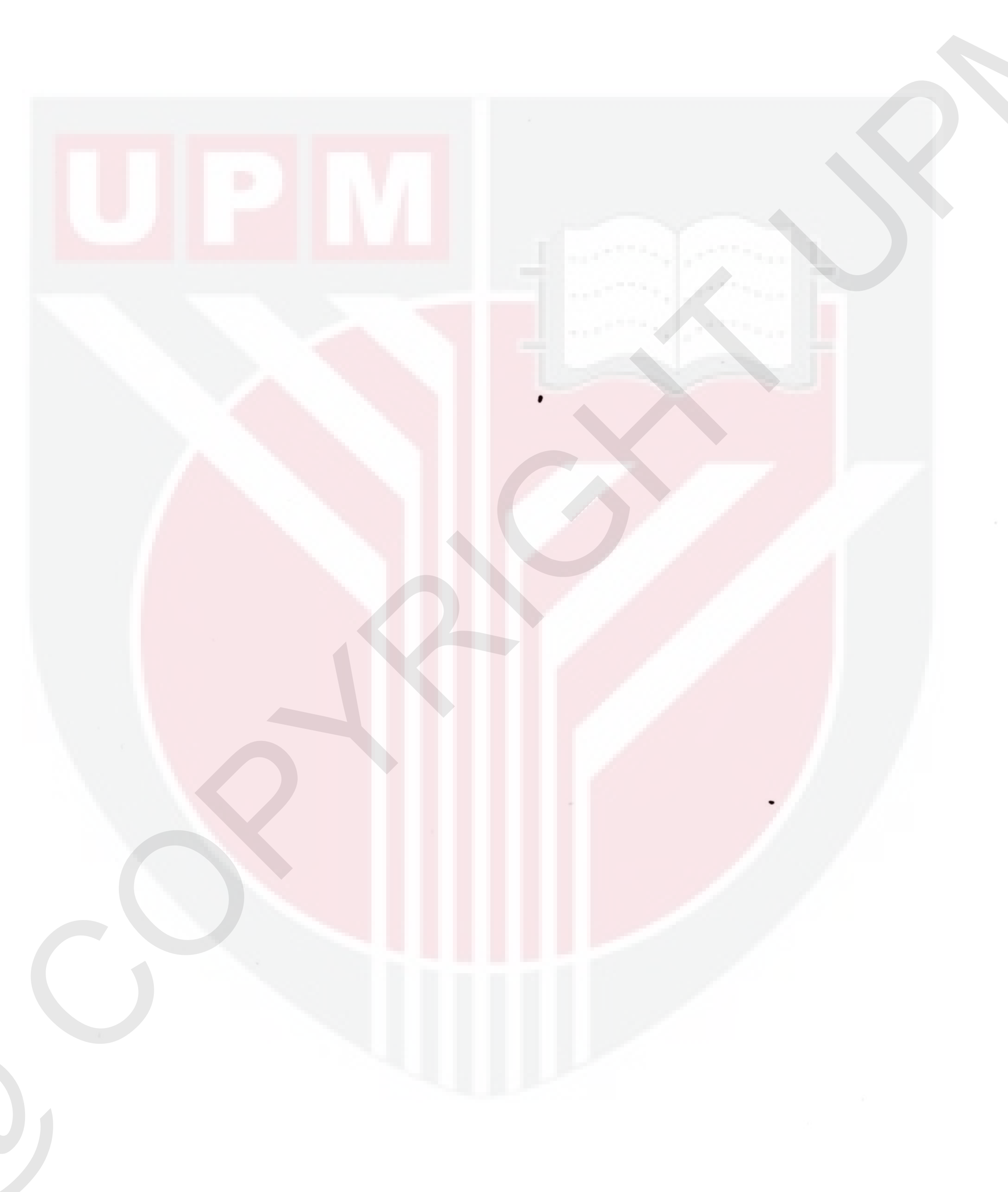
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TABLE OF CONTENTS

APPROVAL i

ACKNOWLEDGEMENT iv

ABSTRACT vi

ABSTRAK viii

TABLE OF CONTENTS x

LIST OF TABLES xiii

LIST OF FIGURES xv

LIST OF APPENDICES xvii

LIST OF ABBREVIATIONS xviii

CHAPTER 1 INTRODUCTION 1

1.1 Background 1

1.2 Problem Statement 5

1.3 Objectives 6

1.4 Scope of Study 6

CHAPTER 2 LITERATURE REVIEW 8

2.1 Essential Oils 8

2.1.1 Extraction of Essential Oils 10

2.1.2 Usage of Essential Oil 15

2.2 *Aquilaria* Species 16

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2.3 Agarwood Oil 19
2.3.1 Agarwood Oil Extraction 19
2.3.2 Physical Properties 21
2.3.3 Chemical Properties 22
2.3.4 Usage of Agarwood Oil 24
2.4 By-product of Hydrodistillation 25
2.4.1 Hydrosol 26
2.4.2 Residual Water 27
2.5 Water Soluble Essential Oil Constituents 28
2.6 Recovery Processes 29
2.6.1 Centrifugation 30
2.6.2 Membrane Separation 31
2.6.3 Redistillation 32
CHAPTER 3 METHODOLOGY 34
3.1 Materials 34
3.2 Research Approach 35
3.3 Storage Condition for the By-products 36
3.4 Recovery Processes 36
3.4.1 Centrifugation 36
3.4.2 Microfiltration 37
3.4.3 Redistillation 38

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3.5 Analysis 39
3.5.1 Chemical Properties 39
3.5.2 Physical Properties 40
3.5.3 Biological Properties 42
CHAPTER 4 RESULTS AND DISCUSSION 45
4.1 Chemical Properties 45
4.1.1 Chemical Profile by GC-MS Analysis 45
4.1.2 pH (Acidity) 52
4.1.3 Total Dissolved Solids (TDS) 55
4.2 Physical Properties 58
4.2.1 Visual Observation 58
4.2.2 Turbidity 62
4.2.3 Particle Size Analysis 69
4.3 Microbiological Properties 72
4.3.1 Microbial Load 72
CHAPTER 5 CONCLUSION AND RECOMMENDATION 79
REFERENCES 83
APPENDICES 101

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LIST OF TABLES

Table 2.1: Distribution of *Aquilaria* Species in Malaysia.. 16

Table 2.2: Commonly used agarwood grades found in the Malaysian market.......... 18

Table 2.3: Physical properties of agarwood oil... 22

Table 2.4: Sesquiterpenes from agarwood oils found Malaysia *Aquilaria* species (Grade C) extracted by hydrodistillation.. 24

Table 2.5: Some medicinal properties of agarwood oil 25

Table 4.1: GC-MS analysis for agarwood residual wate 48

Table 4.2: GC-MS analysis for agarwood hydrosol.. 50

Table 4.3: pH values for different types of water samples 53

Table 4.4: Average total dissolved solids (ppm) for different types of water samples 56

Table 4.5: Visual observations of both hydrodistillation by-products 59

Table 4.6: Visual appearances of different types of recovered residual water samples 61

Table 4.7: Average turbidity values for different types of water samples 64

Table 4.8: Percentage of turbidity reduction for different types of recovery processes 67

Table 4.9: Average particle diameter and average polydispersity index for different types of water samples 71

Table 4.10: Average total plate count (CFU/ml) for different types of water samples 75

Table 4.11: Microbial log reduction for different types of recovery processes 76

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LIST OF FIGURES

Figure 2.1: Illustration of hydrodistillation process 10

Figure 2.2: Illustration of steam distillation process 11

Figure 2.3: Illustration of solvent extraction process 12

Figure 2.4: Illustration of supercritical carbon dioxide extraction process 13

Figure 2.5: Illustration of cold pressing process 14

Figure 2.6: High-quality natural agarwood with dark and dense resinous appearance 17

Figure 2.7: Market prices of agarwood in Kelantan 19

Figure 2.8: A commercial hydrodistillation system for agarwood oil extraction in Gua Musang, Kelantan (Malaysia). (A) Extraction contraption and (B) extracted oil in the oil separating funnel 21

Figure 2.9: Skeleton of hydrocarbons found in volatile agarwood oil 23

Figure 2.10: Skeleton of oxygenated sesquiterpenes found in volatile agarwood oil 23

Figure 3.1: (A) Residual water retained in distillation tank; (B) Hydrosol collected in the separating funnel of the Clevenger set up 34

Figure 3.2: Overview of research approach, methods and analysis 35

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Figure 3.3: (A) Appearance of residual water before and after centrifugation; (B) Precipitate or pellet remained in the centrifuge tube 37

Figure 3.4: Dirt and tiny agarwood chips retained on the filter paper 38

Figure 3.5: Distillation apparatus set up 39

Figure 3.6: Illustration of dynamic light scattering for large and small particle........ 41

Figure 3.7: Illustration of serial dilution 43

Figure 3.8: Illustration of standard spread plate method... 44

Figure 4.1: LACs generated from agarotetrol upon heating 48

Figure 4.2: GC-MS chromatogram for agarwood residual water 49

Figure 4.3: GC-MS chromatogram for agarwood hydrosol..................................... 51

Figure 4.4: Physical appearances for different types of water samples 62

Figure 4.5: Effects of high water turbidity to the ecology 65

Figure 4.6: Size range for different kinds of cells and cellular components............. 78

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LIST OF APPENDICES

Figure A-1: Report of GC-MS operating parameters 103

Figure A-2: GC-MS analysis report for blank 104

Figure A-3: GC-MS chromatogram report for blank 105

Figure A-4: GC-MS analysis report for residual water 106

Figure A-5: GC-MS chromatogram report for residual water 107

Figure A-6: GC-MS analysis report for hydrosol 108

Figure A-7: GC-MS chromatogram report for hydrosol 109

Table A-1: Raw data for pH analysis 110

Table A-2: Raw data for total dissolved solid analysis 110

Table A-3: Raw data for turbidity analysis 110

Table A-4: Raw data for particle size analysis (particle diameter) 111

Table A-5: Raw data for particle size analysis (polydispersity index) 112

Table A-6: Images of plate according to dilution factor 113

Table A-7: Raw data for microbial load analysis according to serial dilution 117

Table A-8: Raw data for colony forming unit 118

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LIST OF ABBREVIATIONS

AAB	Acetic acid bacteria
CFU	Colony forming unit
DO	Dissolved oxygen
GC-MS	Gas chromatography-mass spectrometry
LAC	Low molecular weight aromatic compound
LRV	Log reduction value
NTU	Nephelometric turbidity unit
RW	Residual water
SPME	Solid-phase microextraction
TDS	Total dissolved solid
TSS	Total suspended solid

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CHAPTER 1

INTRODUCTION

1.1 Background

Agarwood tree at first glance is similar to other ordinary plant growing in the tropical forest. However, agarwood tree has very high treasured property. Agarwood or known as gaharu is the resinous heartwood produced by *Aquilaria* species. One of the well-known species is *Aquilaria malaccensis* or karas tree. As a result of pathology or a wounding procedure, resinous heartwood is created. The formation of resin can also be a kind of natural biological respond to fungal infection. It has been found out that the use of agarwood can be traced to ancient cultures or even the earliest civilization. The value of agarwood in food and traditional medicine aspect has been documented in the Chinese, Japanese, Korean and Indian culture. In addition, agarwood is commonly used in South East Asian countries such as Laos, Cambodia, Vietnam, Thailand, Indonesia and Malaysia because of its ancient trade routes and the high demand for agarwood essential oils (Ali, Jin, & Jamil, 2016).

Agarwood oil is an essential oil of *Aquilaria* trees that extracted from its resinous wood. It carries a distinctive scent which is volatile at above room temperature. When the oil is extracted from different parts of the trees such as from the leaves, branches or chips from the trunk of agarwood, the quality of the essential oil will be deviate as well. Generally, most of the oils are extracted from its resinous wood. The identity and quality of the agarwood oil produced are depending on its chemical and physical characteristics. The properties of the agarwood oil are correlated with the presence of chemical components which can be affected by multiple factors such as geographical factors, genetic, variability, ecology factors and extraction process (Bandoni, 2000).

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Hydrodistillation is the most popular method for extracting agarwood essential oil in commercial scale due to easy operation, lower cost, and green water-based process. Hydrodistillation imply the principle of isolating essential oils from aromatic and medicinal plants which involve the process of water distillation in direct contact with fresh or dried macerated plant materials (Hamid et al., 2011). The process of agarwood hydrodistillation extraction starts with filling the distillation pot with fermented agarwood chips or powder which then being heated on a brick furnace. In order to ensure all oil has been extracted, the heating process may continuously take up to 3 days or more. The fundamentals in this step is where the hot vapor from heating goes through a condenser with external running water serve as cooling effect result in condense of vapors that drop into a collecting funnel where oil and water are separated gravitationally (Burfield & Kirkham, 2005). A two-phase system with water and the essential oil is produced, where the oil can be decanted and collected (Boutekedjiret et al., 2003).

Hydrodistillation of agarwood yields 4 distinct products: 1) Essential oil, the principal product of commerce 2) Distilled/spent biomass 3) Residual water remaining in the distillation tank and 4) Hydrosol/condensate water (Rao, 2012). The focus of the study is specified on liquid by-products namely residual water and hydrosol. Agarwood hydrodistillation residual water is an effluent that is being drained out after the distillation of aromatic plant. It is the dark coloured thick water that retained in the distillation tank. The dissolved phytochemicals content and then suspended biomass residue of distilled crop associated in the residual waters are susceptible to microbial contamination and required to be processed before contamination occurs (Rao, 2013). Hydrosol is defined as the distillation water with dissolved essential oil components produced during distillation of aromatic plants. Distillation waters are highly dilute

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acidic (pH 3.5-6.5), mild or pleasant-scented colloids or emulsions. Agarwood hydrosol is unique as it has the aroma of the essential oil of *Aquilaria sp.* even after storage for 1 year. Generally, hydrosol contains a small fraction of the aromatic compounds recovered from the plant in the distillation process (Catty, 2001).

Recovery process is an important step in order to retain the quality and improve the shelf life of the agarwood hydrodistillation by-products (residual water) by clarifying and removing the suspended particles, contaminants and bacteria. Three recovery processes are proposed for this study are centrifugation, membrane separation (microfiltration) and redistillation. Centrifugation is the process of separation that relies on centrifugal force to separate particles in a solid-liquid mixture into two distinct phases made up of sediment (pellet) and centrifugate (supernatant liquid) (Ford & Graham, 1991). The separation during centrifugation is caused as a result of centrifugal force working on the solid-liquid mixture at the selected speed or revolutions per minute (RPM) that move solid particles radially away from the rotation axis. Centrifugation is proposed as one of the recovery processes as it is susceptible to remove suspended undesirable particles such as wood debris as well as reducing the microbial load. When centrifuged at high speed, the bacterial cells might suffer shear damage from shear forces and collisions with suspended particles (Liu et al., 2017).

Membrane separation technology is extensively employed in agro, food, pharmaceutical, chemical and many other industries for clarification, decolouration, concentration, isolation and purification of products/chemicals present in dilute solutions/fluids. Membrane separation is a technology that apply the principle of physical separation of different compounds from a feed solution under a hydrostatic pressure difference applied between the two sides of a perm selective barrier. The

result from membrane separation are permeate and retentate fraction where permeate is which components that can pass through the membrane while retentate are compounds rejected by the membrane (Cassano et al., 2018). Structure, functional groups, particle/molecular sizes determine the acceptance (selectivity) or rejection of the compounds by the membranes. Micro ultra, nano filtration; reverse osmosis (RO), gas separation, pervapouration are some of the commonly practiced techniques. Microfiltration with pore size of 0.2 μm proposed in the study as filtration of liquids through 0.2-μm filter is an often-used and common technique for removing of microorganisms from heat-sensitive solutions. This method is suggested by several microbiological textbooks for the sterilization of heat-sensitive solutions because it is commonly believed that this filtration is able to removes all microorganisms (except viruses) from the filtered solutions whereby such 0.2 μm filtration is often referred to as "sterile filtration" (Hahn, 2004).

Redistillation is the earliest method for recovering the dissolved essential oils and other chemical compounds from liquid by-products of hydrodistillation and it is industrially practiced. Distillation will remove most of the dissolved and suspended materials and kill all kinds of organisms, but simple distillers will not remove organic compounds as they evaporate and condense along with water vapor (Stein, 2008). Although it is a simple and inexpensive method that can be practiced, but it recovers the water-soluble oil component partially and disproportionately. Prolonged heating will results in chemical changes and degradations in the distillation products (Gokhale, 1959).

The attempt to transform these by-products into cosmetics products is an innovative move. This effort not only minimizes waste and generates extra income for

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the industry but also overcomes some of the environmental problems caused by the by-products being discharged directly into stream. Some of the properties of agarwood hydrodistillation by-products are determined in this study in order to have contrast comparison and better understanding among these unique aromatic water before further utilization. Based on the comparison of the properties, the effectiveness of the proposed recovery processes can be studied and hence the one which give the optimum performance among others can be selected to be applied in industry. This initial investigation of the properties can be classified into 3 categories which are chemical, physical and microbiological properties. For chemical properties, chemical profile by GC-MS analysis, pH value and total dissolved solids (TDS) are studied; for physical properties, visual observations, turbidity, particle size analysis are studied; and for microbiological properties, microbial load is studied.

1.2 Problem Statement

This project study is in collaboration with industry as the aim is to solve the current problems and issues faced by the industry. Industry X located at Langkawi, Kedah is processing agarwood oil from wild agarwood by applying hydrodistillation extraction method. One of the issues faced by the industry is that the residual water left after hydrodistillation process is discarded because it contains wood debris, suspended particles, impurities and emitted unpleasant smell within few days of storing in room temperature due to microbial growth. Since there is limited information provided for the properties of agarwood hydrosol, therefore instead of selling it as a product, the employees have used it for their own drinking purposes or added back into the next batch of hydrodistillation as a medium. However, the by-products might have some economical values as it might contain some valuable

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agarwood oil fraction and other water-soluble bioactive compounds which can be utilized in manufacturing of cosmetics products. The general practice of disposing off the liquid by-products especially residual water as an effluent is fraught with environmental problems in the long run as the dissolved essential oil components that escape with the effluent possess antibacterial, antifungal, pesticide, other biological activities and some of them could be toxic (Rao, n.d.). The effects of draining such residual waters into the surrounding fields resulted in enhancement of bacterial populations such as *E. coli* and *Staphylococcus aureus* etc. Therefore, in order to study the properties of residual water for further application, it must be processed immediately after hydrodistillation to remove all the contaminants and microbes that will lead to quality deterioration.

1.3 Objectives

The general objective of this study is to characterise the agarwood hydrodistillation liquid by-products namely residual water and hydrosol whereas the specific objectives of this study are:

1. To determine the chemical, physical and microbiological properties of the agarwood hydrodistillation by-products before considering to apply them for further usage.
2. To analyse the effectiveness of the proposed recovery processes for residual water based on the three properties mentioned above.

1.4 Scope of Study

The scope of this study is to characterise the agarwood hydrodistillation liquid by-products namely residual water and hydrosol based on chemical, physical and

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microbiological properties. The liquid by-products were obtained from Industry X located at Kedah and they are processing agarwood oil from the resinous wood of wild *Aquilaria* species namely *Aquilaria malaccensis*. The residual water that still contains a lot of agarwood chips and debris, suspended particles, impurities and microorganisms has to go through certain recovery processes before further study on its properties. Hence, three recovery processes such as centrifugation, microfiltration and redistillation were proposed to the industry in order to clarify the residual water. The parameters set for each of the recovery process were: centrifugation (9000 g, 25 minutes); microfiltration (0.2 μm syringe filter); and redistillation (100 °C). Chemical properties such as chemical profile by GC-MS analysis, pH value and total dissolved solids; physical properties such as visual observation, turbidity, particle size analysis; and for microbiological properties such as microbial load were studied for the agarwood hydrodistillation by-products and the recovered residual water samples. The effectiveness of the proposed recovery processes for residual water were then studied and compared based on the results obtained. The recovery process which give an optimum performance among others is suggested to the industry. The optimum performance mentioned here means that the selected recovery process should be able to clarify the residual water while retaining its original quality at the same time.

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CHAPTER 2

LITERATURE REVIEW

2.1 Essential Oils

Essential oils also known as essences or volatile oils, are the volatile of secondary metabolites produced by plants either for their nutrition or other purposes such as protectant or attractant. Essential oil can be understood as the complex mixtures of chemical components in plants that has been extracted from steam distillation, solvent extraction, or physically pressed plant material techniques (Guenther, 1948; Leung, 1980). The most accurate definitions of essential oils was summarized by (Mekem Sonwa, 2000): "Essential oils are products or mixtures of products, which are formed in cytoplasm and are normally present in the form of tiny droplets between cells. They are volatile and aromatic". They composed of "mixtures of fragrant substances or mixtures of fragrant and odourless substances", where a fragrant substance is defined as a "chemically pure compound which is volatile under normal conditions and which owing to its odour, can be useful to society".

The aromatic and volatile characteristics of essential oils are obtained from plant material, including flowers, roots, bark, leaves, seeds, peel, fruits, wood, and whole plants (Hyldgaard et al., 2012). In a same plant, obtaining oil from different parts may result in completely different scents and properties. Climate, seasonal and geographical conditions, harvest period and extraction techniques are some of the factors that will determine the quantity of essential oil being extracted. (Panizzi, Flamini, Cioni, & Morelli, 1993). The yield of oils from the plants can also be affected by the stages of the plant growth.

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Chemical constituents of essential oils are complex mixtures of volatile compounds including terpenes (mostly monoterpenes and sesquiterpenes), phenolics and alcohols (Lucchesi et al., 2004). They are also oxygenated derivatives of hydrocarbon terpenes such as aldehydes, ketones, alcohols, phenols, acids, ethers and esters (Bakkali et al., 2008). The origin definition of essential oils mainly focusses on the presence of terpenes compound principally mono- and sesquiterpenes. However, compounds such as allyl and isoallyl phenols are another concern on the identity of essential oils. It has been found out that different terpenes with high number of carbons as well as other substances obtained in volatile oils by distillation has changed the original definition of essential oils. This is because during the distillation process, plant metabolites such as fats, coumarins, anthraquinones, and certain alkaloids are distillable while some compounds are derived from glycosides are transformed. In general, the main components of essential oils are formed by mono- and sesquiterpenes. There is also other derivatives of essential oils such as hydrocarbons (e.g., turpentine, formed by α- and β-pinene) or oxygenated components (Ríos, 2016).

In the aromatherapy use, vegetables oils and essential oils often become confused. Both of them are natural oil having therapeutic properties but composed in different ways. Both vegetable oils and essential oils have a complex chemical composition and the exact chemical composition of each oil is determined by the plant species as well as the extraction process carried out. Vegetable oils also known as fixed oils with its non-volatile properties, insoluble in alcohol and do not evaporate as how essential oils are. In contrast, essential oils are volatile and evaporate at or above room temperature; fragrance of plants are released through heat application and they are soluble in alcohol (Sharon, 2011).

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2.1.1 Extraction of Essential Oils

Essential oils are valuable plant products comprising complex composition of volatile principles that may change during the preparation process. (Bruneton, 1995). Accelerating diffusion through cell wall or crushing the cell wall are the methods that can be used to remove oil droplets that has been stored in the oil glands and sacs. Factors such as part of plants being extracted, stability of oil to heat and susceptibility of the oil constituents to chemical reactions will determine the techniques used for extraction of oil. There is pros and cons in comprising with each method used for extraction specifically on the quality oil extracted oil such as the yield, duration, cost, and ability to extract targeted compounds (Augusto et al., 2003; De Castro, Jimenez-Carmona et al., 1999; Milman, 2005). Common techniques used for the extraction of essential oils are:

- Hydrodistillation

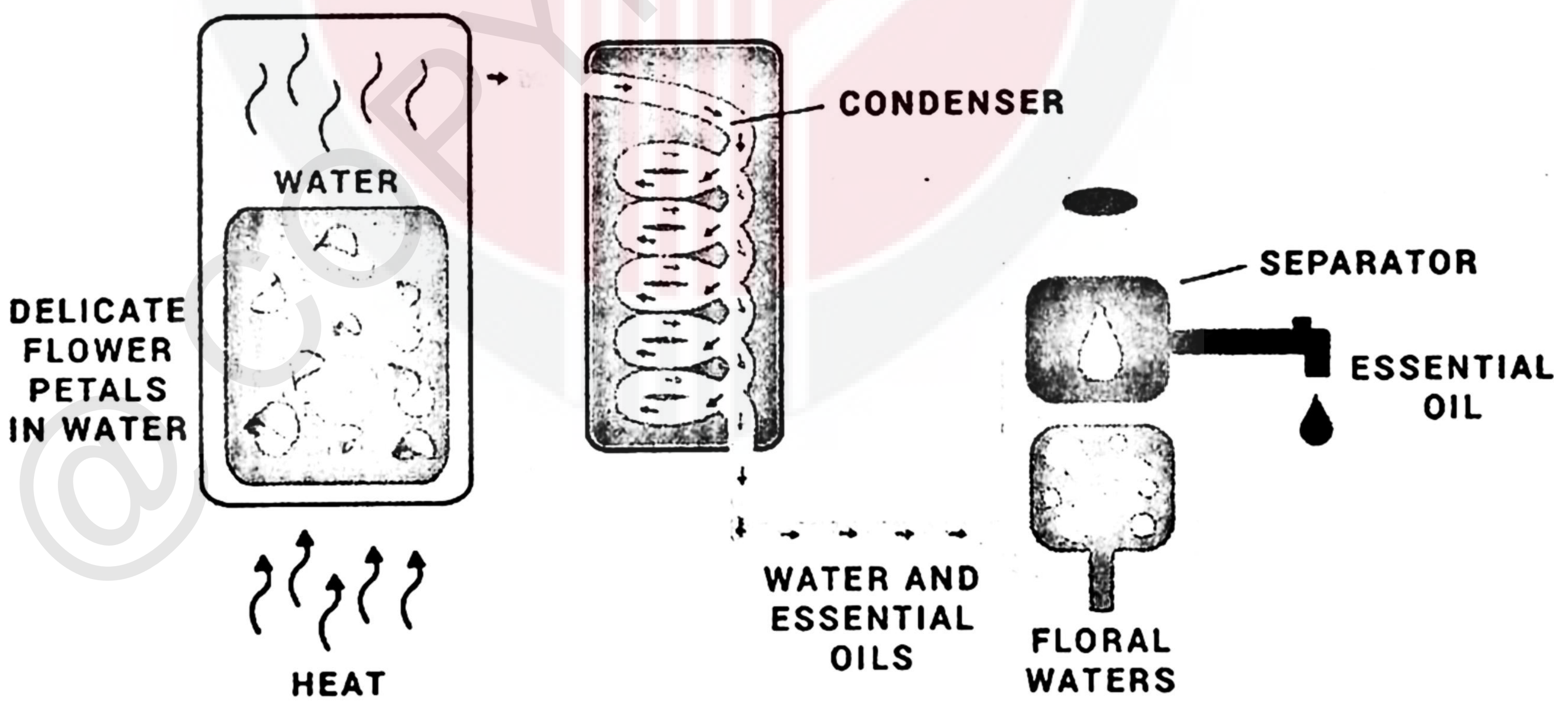

Figure 2.1: Illustration of hydrodistillation process

Water or hydrodistillation is one of the oldest and simplest methods being used for the extraction of essential oils (Meyer-Warnod, 1984). Hydrodistillation imply the principle of isolating essential oils from aromatic and medicinal plants which

involve the process of water distillation in direct contact with fresh or dried macerated plant materials. In this technique, the procedure starts with grinding and weighing of plant material then transferring them into the Clevenger set up. After that, the plant material is heated in two to three times its weight of water with direct steam. Water vapour and oil are removed from the system through a water cool condenser when the distillation vessel is being heated (Hamid et al., 2011).

- Steam distillation

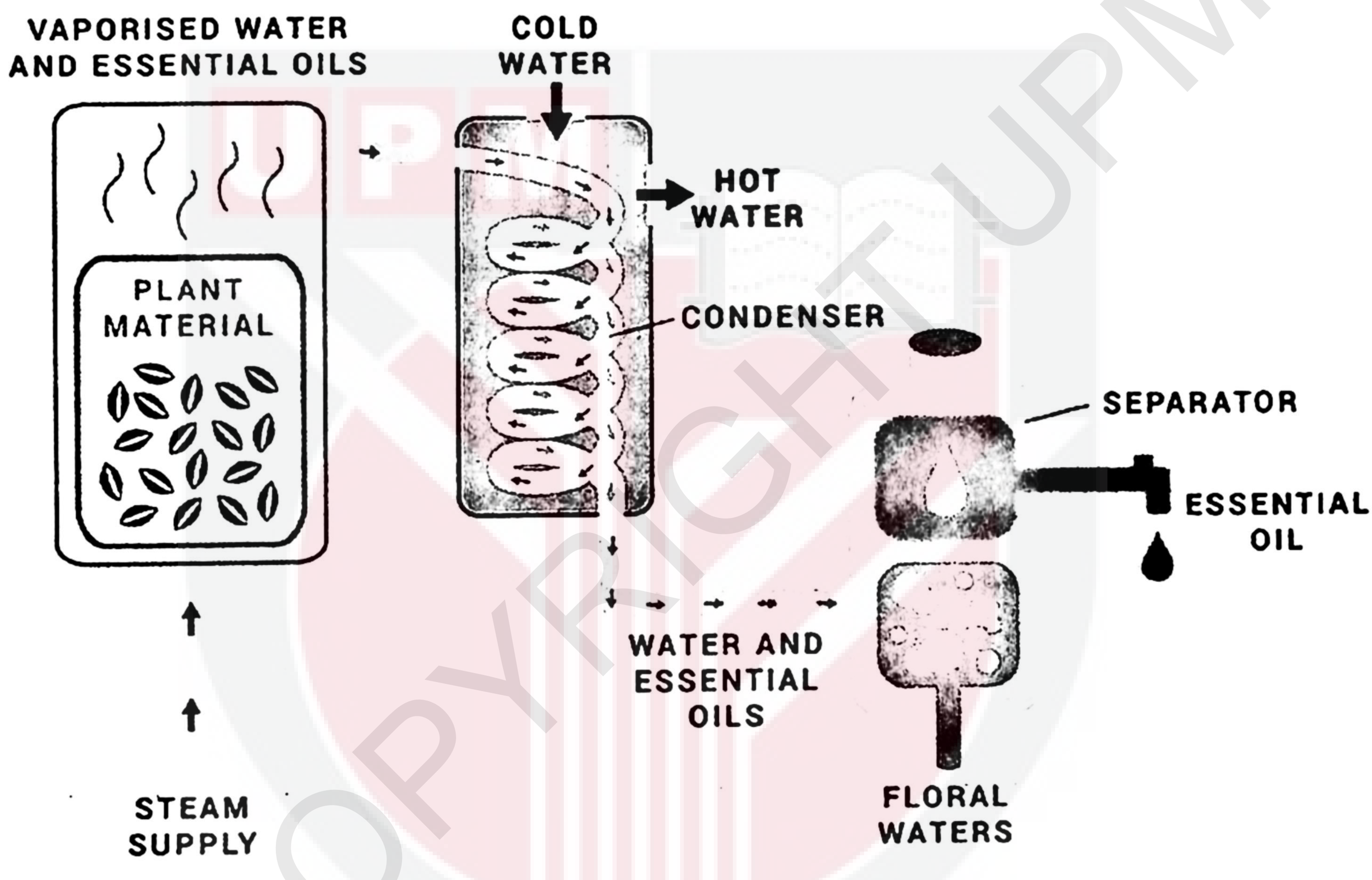

Figure 2.2: Illustration of steam distillation process

Steam distillation is a type of distillation that is suitable and benefit for a temperature-sensitive plant such as natural aromatic compounds. This method has been commonly used as laboratory method of purification of organic compounds but vacuity distillation has yet to replace it in the recent research. However, in certain industrial sectors, steam distillation is still important. (Fahlbusch et al., 2003). The procedure to carry out this technique started with placing the desired plant (fresh or sometimes dried) into the vessel then allowing steam to be added and passed through the plant which contains aromatic molecules or oils. The steam

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added act as an agent that break up the pores of the raw materials and release essential oil from the materials. After that, the system yields a mixture of vapor and desired essential oil of which vapor will be further condensed and result in collection of essential oil. (Badami & Rai, 2004).

- Solvent extraction

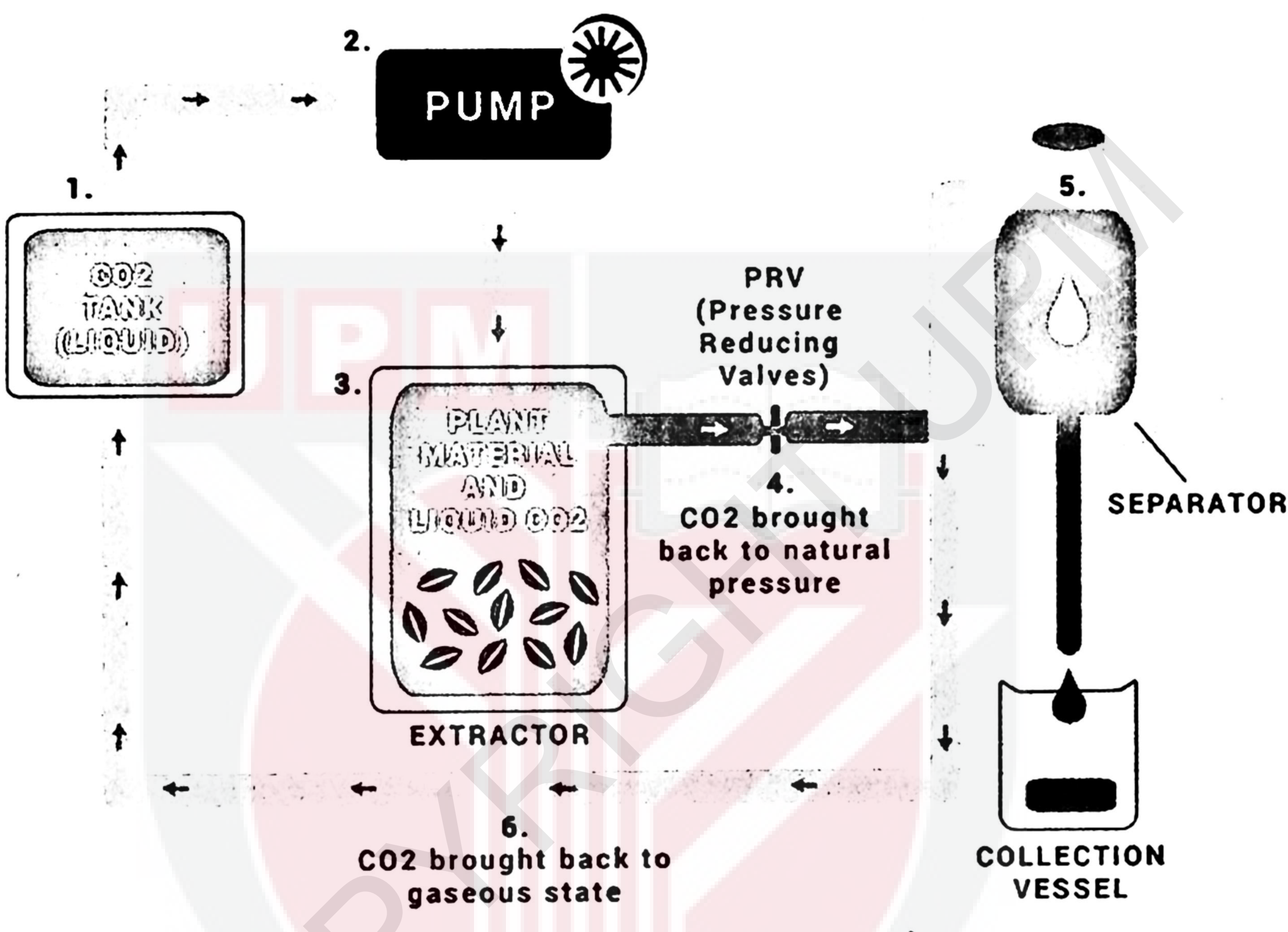

Figure 2.3: Illustration of solvent extraction process

Solvent extraction is an effective method use to obtain higher quantity of essentials oil at lower cost. (Chrissie, 1996). The part of the plant of which is being extracted determine the type of solvent used. For instance, leaves, roots, fruits are extracted with benzene with or without mixture of acetone or petroleum ether, in the cold or at boiling point while flowers are extracted with ethers. Oil waxes on the plant and the plant colour are dissolved by the used of appropriate solvent. To remove the solvent after extraction, it is carried out under reduced pressure that result in leftover of semisolid concentrate that will then be extracted with absolute ethanol. Although this technique

is simple, it has the disadvantages of which long extraction is needed, great amount of solvent is consumed and often unsatisfactory reproducibility (Dawidowicz et al., 2008).

- Supercritical carbon dioxide (CO_2)

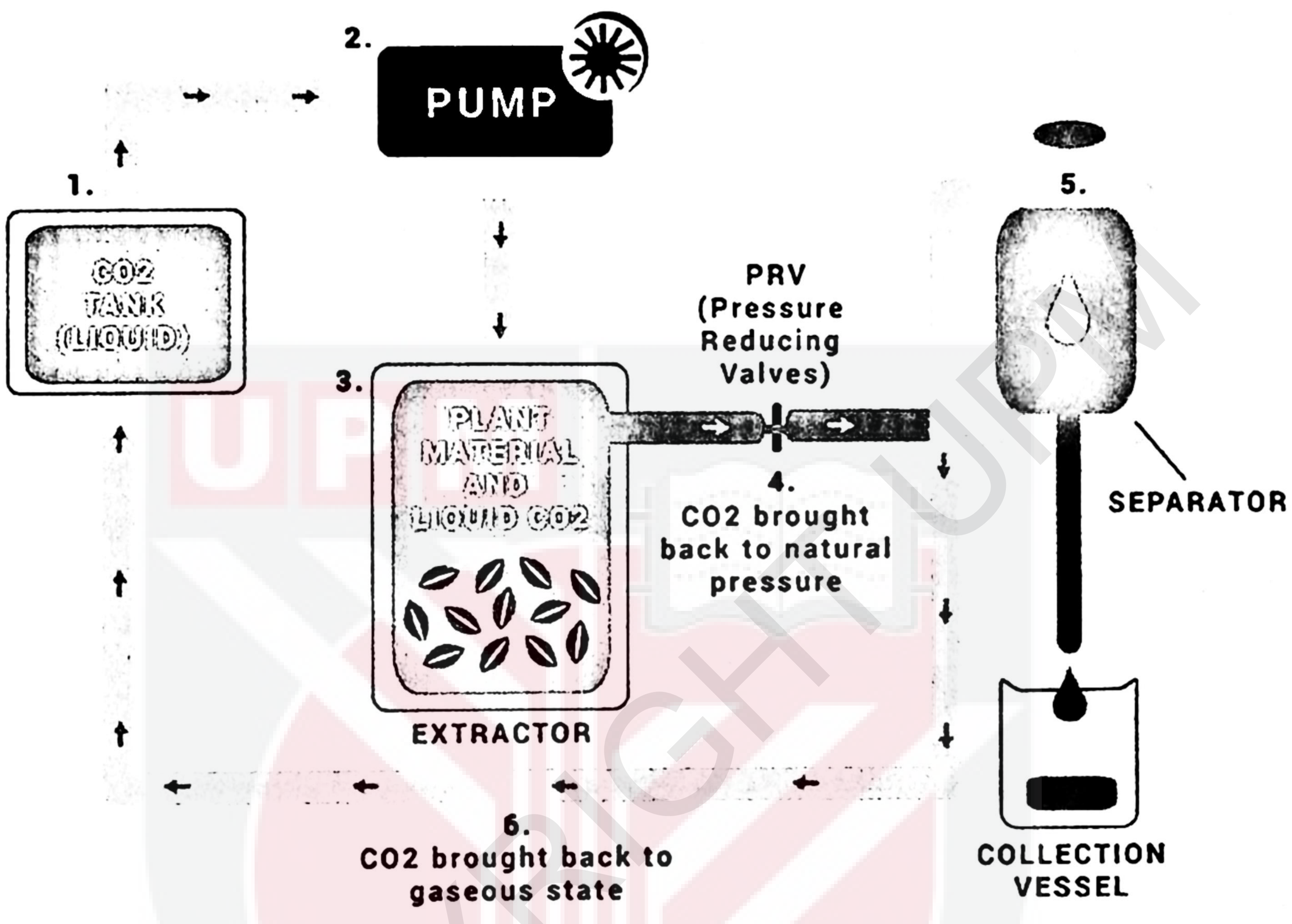

Figure 2.4: Illustration of supercritical carbon dioxide extraction process

Supercritical carbon dioxide (CO_2) extraction is a new extraction approach that apply gas rather than heat in its principles. The advantage of this approach is which labile compound are retained and other principles are unaltered. As instance, purification by means of this approach result in non-volatile flavonoids in its final product (Herzi et al., 2013). The procedure to carry out this technique is firstly placing the plant material in a high-pressure vessel where carbon dioxide is allowed to pass through the vessel. After turning into liquid form, carbon dioxide serves as a solvent used to extract essential oil from the plant materials. The carbon dioxide returns to a gaseous state leaving no residue behind when the pressure is

decreased. Qualities of essential oil extracted with any of the techniques described above depend on the chemical composition of the oil (Hamid et al., 2011).

- Cold pressing

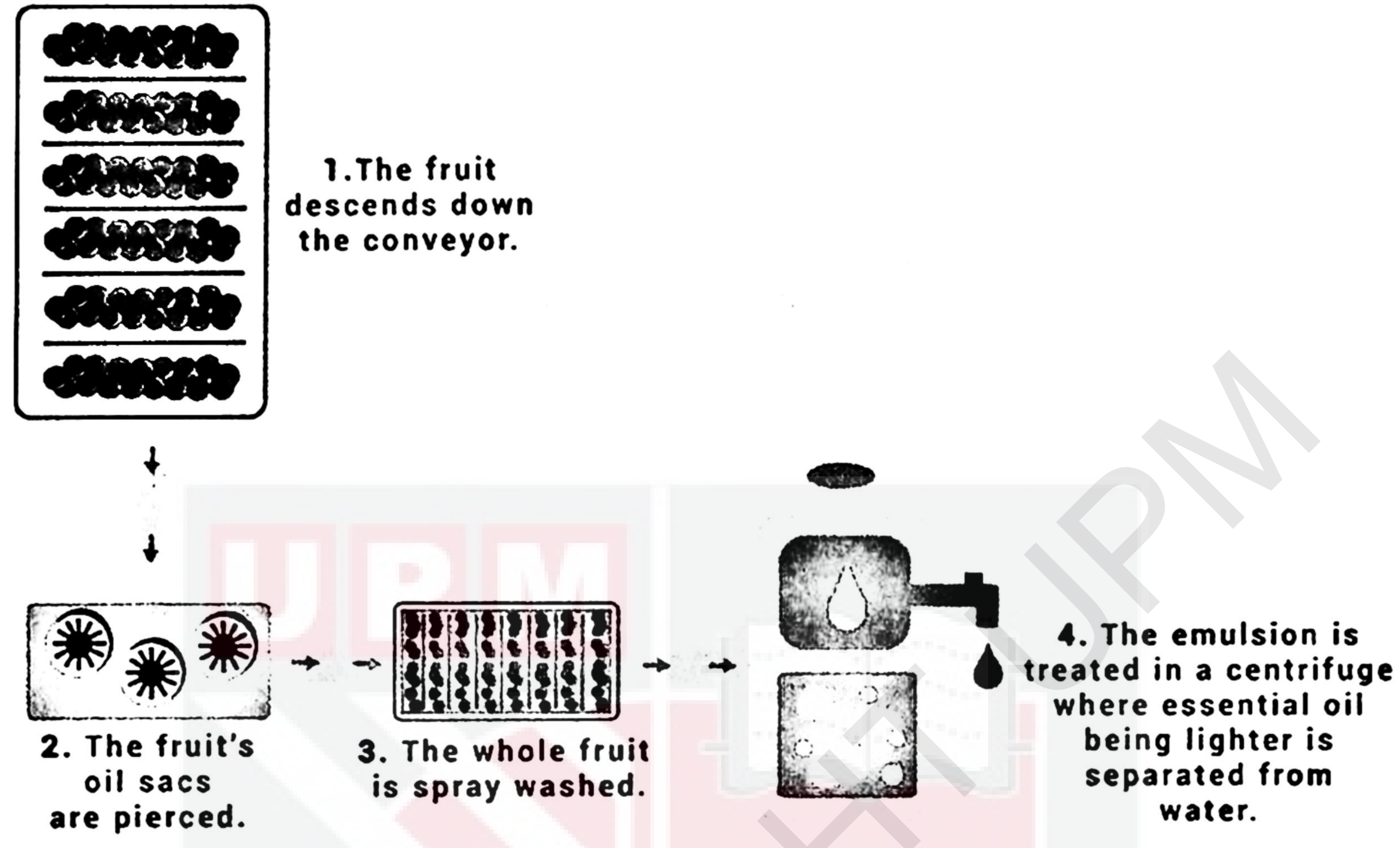

Figure 2.5: Illustration of cold pressing process

Cold pressing is one of the simplest ways to extract essential oil whereby the plant materials is being pressed and the oil is being collection, however, the application of this approach in scientific research is not widely apply. When the essential oil is found to be in the outer rind of a fruit such as in the citrus peel, this technique can be used for easy release of the oil. Citrus fruits, such as grapefruit, orange, lemon, and lime are mechanically pressed to extract their liquid, and then the watery juice is separated from the essential oil (Curtis, Thomas, & Johnson, 2016). The tiny pouches of fruits that contain essential oil are pierced when the fruits are being extracted by rolling over a trough with sharp projections that penetrate the peels. The whole fruit is pressed and the juice is being squeezed out that will then undergo centrifugation to separate the juice and the essential oil content (Hamid et al., 2011).

2.1.2 Usage of Essential Oil

In the fields such as manufacturing of perfumes; cosmetics; aromatherapy; spices and nutrition; phytotherapy; and insecticides, essential oils act as a valuable raw materials (Buchbaure, 2000). The quality and use of essential oils are differ as the source and extraction procedure.

From the economic point of view, the use of essential oils has growing greater interest especially in the cosmetics, soap, detergent, and perfume industry. The world production of essential oils for the preparation of perfumes has clearly increased, with specific groups of aromatic plants being highly sought after in the market (Ríos, 2016). In the context of perfume and cosmetic industry, essential oils play an important role along with its fragrance compounds that serve as natural chemical preservative which offer various benefits for skin and body at the same time. The chemical compounds associated in oils that contribute to pleasant odour enhance the value of the cosmetic products produced. Therefore, these precious substance are vital in the manufacturing of cosmetic and perfume products (Sarkic & Stappen, 2018).

Essential oils has the potential to act as a medicinal agents with its medicinal properties that lead to their used in pharmaceutics and medical field (Harris, 2010; Lis-Balchin, 2010). Aromatherapy is the therapeutic use of fragrances or at least more volatiles to cure or mitigate or prevent diseases, infection and indisposition by means of inhalation (Buchbauer et al., 1993). The benefits of essential oils are that when inhaling the volatile terpenes constituents, it helps to control the central nervous system. The properties of such as fungicidal, antidepressant, antibacterial, stimulating, and relaxant effect in essential oil prompting them to be used as an effective therapeutic agent (Hamid et al., 2011).

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Furthermore, the use of essential oils also covered a wide variety of consumer goods such as confectionery food products, soft drinks, and distilled alcoholic beverages due to its good flavouring properties (Kettenring & Geeganage, 2010). It has been reported that essential oil has benefited nutritional and agricultural fields with its antibacterial, antifungal, antiviral, nematicidal, insecticidal, and antioxidant properties (Lopez-Reyes et al., 2013; Turek & Stintzing, 2013). Because of this, their use as antioxidants and preservatives in food has been suggested (Hyldgaard et al., 2012; Tiwari et al., 2009), either incorporated into the foodstuff packaging material (Kuorwel et al., 2011) or as plant and crop protectants (Adorjan & Buchbauer, 2010).

2.2 *Aquilaria* Species

Agarwood or gaharu is resin-impregnated wood of the tree genus *Aquilaria* (Thymelaeaceae). The distribution of *Aquilaria* species in Malaysia is shown in Table 2.1 (Chang, Azah, & Rashid, 2011). *Aquilaria malaccensis* or karas tree is one of the well-known species.

Table 2.1: Distribution of *Aquilaria* Species in Malaysia (Chang et al., 2011)

Species	Local Name	Distribution
Aquilaria malaccensis	Karas, kekaras, Chandan, engkaras	Peninsular Malaysia, Sabah, Sarawak
Aquilaria microcarpa	Engkaras	Sabah and Sarawak
Aquilaria hirta	Chandan buluh	Peninsular Malaysia
Aquilaria rostrata	-	Peninsular Malaysia
Aquilaria beccariana	Gaharu tanduk	Peninsular Malaysia, Sabah and Sarawak

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Natural agarwood is the pathological product from diseased *Aquilaria* tree primarily because of wounds on the trunk. Consequently, the tree produces a type of resin (known as agar, agarwood, or gaharu) that is both high in volatile organic constituents and fragrant as a response to the attack. It is believed that the fragrant resin assists the tree in suppressing or retarding the microbial growth. The affected wood became dark brown or black due to the increased mass and wood density from resin development, leaving the unaffected wood in its original pale beige colour. The high-quality agarwood is normally recognized from its dark colour and strong aroma as shown in Figure 2.6 (Tajuddin, Aizal, & Yusoff, 2016).

Figure 2.6: High-quality natural agarwood with dark and dense resinous appearance

Agarwood grades in the Malaysian market heavily depended on physical appearances. There is no one grading system but generally, it is based on the ABC system. An example of this system is presented in Table 2.2 and is divided into nine grades (Mazlan & Dahlan, 2010). Each grade may have additional subgrades, for example, grade A breaks down to A1 to A10. The prices are subjected to the perplexing grades and it can begin from anywhere between a few dollars to thousands of dollars per kg (Lim & Awang Anak, 2010; Mokhtar et al., 2007). This huge range price can

be inflated whenever there is increased demand and shrinking supplies. The market prices of agarwood in Kelantan based on different grades which reported by Yip on the year of 2014 are shown in Figure 2.7.

Table 2.2: Commonly used agarwood grades found in the Malaysian market (Mazlan & Dahlan, 2010)

Grade	**Resin coverage on the surface**	**Resin colour**	**Wood shape**
Super king	Entire	Total black and shiny	Solid wood chunks (500 g to 3 kg)
Triple super	Entire	Total black and shiny	Solid wood chunks (200 – 500g)
Double super	90%	Less black and shiny	Solid wood chunks (50 – 200g)
Super	80%	Resin is black and grayish	Solid wood chunks of mixed sizes
A (A1 – A10)	Entire	Black turning into gray	Solid wood chunks of mixed sizes
AB	Entire	Black turning into brown	Solid wood chunks of mixed sizes
B (B1 – B10)	Entire	Black turning into brown	Solid wood chunks of mixed sizes
C	50%	Gray	Varies in shapes and sizes
D	Entire	Gray and whitish	Varies in shapes and sizes

Market prices of agarwood in Kelantan	
Grade	Market Price (per kg)
Double Super Grade	RM10,000 to RM12,000
Super Grade	RM8,000 to RM10,000
Grade A	RM4,000 to RM8,000
Grade B	RM3,000 to RM4,000
Grade C	RM1,000 to RM2,000
Mixed Grade	RM60 to RM250
Essential Oil	RM19,000 to RM38,000

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Figure 2.7: Market prices of agarwood in Kelantan (Yip, 2015)

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2.3 Agarwood Oil

Agarwood oil is an essential oil of *Aquilaria* trees that extracted from resinous wood. It carries a distinctive scent which is volatile at above room temperature. The identity and quality of the agarwood oil produced depending on its chemical and physical characteristics. The properties of the agarwood oil are correlated with the presence of chemical components which can be affected by multiple factors such as geographical factors, genetic, variability, ecology factors and extraction process (Bandoni, 2000)

2.3.1 Agarwood Oil Extraction

Pre-treatment techniques are important prior extraction process to improve agarwood oil. One of the pre-treatments to improve production essential oil is by applying microbial in soaking process known as enzymatic hydrolysis. Enzymatic hydrolysis is defined as a process of breakdown the cell wall in molecule with the presence of water. In this process, enzymes act as biological catalyst serve to break down cellulose into lignicelluloses and was first introduced by Hitze et al. (1972)

emphasis on the use of enzymes as a support in the oil extraction process. Degradation of cellulose and plant cell wall fibres are found to be caused by microorganisms such as fungi and bacteria (Dashtban, Schraft, Syed, & Qin, 2010). In addition, digestibility of agarwood is believed to be improved by presence of fungi. Degradation of agarwood cell walls has to be completed first to facilitate the extraction process and enhance the permeability of oil. Based on a research from (Arsat, 2008), it is claimed that the use of fungi as technical enzyme gave highest yield of oil as compare to the extraction process without enzyme pre-treatment. The main aim of using enzyme to degrade cell wall is to increase oil extractability of agarwood chips.

Hydrodistillation is the most popular method for extracting agarwood essential oil in commercial scale due to easy operation, lower cost, and green water-based process. The copper distillation pot has been traditionally used and it is still in use by some traders. Nowadays, the copper has been replaced by stainless steel. In this approach, the process starts with filling the pot with fermented agarwood chips or powder which then being heated on a brick furnace. In order to ensure all oil has been extracted, the heating process may continuously take up to 3 days or more. The fundamentals in this step is where the hot vapor from heating goes through a steel condenser with external running water serve as cooling effect result in condense of vapours that drop into a collecting funnel where oil and water are separated gravitationally (Burfield & Kirkham, 2005). A two phase system with water and the essential oil is produced, where the oil can be decanted and recovered (Boutekedjiret et al., 2003). The distilled and condensed water phase is known as hydrosol. Cohobation is a process where the hydrosol is being recycled and taken for another cycle of hydrodistillation (Catty, 2001). Cohobation is defined as redistillation or recirculation of distillation water during the distillation process. Earlier literature used

the terms redistillation and cohobation synonymously. The distillation water flowing out of the receiver is directed into the cohobation column. Steam and essential oil vapours rising from the distillation tank clash with the distillation water flowing downstream creating a counter-current flow. The hydrosol ultimately reaches the distillation tank. The essential oil present in the hydrosol volatilizes in the cohobation column as well as in the distillation tank and is collected in the receiver along with the primary oil. Cohobation, like redistillation results in partial and disproportionate recovery of water-soluble oil components (Rao, n.d.).

Figure 2.8: A commercial hydrodistillation system for agarwood oil extraction in Gua Musang, Kelantan (Malaysia). (A) Extraction contraption and (B) extracted oil in the oil separating funnel

2.3.2 Physical Properties

The physical properties of agarwood oil depend on the species of *Aquilaria* trees in the aspect of colour, odour, viscosity and specific gravity which are shown in Table 2.3 The main compounds found in the oleoresin of agarwood are a complex mixture of sesquiterpenes and 2-(2-phenylethyl) chromones (Naef, 2011). The impressive pleasing odour that some described as balsamic, spicy, woody, and sweet

are created by having some simple volatile aromatic compounds together. For example, β-elemol is a fragrant sesquiterpene, which contributes to peppery and lemony odours. Other sesquiterpene like β-caryophyllene contributes to sweet, spicy, and fruity odours (Breitmaier, 2006).

Table 2.3: Physical properties of agarwood oil (Nadia, 2012)

Properties	Description
Odour	Sweet aromatic scent
Appearance	Lightly yellow to brownish liquid
Specific gravity at 25°C (g/cm^3)	0.89 – 1.08
Refractive index at 20°	1.4910 – 1.6090
Optical rotation at 20°	-13.20 until -17.80
pH value	6.8 – 13.2
Esterification index	18.3 – 27.1
Solubility	Soluble in alcohol

2.3.3 Chemical Properties

Agarwood contains several types of sesquiterpenes, and it has become the subject of active research in the past 40 years or more. It is believed that Agarol was the first sesquiterpene isolated from agarwood (Bhattacharyya et al., 1952; Buechi & Wueest, 1979; Maheshwari et al., 1963). Nowadays, more than 70 sesquiterpene compounds have been identified from agarwood (Naef, 2011). The basic molecule of terpenes is the isoprene unit C_5H_8. Chemically, the terpenes can be divided into several classes including mono-, sesqui-, and diterpenes. Monoterpenes are comprised of two isoprene units, $C_{10}H_{16}$, whereas sesquiterpenes, $C_{15}H_{24}$, contained three isoprene units. Sesquiterpenes that are important in agarwood oils are sesquiterpene hydrocarbons ($C_{15}H_{24}$) and oxygenated sesquiterpenes ($C_{15}H_{26}O$). They are derived from the sesquiterpene skeletons and grouped as agarofuran, agarospirane/vetispirane,

cadinane, eremophilane/valencane, eudesmane/selinane, guaiene, and prezizane types as shown in Figure 2.9. Important oxygenated sesquiterpenes including agarospirol, jinkohol, jinkohol-eromol, and kusenol that may contribute to the characteristic woody aroma of agarwood are shown in Figure 2.10.

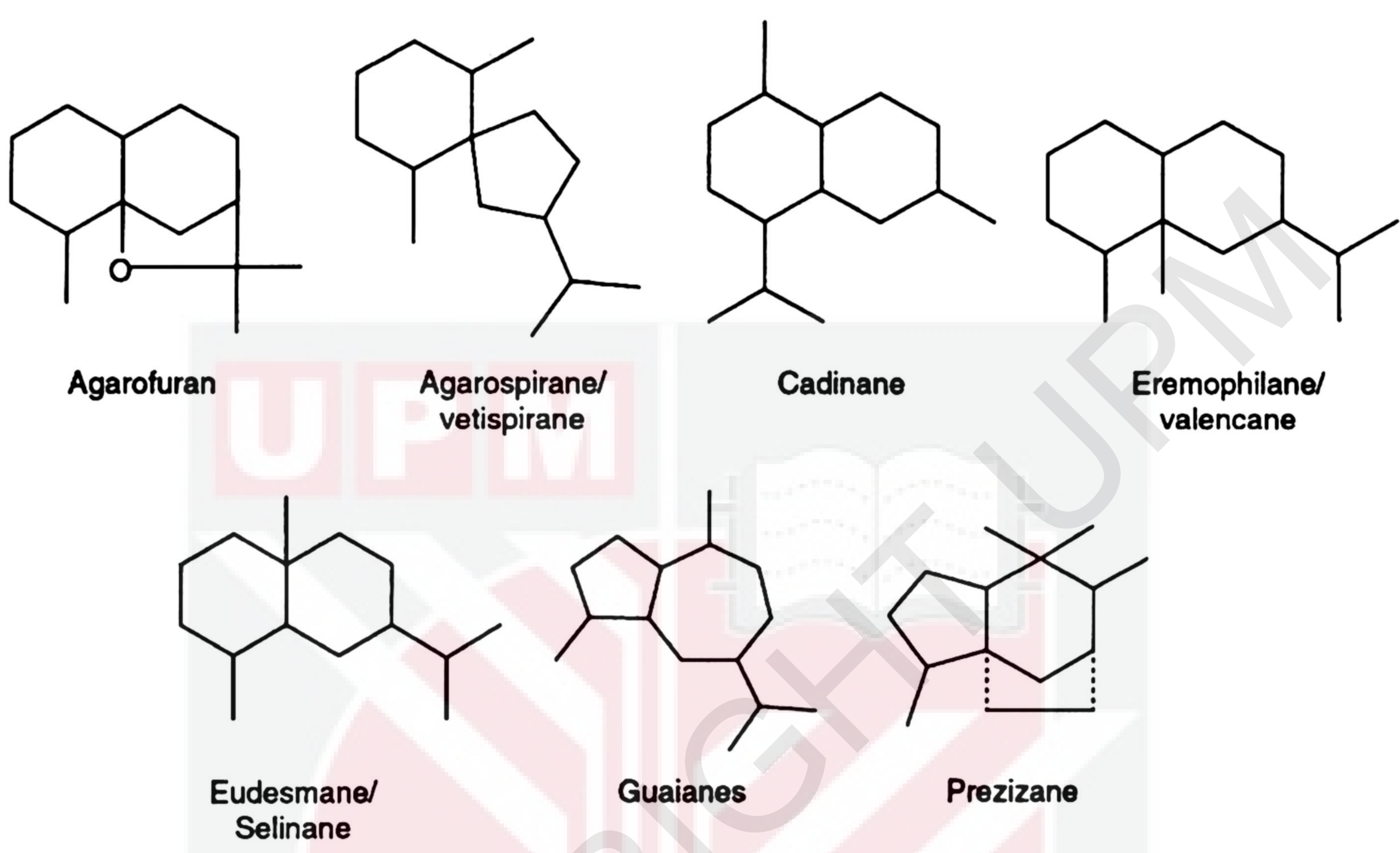

Figure 2.9: Skeleton of hydrocarbons found in volatile agarwood oil (Naef, 2011; Chen et al., 2012)

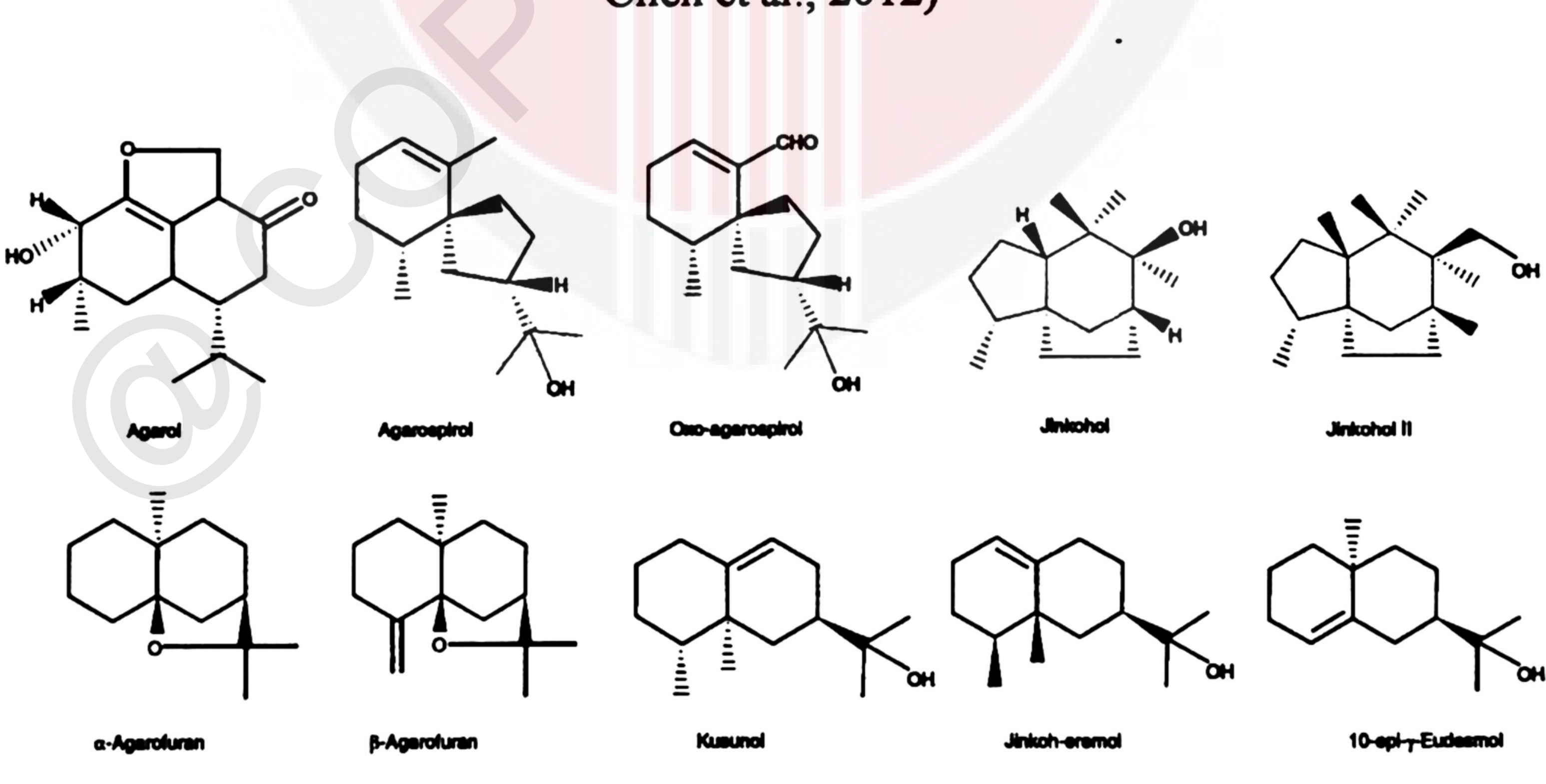

Figure 2.10: Skeleton of oxygenated sesquiterpenes found in volatile agarwood oil (Varma, Maheshwari, & Bhattacharyya, 1965)

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Generally, agarwood oils are mixtures of sesquiterpenes, oxygenated sesquiterpenes, oxygenated phenyls, carboxylic, and carbonyl hydrocarbons. Table 2.4 shows the main sesquiterpene hydrocarbons and oxygenated sesquiterpenes, which have been identified by previous agarwood research.

Table 2.4: Sesquiterpenes from agarwood oils found Malaysia *Aquilaria* species (Grade C) extracted by hydrodistillation (Tajuddin, 2010; Naef, 2011)

Aquilaria species	Extraction mode	Main constituents	Reference
A. malaccensis from:			
India (Hojai in Assam province)	Hydrodistillation	Aromadendrene; (+)-calarene; 6-guaiadiene; valencene	Jayachandran et al. (2014)
Indonesia (type B)	Benzene	Jinkohol	Nakanishi et al. (1981)
Indonesia (type B)	Benzene	Jinkohol II	Nakanishi et al. (1983)
Indonesia (type B)	Benzene	Agarol, 3,4-dihydroxydihydro-agarofuran, oxo-agarospirol, jinkoh-eremol	Nakanishi et al. (1984)
Malaysia (grade C)	Hydrodistillation	α-Agarofuran; β-agarofuran; *nor*-ketoagarofuran; agarospirol; 3-phenyl-2-butanone; β-eudesmol; 10-*epi*-γ-eudesmol; α-guaiene; jinkoh-eremol; jinkohol II; kusunol	Nor Azah et al. (2008)
Vietnam (Kyara, Kanankoh)	Acetone	Oxo-agarospirol, α-guaiene, (-)-Guaia-1(10),11-dien-15-al	Ishihara et al. (1991a)
Vietnam (type A)	Benzene	α-Agarofuran, 3,4-dihydroxydihydro-agarofuran, oxo-agarospirol, jinkoh-eremol, jinkohol, jinkohol II, kusunol	Yoneda et al. (1984)
A. sinensis from			
China	Hydrodistillation	Agarospirol; caryophyllene oxide; α-copaen-11-ol; eremophila-7(11),9-diene-8-one; eudesm-7(11)-en-4α-ol; γ-eudesmol; guaia-1(10),11-dien-9-one; guaiol; selina-3,11-dien-14-al; α-selinene	Chen et al. (2011)
China (Hainan, Guangdong)	Ethanol, hydrodistillation	Agarospirol; aristolene; caryophyllene oxide; α-copaen-11-ol; eudesm-7(11)-en-4α-ol;	Xing et al. (2012)

2.3.4 Usage of Agarwood Oil

Essential oils obtained from agarwood resins are commonly used as perfumery ingredients. The unique aroma of the agarwood oils is the result of complex mixtures of sesquiterpenes, oxygenated compounds, and chromone derivatives, such as α-agarofuran, β-agarofuran, agarospirol, jinkohol, jinkohol II, and valerianol (Naef, 2011). Research studies have also shown that *Aquilaria* species possessed antioxidant, antimicrobial, and laxative properties (Huda, Munira, Fitrya, & Salmah, 2009; Dash, Patra, & Panda, 2008; Hara et al., 2008). Agarwood has also been studied for its relaxing and sedative effects.

Agarwood oil has many medicinal properties which some of them are yet to be discovered. The common medicinal properties of agarwood oil can be summarized in the Table 2.5 (Plantation International, n.d.).

Table 2.5: Some medicinal properties of agarwood oil (Plantation International, n.d.)

Properties	Function
Stimulant	quickens physiological function
Tonic	strengthens the body
Anti-inflammatory	reduces inflammation
Aphrodisiac	increases sexual desire
Diuretic	stimulates the production of urine
Antimicrobial	kills or resists pathogenic microorganisms
Carminative	settles the digestive system and relieves gas
Febrifuge	combats fever
Digestive	aids digestion of food
Expectorant	loosens mucus in the respiratory tract and aids expulsion
Muscle relaxant	eases tension in muscles
Demulcent	protects mucus membranes and calms irritation
Emmenagogue	stimulates menstrual flow
Analgesic	relieves pain
Anti-arthritic	combats arthritis
Antipruritic	relieves or prevents itching
Stomachic	digestive aid and tonic, improves appetite

2.4 By-product of Hydrodistillation

Distillation of perfumery plants yields 5 distinct products: 1) Essential oil, the principal product of commerce 2) Distilled/spent biomass, a useful by-product 3) Ash from the furnace, cheap manure 4) Residual water remaining in the distillation tank and 5) Distillation/condensate water, another useful by-product (Rao, 2012). The aim

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of the experiment is focusing on the liquid by-products formed which will be further explained as below.

2.4.1 Hydrosol

Hydrosol is defined as the distillation water with dissolved essential oil components produced during distillation of aromatic plants. Distillation waters are highly dilute acidic (pH 3.5-6.5), mild or pleasant-scented colloids or emulsions. During distillation the time of collection will cause the odour of hydrosol to be varied from mild to strong, pleasant to unpleasant and similar to dissimilar to the essential oil extracted. As instance, hydrosols collected at the early stage of the distillation are pleasant-scented comparing to those collected in the latter stage which give grassy or vegetative notes. This is because there is difference in their composition brought out by low or high boiling terpenoids present in the hydrosols. Hydrosols collected in the early and late periods of distillation are considered therapeutically more useful than the entire distillation water (Catty, 2001; Rose, 1999). They do not have the ill effects of essential oils such as strong scent that causes headache, skin and eye irritations etc. Other than that, after appropriate dilution, they are safe to be apply on skin or taken orally. They can also be used in many ways on humans and their pets (Catty, 2001; Price & Price, 2004; Rose, 1999). Due to the fact that hydrosols are cheap and affordable flavouring, good perfumery ingredients, aromatherapy products that caught the fancy of global consumers, aroma therapists and researchers, it has been widen its marker to the international level. Several aroma therapists and aromatherapy companies are aggressively marketing hydrosols of diverse aromatic crops.

In addition to natural hydrosols, man-made synthetic hydrosols by dissolving desired synthetic or natural aroma chemicals or natural or reconstituted or synthetic

essential oils in water are also in the market. Through fortifying of natural hydrosols with desired chemical substances, it is able to produce reconstituted or semi-synthetic hydrosols. It is common to adulterate costly hydrosols with water or alcohol. According to aromatherapy practitioners, hydrosols must be carefully produced in distillation units as the main products of distillation. The precaution related to hydrosols are which it should be collected in aseptic containers, sealed and stored in a cool place to avoid any contamination to prolong its shelf life of one year or more. Food hydrosols are formed by which it is a uniform without any visible oil droplets where oil droplets that formed on the surface of hydrosol are to be decanted (Rao, 2012).

Agarwood hydrosol is a by-product produced during the hydrodistillation of the resinous wood part of *Aquilaria sp.* This by-product is unique as it has the aroma of the essential oil of *Aquilaria sp.* even after storage for 1 year. Generally, hydrosol contains a small fraction of the aromatic compounds recovered from the plant in the distillation process. Every aromatic substance has a maximum solubility in water. The essential oil separate into a distinct layer on top of the distillation water after the point where the aromatic compounds is dissolved in the distillation water. (Catty, 2001).

2.4.2 Residual Water

Residual water is an effluent that is being drained out after the distillation of aromatic plant. It is the dark coloured thick water that retained in the distillation tank before draining. The residual water is contaminated with microbes and emitted disagreeable smell within few days being kept under ambient conditions. The dissolved phytochemicals content and then suspended biomass residue of distilled crop

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associated in the residual waters are susceptible to microbial contamination and required to be processed and utilized before contamination occurs (Rao, 2013).

Undiluted decoction (residual water) of *Satureja thymbra* from Greece was found to be as effective as chemical sanitizers in controlling biofilms formation by food-spoilage and pathogenic bacteria *Pseudomonas putida*, *Salmonella enteric* and *Listeria monocytogenes* on open surfaces of food processing industry (Chorianopoulos et al., 2008). Besides, a research has shown that marinating turkey meat products with residual waters of rosemary, sage and thyme able to retain their spicy, acidic odour and flavour after storage. This is because the spice residual waters contribute to the lipid oxidation retardation and antioxidant effects. These products can be utilized to prevent rancidity in marinated, stored, heat-treated meat products (Mielnik et al., 2008).

2.5 Water Soluble Essential Oil Constituents

Essential oils are sparingly water-soluble. The phenomenon of water solubility of essential oil constituents is complex and is poorly studied. During distillation, oil constituents remain in intimate contact with a large volume of steam/condensate water for a long time. Depending on their solubility, essential oil constituents are selectively and disproportionately partitioned into the water phase (Fleisher, 1990). Polar, hydrophilic (oxygen containing) compounds that can form hydrogen bonds with water (polar solvent) are relatively more water-soluble than nonpolar, monoterpene and sesquiterpene hydrocarbon (soluble in oil or nonpolar solvent) fractions that are either suspended (as in emulsion) or held in the interspaces of water molecules (like sugar in water) depending on their size and density. Alcohols, ketones, aldehydes, phenols, esters that impart aroma character to the oils are water-soluble to the extent of <0.05-

1.0% (Fleisher, 1990). Due to their strong hydrogen bonding capability alcohols are more water- soluble than esters that get hydrolysed into corresponding alcohols in response to existing congenial temperature regime during distillation. Some of these compounds in complex mixtures such as essential oils are capable of carrying other compounds with them into the aqueous stream through electrostatic attractive forces (such as hydrogen bonding). However, this pairing of aroma chemicals depends on their structure and functional groups (Bohra, Vaze, Pangarkar, & Taskar, 1994). For example, linalool pairs with phenylethyl alcohol, but not with eugenol possibly due to lack of bonding between the phenolic OH group of eugenol with tertiary alcohol group of linalool (Bohra et al., 1994). Structure and functional groups of aromatic compounds therefore, play a pivotal role in their water solubility. Water solubility depends on the conditions of distillation (method, water pH, steam temperature etc), condensation (temperature, speed of condensation, volume of condensate, time allowed for separation of oil etc.) and the nature of the plant material distilled (plant part, age, geographical location, relative percentages of compounds present etc.). Water solubility of the essential oils is generally in the range of <0.1-0.2% (Hart et al., 2000), though other investigators have reported solubilities of 0.2-0.4% (Spencer, 1992) or even higher (Fleisher, 1990) for specific species. There is a need to study the factors that influence the water solubility of essential oil components in distillation water during distillation and devise technologies to minimize it.

2.6 Recovery Processes

Microbial contamination is one of the serious problems of the by-product (hydrosol or residual water) that may occur during storage in unsterile containers, keeping in inappropriate temperature and sometimes use of unsterile water for dilution

before packing. Inadequate storage conditions lead to metabolize aromatic and other compounds by microorganisms (Sabahi, Zebarjad, & Moein, 2019). They must be undergone recovery process to be separated from suspended particles, contaminants and bacteria in order to increase the shelf life of the product.

2.6.1 Centrifugation

Centrifugation is a technique applied to the removal of a small concentration of solid particles from fluids. The particles size differ greatly from larger particles to colloidal size where larger particles can be easily removed through sedimentation. For solid-liquid systems, Purchas (1967) classifies these processes according to the type of driving force applied: (1) gravity, (2) vacuum, (3) pressure and (4) centrifugal force.

Rotation of materials occurred when a centrifugal force is applied. The factor that will be influencing the amount of force required are the radius and speed of rotation as well as the density of the materials that is being centrifuged. In the working principle of centrifugation on separation of immiscible liquids (such as emulsion), denser liquid tends to move to the bowl wall while lighter liquid is displaced to an inner annulus. Density of liquids, pressure difference and speed of rotation contribute to variation on the thickness of layers. As instance, separation of dense liquid from a mass of lighter liquid such as removal of water from oils caused the radius of outer layer to be increased. When particles are removed from liquids, the particles move to the bowl wall under centrifugal force. Centrifugation is classified into three groups (Erkmen & Bozoglu, 2016):

1. Separation of immiscible liquids (liquid–liquid centrifuges).
2. Clarification of liquids by removal of small amounts of solids (centrifugal clarifiers).

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3. Removal of solids.

The application of centrifugation on food such as milk, fruit juices and syrups are mainly on the removal of suspended undesirable particles (such as dust, leukocytes, and food particles), bacteria, spores, yeasts, and molds. For example, centrifugation apply on milk is to take out milk materials and left behind microorganisms. It has been found out that approximately 90% of microbial population can be removed under high force of centrifugation. This is advantages for the milk production industry where the thermoduric microorganisms that survived at pasteurization stage can be removed by application of centrifugation (Erkmen & Bozoglu, 2016).

Furthermore, based on research, herb decoctions where the aqueous extract of rosemary, sage and thyme that left as by-product after steam distillation of essential oils found to be a source of antioxidants in marinades for turkey thigh meat. In this research, the decoction (by-products) was centrifuges for 25 minutes at 9000g to remove small particles such as residual plant materials and debris from the liquid before proceeding for further analysis (Mielnik et al., 2008).

2.6.2 Membrane Separation

Membrane separation technology is extensively employed in agro, food, pharmaceutical, chemical and many other industries for clarification, decolouration, concentration, isolation and purification of products/chemicals present in dilute solutions/fluids. Ultra, micro, nano filtration; reverse osmosis (RO), gas separation, pervapouration are some of the commonly practiced techniques. Other applications include dialysis, osmosis, forward osmosis, electrodialysis, membrane electrolysis, electrophoresis etc. A wide array of polar (hydrophilic) nonpolar (hydrophobic) and organophilic; natural and synthetic membranes are available in the market as flat

sheets, tubes or fine hollow fibres. The driving force employed for displacement of specific substances from the feed solution into permeate (solution that passes through the membrane or filtrate) is concentration, temperature, pressure or electrical gradients. As a result, the retentate (feed rejected by the membrane) gets either concentrated or depleted of specific substances leading to concentration polarization. Mass transfer of solutes takes place either through convection or diffusion. Structure, functional groups, particle/molecular sizes determine the acceptance (selectivity) or rejection of the compounds by the membranes. Decrease in permeation flux rates with time and membrane fouling are routinely encountered problems (Rao, n.d.).

Based on the research done by (Sabahi et al., 2019), the combination of ultrafiltration and ultraviolet treatment (F-U treatment) was used to reduce rosewater contamination in traditional and industrial rosewater products as rose water contamination can limit its quality and applications. The application of ultrafiltration and ultraviolet (UV) radiation not only can diminish microbial contamination but also prevent decomposition of the chemical compounds of samples. Comparing between the combination of ultrafiltration and UV radiation beside other physical methods, this approach is believed to be as a valuable alternative technique in obtaining high quality rose water with preserving its constituents that may lost in traditional thermal techniques.

2.6.3 Redistillation

Redistillation is the earliest method of recovering the dissolved essential oils from hydrosols and is industrially practiced. The hydrosol is redistilled separately or along with the next batch of aromatic plant distillation. Though, it is a simple and inexpensive method that can be practiced by the farmers, it recovers the water-soluble

oil components partially and disproportionately. Prolonged heating results in chemical changes and degradations (Gokhale, 1959). The ensuing redistilled oil exhibits a different or even inferior organoleptic profile (Fleisher, 1990). The resultant hydrosol contains the unrecovered constituents from the redistilled hydrosol as well as the water-soluble oil constituents from the current batch of distillation. Distillation will kill all kinds of organisms, but simple distillers will not remove organic compounds as they evaporate and condense along with water vapor (Stein, 2008).

Hydrodistillation of rose petals yields rose oil, rose water and residual water (water remaining in the distillation tank). Rose water and residual water are strongly aromatic. Rose water is widely used in food flavouring, soaps, cosmetics, toiletries and perfumes. Residual water has limited application. One of the major components of rose petals is 2-phenylethyl alcohol. During hydrodistillation, a major portion of this chemical is partitioned into the rose water due to its high-water solubility. Commercial rose oil producers therefore, resort to redistillation of rose water (Eikani, et al., 2005). have examined the composition of Iranian rose *(Rosa damascena Mill.*) oil produced by hydrodistillation (first oil) and redistillation (in a commercial hydrodistillation unit) of rose water (second oil). The profiles of the oils showed marked differences in the percentages of their compounds. Redistillation failed to recover 2- phenylethyl alcohol which was lost into second distillation (redistilled) rose water.

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CHAPTER 3

METHODOLOGY

3.1 Materials

By-products of agarwood hydrodistillation process namely residual water and hydrosol as shown in Figure 3.1 were obtained from Industry X located at Langkawi, Kedah, Malaysia. The residual water was the hot water extract of agarwood remaining in the distillation tank after extraction process whereas hydrosol is the distilled water in contact with agarwood oil obtained in the separating funnel of Clevenger set up. Spritzer distillation drinking water was used as a controlling variable in this study. Difco ™ Plate Count Agar powder was used to perform microbial plate counts for the by-products and recovered residual water samples.

Figure 3.1: (A) Residual water retained in distillation tank; (B) Hydrosol collected in the separating funnel of the Clevenger set up

3.2 Research Approach

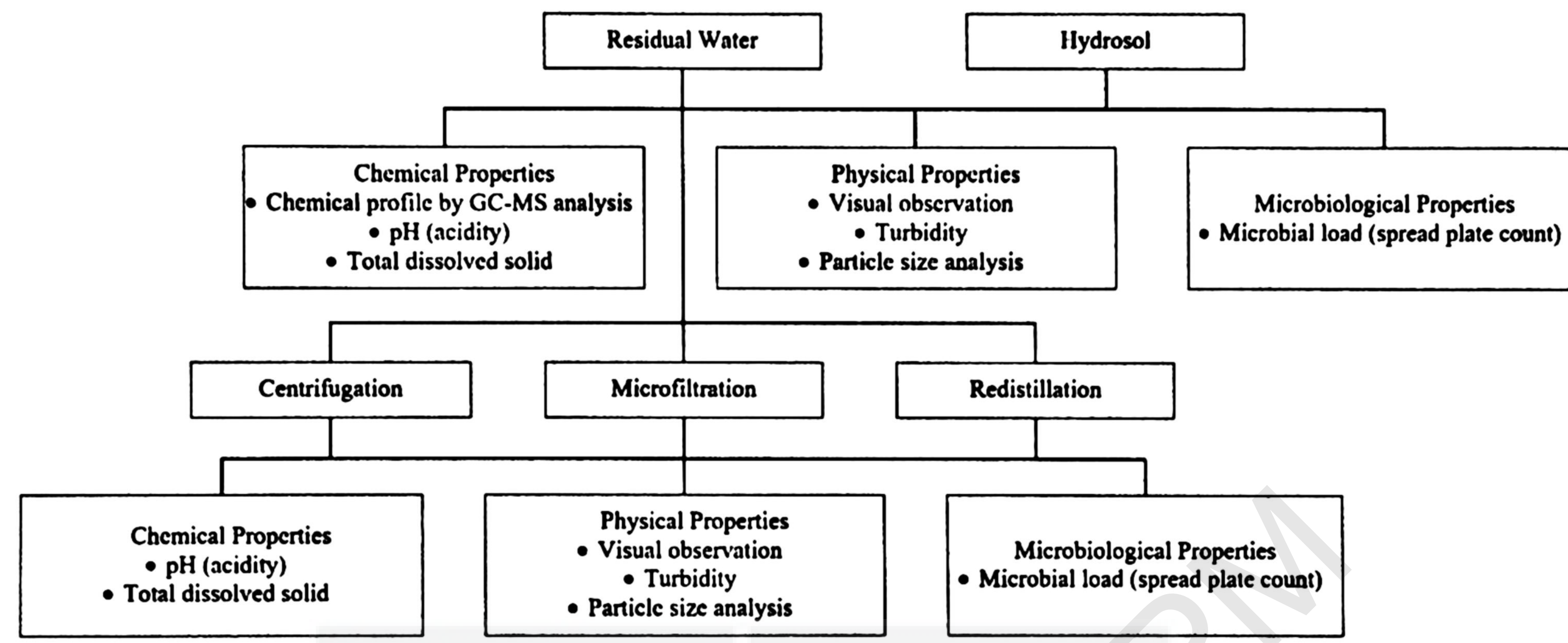

Figure 3.2: Overview of research approach, methods and analysis

Figure 3.2 shows the overview of research approach, methods and analysis in this study. For the first objective, chemical, physical and microbiological properties of agarwood hydrodistillation by-products namely residual water and hydrosol were determined. For chemical properties analysis, the by-products were sent to external laboratory for GC-MS analysis in order to obtain their chemical profiles. Besides, pH and total dissolved solids (TDS) of the by-products were also measured with benchtop pH and TDS meter. For physical properties analysis, particle size analysis was carried out to study the average particle size diameter and polydispersity index of the by-products. For biological properties analysis, microbial load for the by-products were studied by using standard plate count method in order to study their average total plate count. For the second objective, three recovery processes such as centrifugation, microfiltration and redistillation were proposed in order to clarify the residual water. The three properties mentioned above were also determined for the recovered residual water samples. The analysis for each property was almost similar to that stated in the first objective whereby only chemical profile with GC-MS analysis was not carried

out for the recovered residual water samples. The effectiveness of each recovery process was then studied based on the results obtained for the analysis and the one which give an optimum performance was selected and proposed to the industry.

3.3 Storage Condition for the By-products

The agarwood hydrodistillation by-products were collected in plastic containers once they were cooled down after the hydrodistillation process. The by-products contained in the plastics container was sealed with tape and stored in a chiller of temperature between 5 to 8 °C before analysis was carried out. This is to redundant the growth of microbial and fungi which will cause degradation and spoilage of the by-products.

3.4 Recovery Processes

3.4.1 Centrifugation

Centrifugation was carried out by using a laboratory benchtop centrifuge (Hettich Universal 320 Benchtop Centrifuge, Germany) with a capacity of 6 centrifuge tubes per run and each tube has a volume of 50 ml. The residual water was first swirled and mixed evenly to allow homogenous mixture before pouring into the tubes up to 50ml. 1 liter of residual water is required to be centrifuged and hence it has to be carried out in 4 runs with 5 centrifuge tubes per run. It is important to take note that the tubes containing sample have to be placed in proper weightage to prevent error of the machine cause by imbalance of sample. The time and speed of centrifuge were set to be 25 minutes and 9000g respectively. The supernatants formed (Figure 3.3A) after centrifuge process were poured into a sterilized blue cap laboratory bottle and stored

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in a chiller for further analysis whereas the precipitate or known as pellet (Figure 3.4B) remained at the bottom of the tubes were discarded.

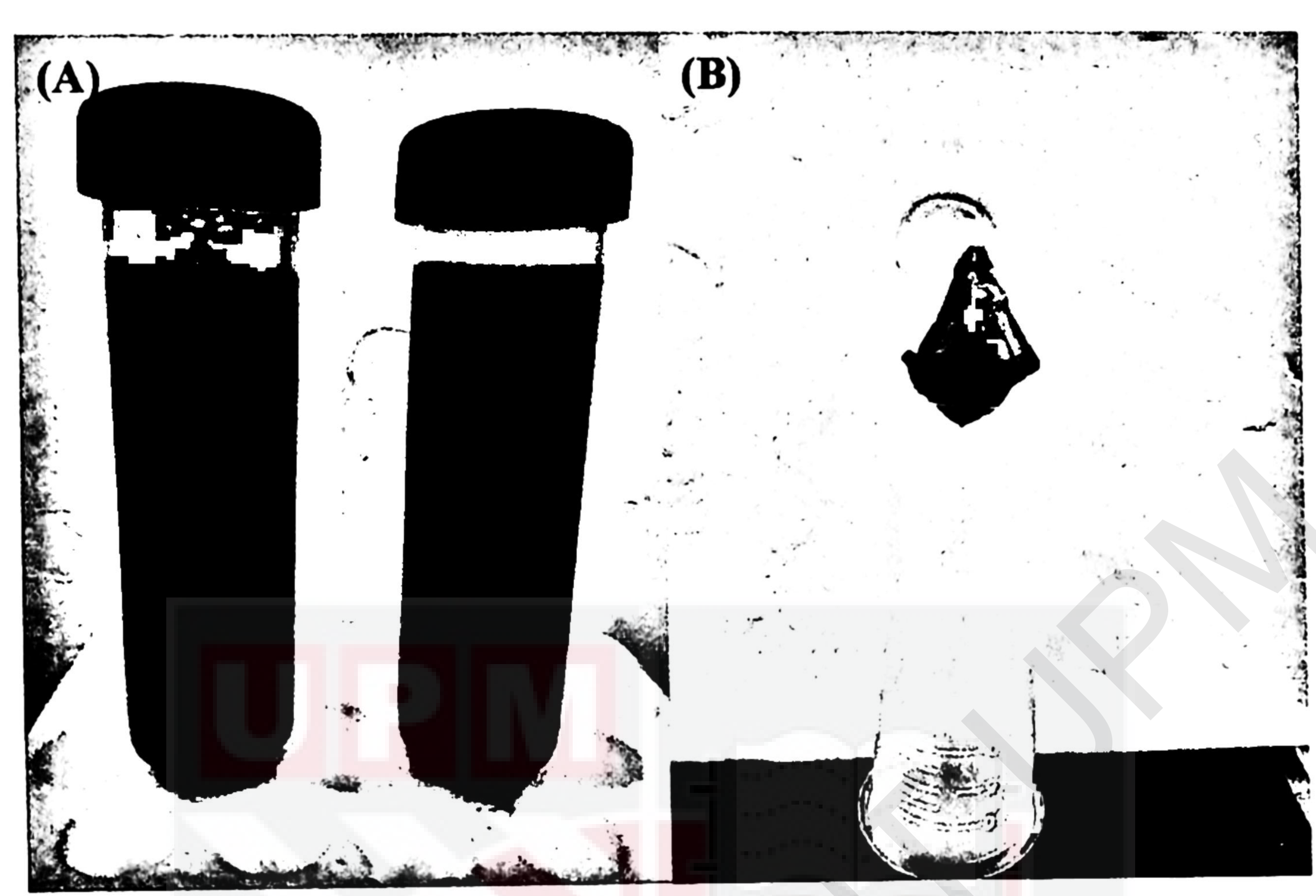

Figure 3.3: (A) Appearance of residual water before and after centrifugation; (B) Precipitate or pellet remained in the centrifuge tube

3.4.2 Microfiltration

In this study, microfiltration of residual water was carried out in laboratory scale by using Acrodisc GHP syringe filter with a membrane of 0.2 μm pore size. Preliminary filtration was performed by using vacuum Buchner funnel set up with Whatman Grade 1 Filter Paper of pore size of 11 μm before microfiltration process. This is to ensure that larger particles such as some of the dirt and tiny agarwood chips which will cause blockage to the microfiltration membrane have been effectively filtered away (Figure 3.4). The microfiltration was then performed by first taking up 50 ml of residual water in a 50 ml syringe and then attached to the 0.2 μm syringe filter. The syringe was pressed manually until all the residual water in the syringe has

been filtered out. The filtered residual water was then poured into a sterilized blue cap laboratory bottle and stored in a chiller for further analysis.

Figure 3.4: Dirt and tiny agarwood chips retained on the filter paper

3.4.3 Redistillation

Distillation apparatus were set up as shown in Figure 3.5. A Clevenger unit was used since the laboratory has insufficient glassware to perform the traditional distillation apparatus set up. Heating mantle was used as heating source and condenser tube with water in and out was used to condense the steam. 1 liter of residual water was poured into a round bottom flask and placed on a heating mantle. The residual water was heated up to its boiling point (approximately 100°C) and its temperature was monitored by using thermometer. When the residual water was boiled and start to evaporate, the opening end of the round bottom flask was connected to the Clevenger unit. Tap water was then allowed to pass through the distillation column to promote the condensation of the evaporated steam. The steam will then be condensed and collected in a conical flask. The distillation process was carried out until 80 % of the

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residual water has been evaporated. The redistilled residual water sample was then poured into a sterilized blue cap laboratory bottle and stored in a chiller for further analysis.

Figure 3.5: Distillation apparatus set up

3.5 Analysis

3.5.1 Chemical Properties

The chemical profile of agarwood hydrodistillation by-products namely residual water and hydrosol were analysed by using GC-MS. The GC-MS column used in this study was HP-5ms (Agilent Technologies; 60 m × 0.25 mm × 0.25 µm, 7-inch cage). The instrument used was a 5977A GC/MSD system (Agilent Technologies) the manufacturer's software (MSD ChemStation) was used. The carrier gas used was helium at a flow rate of 3 mL/min. The oven temperature program used was 60 °C for 10 min then 3 °C/min to 210 °C for 1 min. The run time for residual water and hydrosol were 60 mins and 90 mins respectively. The full GC-MS parameters report can be seen from Figure A-1 in the appendix. The compounds obtained were then matched using

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the NIST 11 library. The pH values and total dissolved solid (TDS) of the by-products and recovered residual water samples were measured by using benchtop pH meter (Sartorius PB-10 pH Meter, Germany) and portable TDS meter (Senz TDS meter, Singapore). The benchtop pH meter was first calibrated by using buffer solution of pH 7 and 4 before measurement. Three repetition of measurements were carried out for both pH and total dissolved solid analysis and average values were obtained.

3.5.2 Physical Properties

The agarwood hydrodistillation by-products and recovered residual water samples were first poured into respective scientific glass vial until they were 3/4 filled. The photos of the vial contained each water samples were snapped by using smartphone and visual observations were discussed based on the photos. Besides, the turbidity values of the water samples were analysed by using turbidimeter (Eutech Instruments TN-100 Turbidimeter, United States). The turbidimeter was first calibrated by the standard solution of 800 NTU, 100 NTU, 20.0 NTU and 0.02 NTU. The water sample was poured into the provided glass vial until the level shown and then placed into the turbidimeter by aligning the vial's index mark with the meter's index mark. The vial is then fully snapped in and covered with light shield cap before analysis was started. Particle size analysis for the water samples were carried out using the zeta/nano analyser (Nanoplus Particulate Systems, United States). The mean particle size diameter and polydispersity index values displayed on the Nanoplus software were recorded. The analyser applied dynamic light scattering (DLS) technique that is based on the Brownian motion of scattered particles. Particles suspended in a fluid are in steady Brownian motion as a result of interaction with the suspended fluid molecules. In Stokes-Einstein theory of Brownian motion theory,

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particle motion of smooth spheres at very low concentration is measured by the viscosity and temperature of the fluid, as well as the particle size. Therefore, the particle size can be calculated from a measurement of the particle motion in a fluid of defined temperature and viscosity. The Brownian motion theory is that particles collide with solvent molecules all the time. These collisions cause the transfer of a certain amount of energy which induces the movement of particles. The flow of energy is more or less constant, and thus has a greater effect on smaller particles. As shown in Figure 3.6, smaller particles are moving at higher speeds than larger particles (International Organization for Standardization, 2017). Three repetition of measurements were carried out for the turbidity and particle size analysis. The particle size analysis will be carried out in two analysis per run therefore average values are computed from 6 sets of reading.

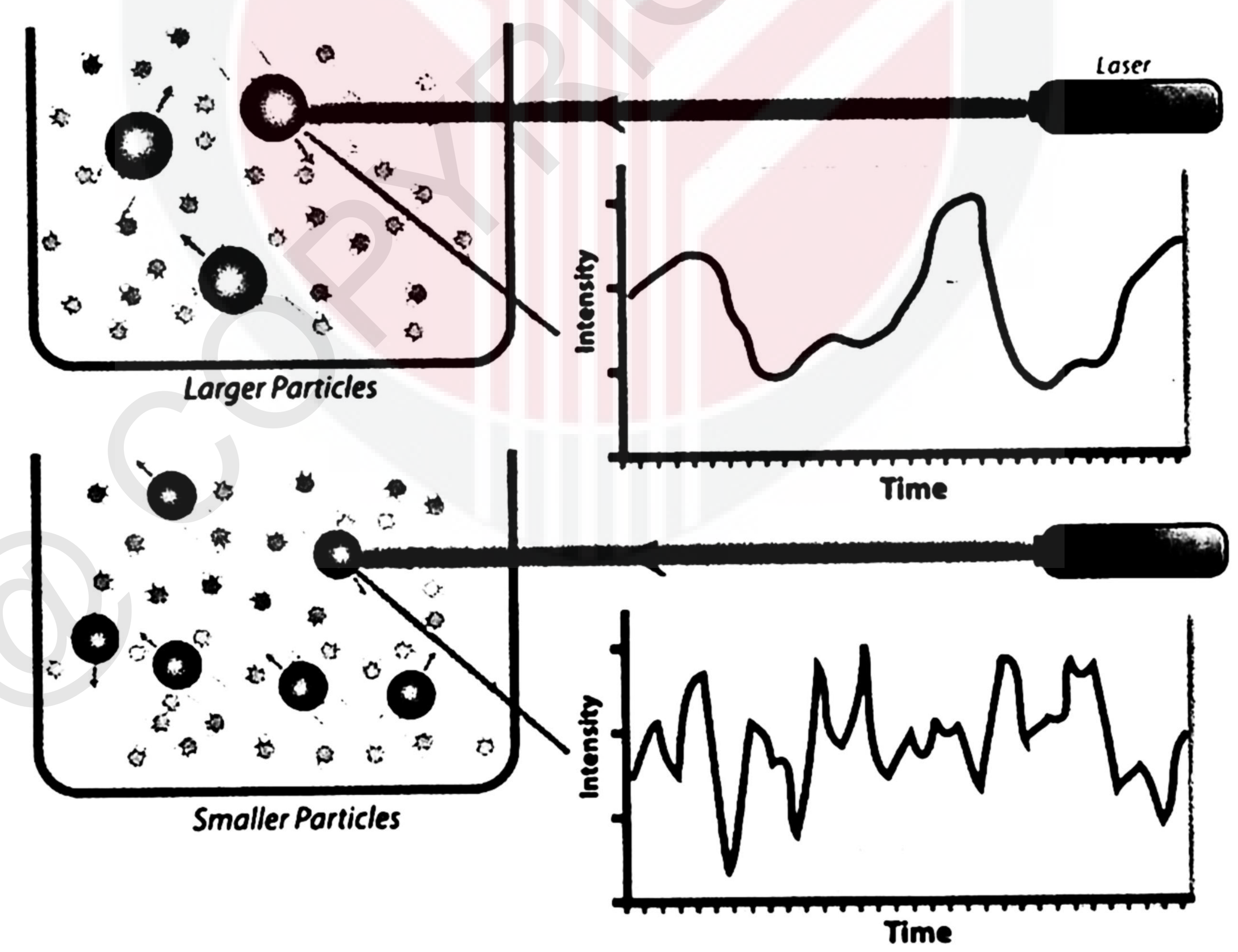

Figure 3.6: Illustration of dynamic light scattering for large and small particle

3.5.3 Biological Properties

Microbial load of the by-products and recovered solutions were studied by applying the spread plate method. The whole process of microbial load analysis was carried out in a laminar flow cabinet (ESCO Laminar Flow Cabinet, Singapore) to prevent any external contamination from the surrounding. Agar plates were first prepared by suspending 23.5 g of Difco Plate Count Agar powder in 1 liter of distilled water and the mixture was mixed thoroughly. The mixture was then heated with frequent agitation and boil for 1 minutes on hot plate with magnetic stirrer to completely dissolve the powder. The mixture was then autoclaved (Hirayama HVE-50 Autoclave, Japan) at temperature of 121°C for a period of 15 minutes. The mixture was then cooled and poured into petri dishes until it was 2/3 filled. The solidified agar plates were then arranged in a row with an upside-down position (agar facing upward) to prevent accumulation of water droplets on the agar which will affect the performance during spread plate count. The plates were then placed into a sterilized plastic and sealed with tape before storing them in a chiller with temperature maintained between 5 to 10°C.

Before performing spread plate method, a serial dilution was carried out as the more concentrate the sample is, the more the bacteria formed in the plate and thus the more difficult the process of calculating the colony formed. The procedure of serial dilution (Figure 3.7) and spread plate method (Figure 3.8) are listed as below (ASTM D5465-93 (1998), 2012).

Procedures for serial dilution:

A series of at least 6 test tubes containing 9 ml of sterile distilled water (Spritzer distillation drinking water) were prepared. Using a sterile pipette, 1ml of sample was

pipetted using a sterile pipette tip in the first tube of the set. It was labelled as 10^1. The contents were mixed well by vortex mixer (WiseMix VM-10 Vortex Mixer, Portugal). 1ml of the sample from first tube was pipetted and transferred to second tube. It was labelled as 10^2. The procedures were repeated with all the remaining tubes and labelled them until 10^5. Step 1 to 5 were carried out for agarwood hydrodistillation by-products and recovered residual water samples with 2 repetitions for each sample.

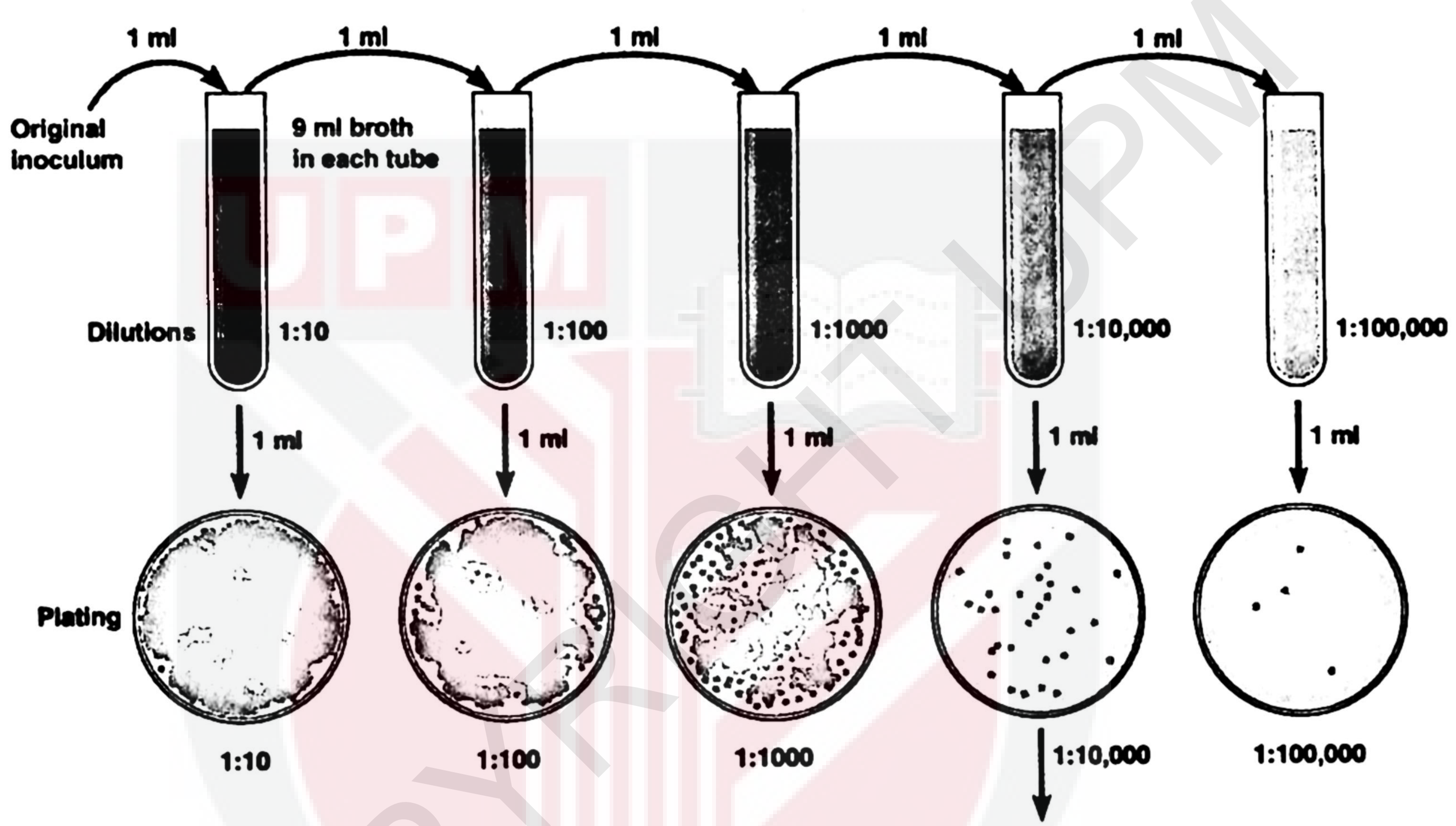

Figure 3.7: Illustration of serial dilution

Procedures for spread plate method:

0.1 ml from the dilution series (10^0 - 10^5) was pipette out onto the centre of the surface of an agar plate. The L-shaped glass spreader (hockey stick) was dipped into alcohol. The glass spreader was flamed over a Bunsen burner. The sample was spread evenly over the surface of agar using the sterile glass spreader, carefully rotating the Petri dish underneath at an angle of 45° at the same time. The plate was incubated at 37°C for 24 hours in an incubator (Memmert Incubator IN55, Germany). Step 1 to 5

were carried out for agarwood hydrodistillation by-products and recovered residual water samples with 2 repetitions for each sample. The colony forming units (CFU) value of the sample was calculated. Once the colonies are counted, multiply it by the appropriate dilution factor to determine the number of CFU/mL in the original sample. An average of total plate count will then be obtained from the results of two repetitions. The log reduction values (LRV) for recovered residual water samples were computed by using the formula:

$$LRV = \frac{Number\ of\ colonies\ formed\ in\ residual\ water}{Number\ of\ colonies\ formed\ in\ recovered\ residual\ water\ samples}$$

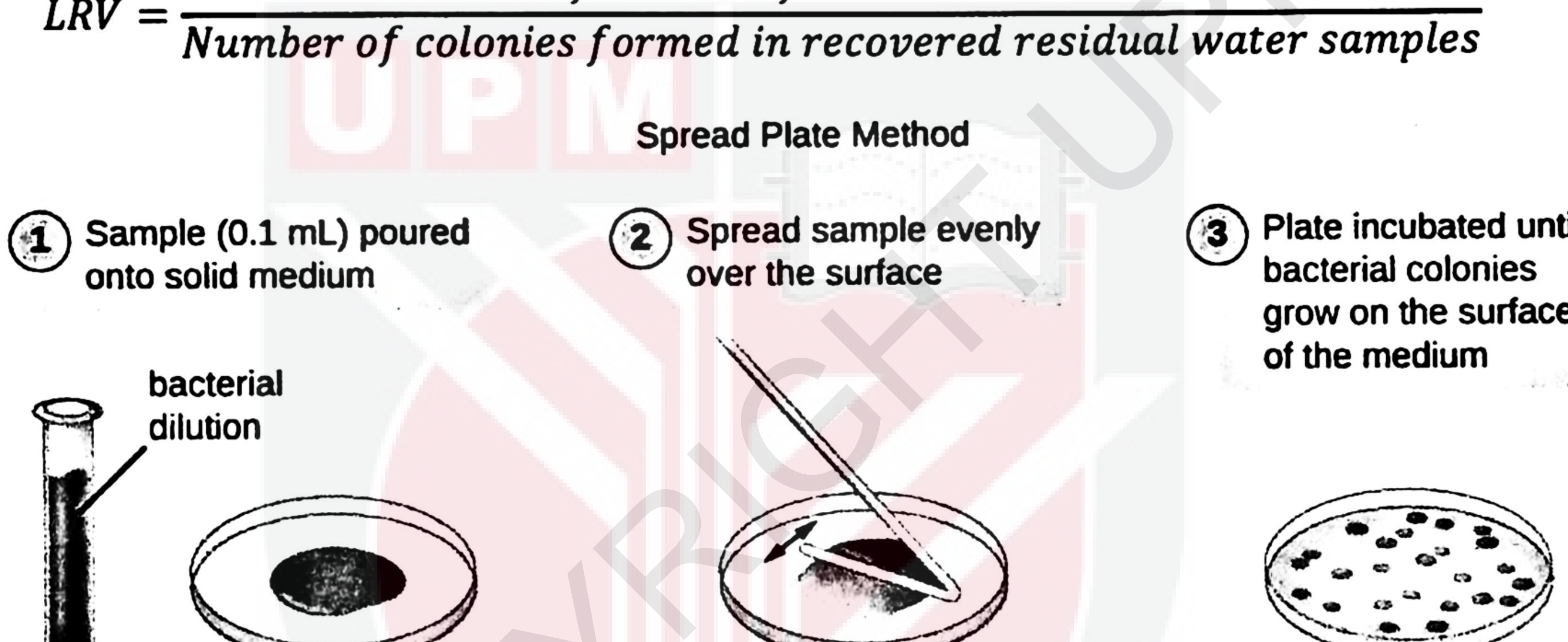

Figure 3.8: Illustration of standard spread plate method

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CHAPTER 4

RESULTS AND DISCUSSION

4.1 Chemical Properties

4.1.1 Chemical Profile by GC-MS Analysis

GC-MS is the analytical method of choice for smaller and volatile molecules like benzenes, alcohols and aromatics and simple molecules like steroids, fatty acids and hormones. It can also be used to analyse liquid, gaseous, and solid samples. The use of GC-MS for compound analysis has many benefits, including the ability to isolate complex mixtures, quantify analytes and assess trace levels of organic contamination (Thermofisher, n.d.).

Table 4.1 and Table 4.2 show the GC-MS chemical profile for the agarwood residual water and hydrosol respectively. Based on the tables, it shows some similarity of chemical compounds presence in the agarwood hydrodistillation by-products such as dimethylsilanediol, methoxy-phenyl-oxime, 4-phenyl-2-butanone or commonly known as benzylacetone. Of these compounds, dimethylsilanediol (DMSD) gives the highest peak in the GC-MS results of residual water and hydrosol. Besides, the blank result obtained by using distilled water has showed a 100 % area of DMSD (blank result can be referred from Figure A-2 and A-3 in appendices). Therefore, it can be confirmed that DMSD is one of the contaminants of the GC-MS system used in this analysis. As stated by Agilent Technologies, (n.d.), contamination is usually identified by excessive background in the mass spectra and it can be originate from the gas chromatography or the mass spectrometry detector. The source of the contamination can be determined by identifying the type of contaminants presence in the mass

spectra. The occurrence of DMSD contaminant can be traced back to the type of column used in this analysis which is the Agilent J&W HP-5ms and it is a (5%-phenyl)-methylpolysiloxane phase (known as polydimethylsiloxanes, PDMS). When PDMS in contact with organic or inorganic material, it will experience abiotic degradation over time to smaller and more water-soluble species. The principal degradation product of PDMS is DMSD, a very water water-soluble compound (Carmichael, 2011). Thus, it can be further confirmed that the highest area percentage given by DMSD is probably due to the column or septum bleeding (Agilent Technologies, n.d.).

Methoxy-phenyl-oxime compound, a nitrogen-containing compound with both phenyl and methoxy groups is found to be presence in both of the agarwood by-products. Based on the research done by Guneser et al. (2017), methoxy-phenyl-oxime is detected by the GC-MS in the fermented olive oil mill waste and it is classified as one of the volatile aromatic compounds that contribute to the odour. However, there is limited information on the flavour characteristics of methoxy-phenyl-oxime (Menotta et al., 2004). Two possible sources will be discussed for the presence of this compound in the agarwood by-products. Some researchers classified it as the contaminant originate from the glue used in solid phase microextraction (SPME) (Grimm & Champagne, 2001). This contaminant could be coming from the previous analysis that carried out associated with SPME where the contaminant can be leftover in GC septa, inlet liner, injection syringes and other part of the GC-MS system. However, the possibility of contamination by this compound is very low since the blank sample result do not show any presence of such compound. Another possibility is that this compound is the secondary metabolite of myxobacteria (Xu et al., 2011) that presence in the agarwood by-products. Besides, this compound also found

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naturally in some food products such as rice cultivars (Bryant & McClung, 2011), reconstituted milk, reduced-fat cheese (Wang et al., 2012) and pickled bamboo shoots (Zheng et al., 2014).

The 4-phenyl-2-butanone or commonly known as benzylacetone detected for both agarwood by-products is a sweet flowery smelling liquid and it is commonly used in manufacturing of soap or perfumes. This compound has been reported to be one of the major chemical constituents presence in the agarwood oil extracted from *Aquilaria* species found in Malaysia (Boon, 2017; Deep & Tajuddin, 2019; Nurlaila et al., 2013; Tajuddin et al., 2016; Tajuddin et al., 2013). Benzylacetone, a type of carboxylic acid derivative is found to be presence in highest percentage among other compounds in agarwood oil, for example, the laboratory extracted agarwood oil has yield 32.1 % of benzylacetone (Tajuddin et al., 2016). Takamatsu and Ito (2020) also reported that the benzylacetone is the major compound found in the hot water extract of agarwood remaining in the distillation flask after distillation (or known as residual water) as it occupied approximately 60 % of the total area of the high-performance liquid chromatography (HPLC) chart. In order to prove that agarotetrol (a chromone derivative) is the main compound that produce low molecular weight aromatic compound (LAC) namely benzylacetone, the purified agarotetrol was heated in a glass vial and its headspace vapor was analysed by SPME-GCMS and the result shows that benzylacetone was highly presence (shown in Figure 1). The structure of agarotetrol was first clarified by Yoshii (1978) and it was then discovered to be widely presence in agarwood regardless of the species or grade (Konishi et al., 1991; Li et al., 2013). Besides, some researchers also detected the presence of major percentage of benzylacetone in the agarwood hydrosol (Gameil et al., 2019; Takamatsu & Ito, 2020).

The study of the hydrosol fraction of agarwood is limited but it is found to be a good source of agarwood LACs (Takamatsu & Ito, 2020).

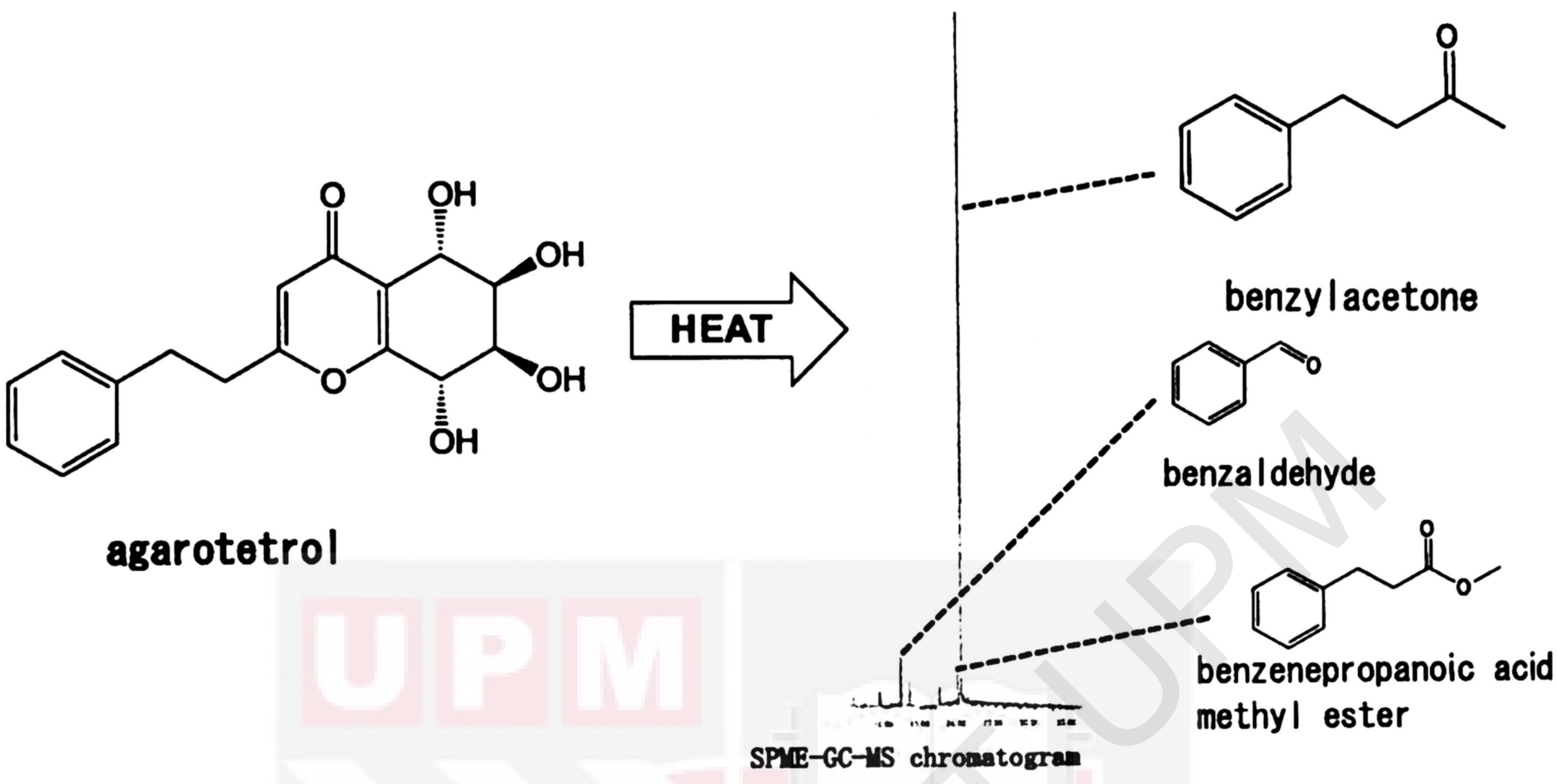

Figure 4.1: LACs generated from agarotetrol upon heating (Takamatsu & Ito, 2020)

Table 4.1: GC-MS analysis for agarwood residual water

Peak Number	Chemical Compound	Chemical Formula	Molecular Weight (g/mol)	Retention Time (min)	Area (%)
1	Acetic acid*	$C_2H_4O_2$	60.05	5.863	20.75
2	Dimethylsilanediol*	$C_2H_8O_2Si$	92.17	6.923	28.42
3	Butanoic acid	$C_4H_8O_2$	88.11	9.250	13.42
4	Methoxy-phenyl-oxime*	$C_8H_9NO_2$	151.16	14.952	17.37
5	4-Phenyl-2-butanone/ Benzylacetone*	$C_{10}H_{12}O$	148.2	34.076	12.77
6	2,2,5,5,8,8-Hexamethyltricyclo [4.3.0.07,9]nonane	$C_{15}H_{26}$	206.37	59.999	7.28

* Compounds with quality (confidence measure) ≥ 70 %

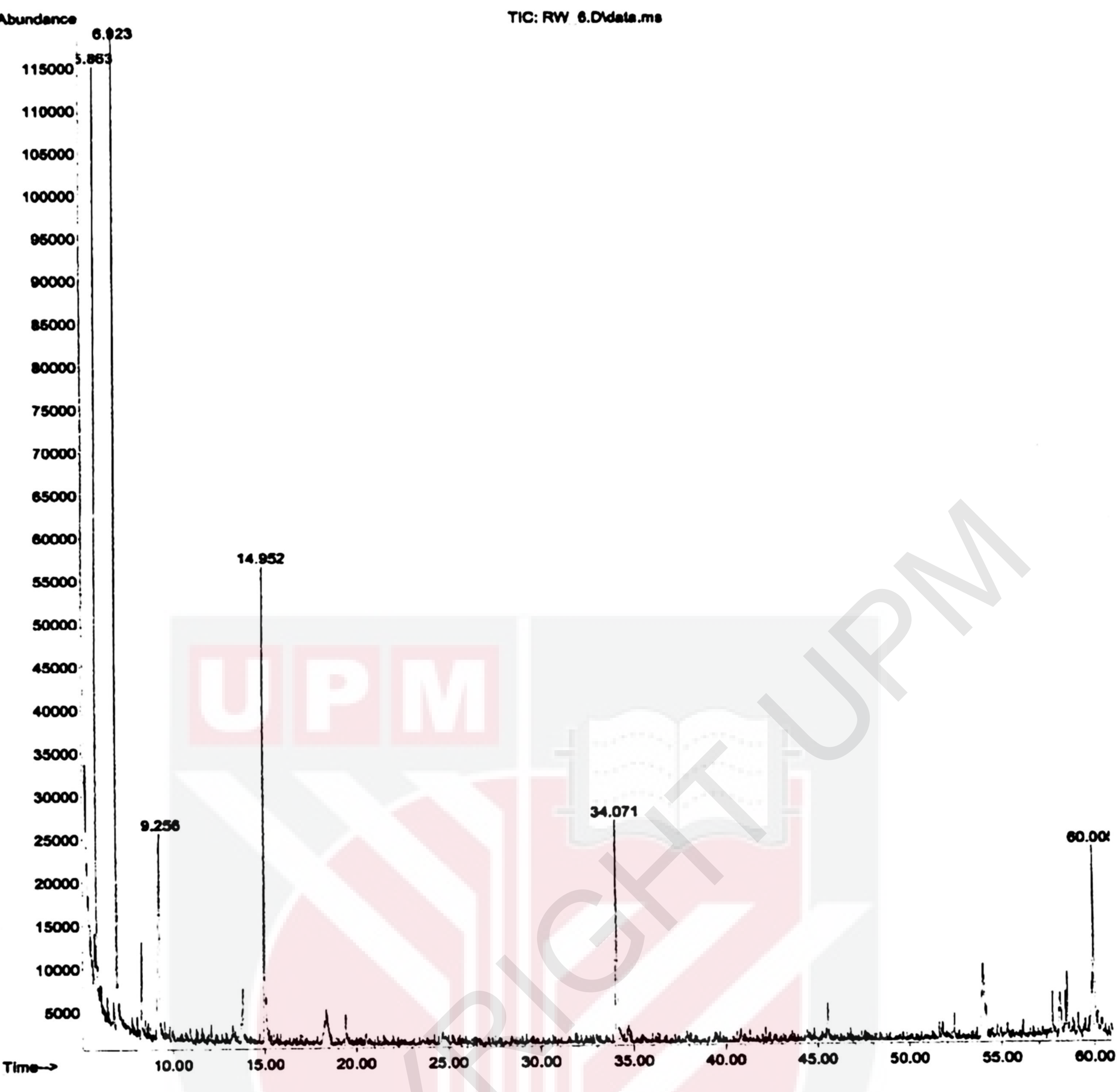

Figure 4.2: GC-MS chromatogram for agarwood residual water

By referring to the results of residual water in Table 4.1 and Figure 4.2, after excluding the peak for DMSD contaminant, it shows that acetic acid is found to give the highest peak followed by methoxy-phenyl-oxime, butanoic acid, benzylacetone and lastly 2,2,5,5,8,8 hexamethyltricyclo[4.3.0.07,9]nonane. Acetic acid and butanoic acid are known as carboxylic acid, derivatives of hydrocarbons in which one or more of the hydrogen atoms in the hydrocarbon have been replaced by a carboxyl group (Badea & Radu, 2018). Xu et al (2011) has claimed that acetic acid, butanoic acid and methoxy-phenyl-oxime (mentioned previously) were presence in the volatile compounds released by myxobacteria. Myxobacteria are identified as Gram-negative bacteria which are well-known for their gliding behaviour and ability to form fruiting

bodies upon starvation conditions (Reichenbach, 2001). Besides, acetic acid is known to be produced and excreted by certain bacteria notably acetic acid bacteria. It has a smell of overripe fruit and it is produced naturally as fruits and some other foods are spoiled (NCBI, 2020a). Butanoic acid might be the product of anaerobic bacteria and it has a taste somewhat like a butter and unpleasant odour. It is a fatty acid occurring in the form of esters in plant oils and animal fats (NCBI, 2020b). Therefore, it can be assumed that the pungent and strong smell given by the residual water is due to the presence of these acids. Tricyclo[4.3.0.0(7,9)]nonane,2,2,5,5,8,8-hexamethyl-,(1.alpha.,6.beta.,7. alpha.,9.alpha.)- or known as 2,2,5,5,8,8 hexamethyltricyclo[4.3.0.07,9]nonane has given the smallest peak among all and appeared at the longest retention time. There is no information to show the presence of such compounds in any agarwood related products. However, the same compound is detected in the analysis of other essential oil particularly *tasmannia lanceolata* extracts (Wright et al., 2017).

Table 4.2: GC-MS analysis for agarwood hydrosol

Peak Number	Chemical Compound	Chemical Formula	Molecular Weight (g/mol)	Retention Time (min)	Area (%)
1	Dimethylsilanediol*	$C_2H_8O_2Si$	92.17	7.181	41.04
2	Methoxy-phenyl-oxime	$C_8H_9NO_2$	151.16	15.506	5.16
3	4-Phenyl-2-butanone/ Benzylacetone	$C_{10}H_{12}O$	148.2	40.640	23.51
4	2-Methyl-3-phenylpropanol*	$C_{10}H_{14}O$	150.22	41.613	23.19
5	4-Hydroxy-3a,7a-dimethyl-4,5-dihydro-3H-2-benzofuranone	$C_{10}H_{14}O_3$	182.22	67.547	7.11

* Compounds with quality (confidence measure) ≥ 70 %

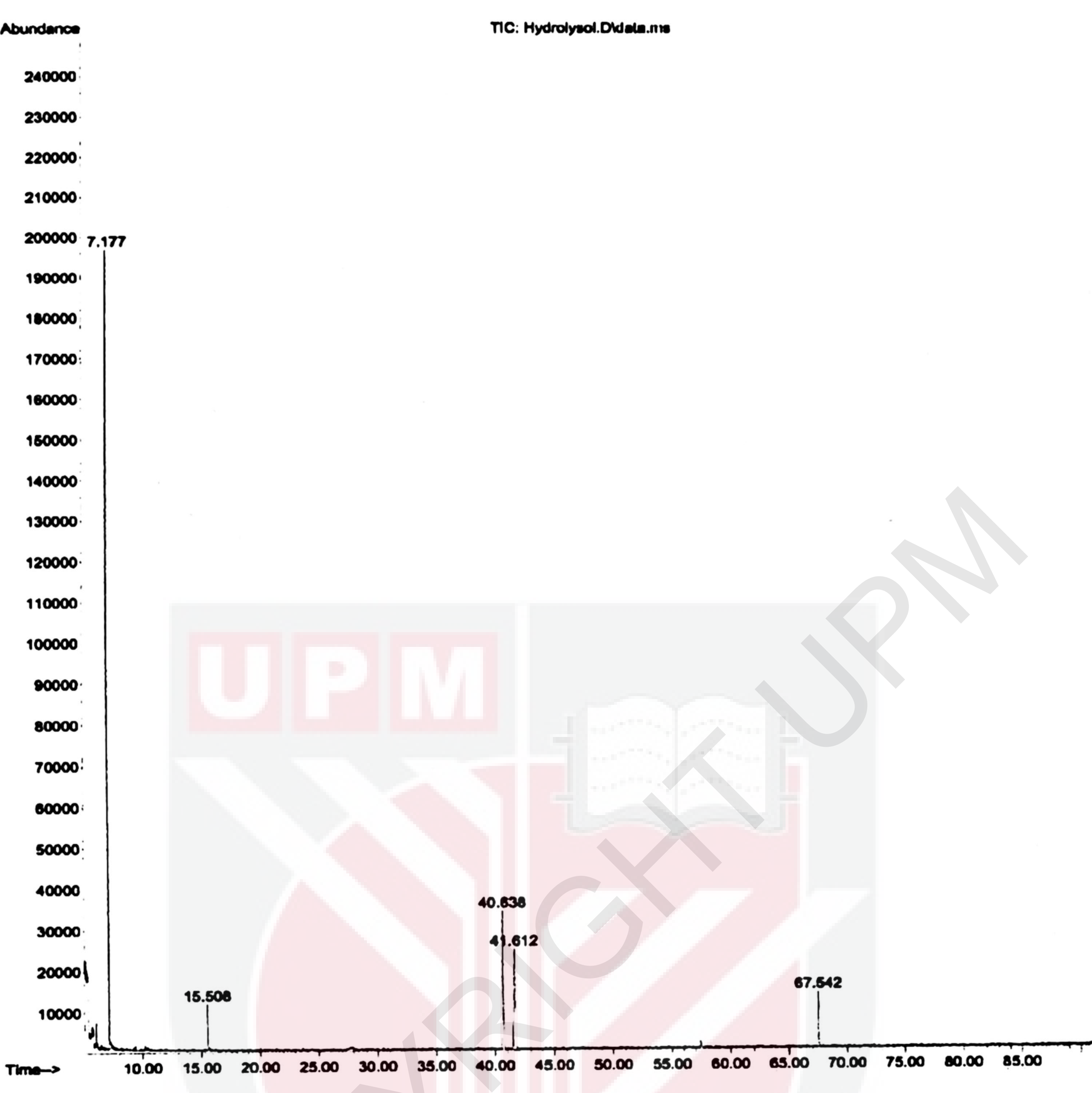

Figure 4.3: GC-MS chromatogram for agarwood hydrosol

By referring to the results of hydrosol in Table 4.2 and Figure 4.3, it shows that other than the DMSD contaminant, the highest peak is given by benzylacetone, followed by 2-methyl-3-phenylpropanol, 4-hydroxy-3a,7a-dimethyl-4,5-dihydro-3H-2-benzofuranone and lastly methoxy-phenyl-oxime. Since the benzylacetone is the major constituent, therefore it is assumed to be the compound that give arise to the sweet smell of agarwood hydrosol. Benzylacetone is known to be potent sedative when inhaled (Miyoshi et al., 2013; Takemoto et al., 2008). Benzenepropanol, .beta.-methyl- or known as 2-methyl-3-phenylpropanol also shows a significant area in the mass spectra of hydrosol. It is predicted as a metabolite that is produced by the metabolism of 2-methyl-3-phenylpropanal (NCBI, 2020c). There is no information to show this

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compound belongs to any agarwood product but the 2-methyl-3-phenylpropanal does show existence in *Ballota nigrafrom* essential oil from Serbia. The presence of small amount of 1(3H)-isobenzofuranone, 3a,4,5,7a-tetrahydro-4-hydroxy-3a,7a-dimethyl-, (3a.alpha.,4.beta.,7a.alpha.)-(.+/-.)- or known as 4-hydroxy-3a,7a-dimethyl-4,5-dihydro-3H-2-benzofuranone that appeared at the longest retention time also found to have no relationship with any agarwood product.

It can be concluded that no agarwood significant compounds (sesquiterpene or terpenoid) were found in agarwood hydrodistillation by-products. Based on the previous research carried out in collaboration with industry X, the agarwood oil that hydrodistilled in laboratory with different parameters has showed major constituents such as benzylacetone, 7-epi-γ-eudesmol, β-eudesmol, α-eudesmol, kusunol, epi-α-bisabolol, rotundone and n-hexadecanoic acid. However, in this study, only benzylacetone is found to be presence in both of the by-products. This could probably due to that all of the agarwood significant compounds have been extracted into oil fraction as the sesquiterpenes or terpenoids are known to have high volatility. Another possible reason is that even though there are presence of agarwood significant compounds in the by-products, but the quantity should be relatively low and they might be degraded or decomposed due to the long period of storage. The agarwood by-products have been stored in refrigerator for more than 3 months before the analysis is conducted as due to the announcement of Movement Control Order in response to the outbreak of COVID-19.

4.1.2 pH (Acidity)

pH is a measure of the relative amount of free hydrogen and hydroxyl ions in the water. Water that has more free hydrogen ions is acidic, whereas water that has

more free hydroxyl ions is basic. pH is an important indicator of water that is changing chemically since it can be affected by chemicals in the water (USGS, n.d.). The pH can control the availability of nutrients, biological functions, microbial activity, and the behaviour of chemicals (APERA Instruments, 2017). In this study, pH of the agarwood hydrodistillation by-products such as residual water and hydrosol as well as the recovered residual water were measured and tabulated in Table 4.3. The recovered residual water samples mentioned are the residual water which undergoes different types of recovery processes such as centrifugation, microfiltration and redistillation.

Based on the results obtained, it shows that Spitzer distillation drinking water which acts as the controlling variable has an almost neutral pH value. The agarwood hydrodistillation residual water shows a pH value of 6.40 ± 0.08 which indicates that it is slightly acidic whereas the agarwood hydrosol with a lower pH value of 4.26 ± 0.04 has a higher acidity compared to the residual water. By comparing the pH results of recovery processes, it can be observed that the physical separation processes such as centrifugation and microfiltration have an insignificant effect to the pH whereas the redistillation process which involves boiling and condensation process has reduced the pH value of residual water to 4.39 ± 0.03 which is considered to be acidic.

Table 4.3: pH values for different types of water samples

Types of Water Samples	pH
Spritzer Distillation Drinking Water (control)	7.02 ± 0.04
Hydrosol	4.26 ± 0.04
Residual water	6.40 ± 0.08
Centrifuged RW	6.41 ± 0.03
Microfiltration RW	6.43 ± 0.03
Redistilled RW	4.39 ± 0.03

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The agarwood residual water appears to be slightly acidic as the GC-MS result has profiled the presence of weak acid such as acetic acid and butanoic acid. The acidic properties of agarwood hydrosol will be discussed more detailed in this section instead of residual water as there is insufficient reference related to the agarwood residual water. As reported by Catty (2001), hydrosols have a wide range of pH but are always acidic, ranging from a low of 2.9 to a high of around 6.5 depending on the types of plant used. The pH value documented for woody plant hydrosol such as sandalwood hydrosol is in the range of 5.9 - 6.0 whereas the cedarwood hydrosol is in the range of 4.1 - 4.2 (Catty, 2001). Based on the research done by Abdullah et al. (2010), it shows that all the agarwood hydrosol samples obtained from two different extraction facilities have displayed acidic nature, with pH in the range of 3.62 - 4.53. The factors that affect the shelf life of a hydrosol are its botanical specific, pH level, conditions of distillation process, techniques of handling and bottling and the storage conditions (Aromaweb, n.d.). As a very broad rule of thumb, Catty (2001) stated that hydrosols with a pH under 5.0 have a shelf life up to two years whereas they will be good for 12 to 18 months with pH over 5.0.

The hydrosols with acidic nature are suitable for manufacturing great toners because they are able to restore the acid mantle of skin after alkaline soap cleansing. This can ensure that the skin is protected from bacteria that are not favourable to live in acidic condition. The alcohol-free toner made from hydrosol can act as a substitute for those who do not prefer alcohol in their toner, since alcohol will since alcohol removes too much of the acid mantle and causing the pores to overproduce oil in an effort to rebalance the skin. Another benefit of applying hydrosol as toner is that it contains natural fragrances whereby other toners might contain mainly synthetic fragrances which can be possible irritants to the skin.

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4.1.3 Total Dissolved Solids (TDS)

Both inorganic and organic substances found in water that can pass through a 2-micron filter are known as total dissolved solids (TDS). This includes anything present in water other than the pure H_2O molecules. TDS, in general, is the sum of the water cations and anions. Ions and ionic compounds that make up TDS are typically carbonate, bicarbonate, chloride, fluoride, sulfate, phosphate, nitrate, calcium, magnesium, sodium, and potassium, but any ion present will contribute to the total. TDS is a non-specific quantitative indicator of the quantity of dissolved inorganic chemicals but does not provide any information about its nature. TDS is not known to be a primary pollutant with any related health effects in human drinking water standards, but rather is used as an indication of the aesthetic characteristics of drinking water and as a broad indicator of a variety of chemical contaminants (University of Wyoming Extension, n.d.).

The values of total dissolved solids for each water sample were measured in the unit of ppm and tabulated in Table 4.4. The table shows that the Spitzer distillation drinking water which act as the controlling variable is having a TDS value of 0 ppm. Wecofilters (n.d.) has stated that distilled water normally has a TDS of 0 ppm due to the purity. The function of this control variable is to ensure the accuracy and reliability of the portable TDS meter as well as the experimental data obtained. By comparing the results for agarwood hydrodistillation by-products, it shows that residual water has a very high value of TDS which is 435 ± 1 ppm when compared to hydrosol with a value of only 4 ± 1 ppm. Among all recovery processes, it shows that redistillation has the highest effectiveness in removing the TDS as it gives a value of 12 ± 1 ppm. Centrifugation process shows a moderate result as it is only able to remove a small

amount of TDS which gives the value of 403 ± 1 ppm. However, microfiltration shows the least effectiveness since it has an insignificant effect on the removal of TDS as it gives a value of 433 ± 2 ppm which is still within the TDS range of residual water.

Table 4.4: Average total dissolved solids (ppm) for different types of water samples

Types of Water Samples	Average Total Dissolved Solids (ppm)
Spritzer Distillation Drinking Water (control)	0
Hydrosol	4 ± 1
Residual water	435 ± 1
Centrifuged RW	403 ± 1
Microfiltration RW	433 ± 2
Redistilled RW	12 ± 1

Since all the organic compounds are considered to be total dissolved solids, therefore the useful water-soluble chemical compound can contribute to the TDS value. However, in this study, it is important to retain all the organic substances as they might be the useful water-soluble compounds that originate from the agarwood. Thus, in term of retaining the original quality of residual water, it shows that microfiltration has the best performance followed by centrifugation and redistillation. Cole-Parmer (2017) has claimed that one of the disadvantages of membrane filtration is that it does not remove dissolved inorganic or organic substances. Centrifugation process can separate suspended solids effectively, but cannot be expected to remove dissolved solids (Records & Sutherland, 2001). Cole-Parmer (2017) also stated that distillation is advantageous in removing all types of contamination except dissolved gases and organic compounds with boiling points below 100 °C. Both the distillation products such as hydrosol and redistilled residual water still contain a small amount of TDS as the effectiveness of distillation in removing organic compounds varies, depending on

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boiling point of the organic compound such. Organic compounds that boil at temperatures greater than the boiling point of water can be effectively removed from the water. Organic compounds that boil at temperatures lower than the boiling point of water will be vaporized along with the water (Kamrin et al., 1990)

4.2 Physical Properties

4.2.1 Visual Observation

Visual observation is a crucial step for determining physical properties of water samples as it provides the information on physical appearance which enables the ease of preliminary analysis. In this experimental analysis, there were three parameters studied for the visual observation which are the colour, visible suspended solid particles and clarity. Two terms are used to describe the colour of water sample which will be the true and apparent colour. 'True colour' is the colour of the sample after the removal of particulate matter whereas 'apparent colour' is the colour resulting from the combined effect of true colour and any particulate matter, or turbidity. In turbid water samples, the true colour is substantially less than the apparent colour (AWWA, 1990). Suspended solids are defined as the amount of fairly small solid particles that stay suspended in water and behave as a colloid due to the water's motion or because the particle density is lighter or equal to the water (Grundfos, n.d.). Water clarity is a physical feature defined by the transparency of the water and it is a subjective measurement that usually determined by human observation. The transparency of water is affected by the amount of sunlight available, suspended particles in the water and dissolved solids such as coloured dissolved organic material (CDOM) present in the water (Kemker, 2014).

The images of the hydrodistillation by-products (residual water and hydrosol) and their respective visual observations are shown in Table 4.5. Based on the table, it can be observed that there is a huge contrast between the colour, visible suspended solid particles and clarity for both residual water and hydrosol. This is due to that both of the by-products are obtained from different parts of the hydrodistillation apparatus

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set up. Agarwood hydrodistillation residual water is known to be a dark coloured thick water that retained in the distillation pot with all the agarwood chips contained within. The residual water was only decanted to remove larger agarwood chips and collected in a container. The dark brown colour of the residual water can be known as the apparent colour as it still contains particulate matters and the source of colour might mainly due to the presence of coloured dissolved organic matter (CDOM) which originate from decaying agarwood chips. The presence of large amount of visible suspended solids is due to the improper separation process carried out by the worker. On the other hand, agarwood hydrosol is known to be the clear distillate product of the hydrodistillation process which was obtained when the steam condensed into liquid together with agarwood oil and collected in the separating funnel at the end of the process.

Table 4.5: Visual observations of both hydrodistillation by-products

Types of Hydrodistillation By-Products	**Visual Observations**
Residual Water	Dark brown coloured murky and opaque solution concentrated with large quantity of visible suspended solid particles such as agarwood debris and other impurities. Some denser particles were observed to be deposited at the bottom of the solution.

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Hydrosol	Clear and transparent colourless solution without any visible suspended solid particles.

The images of the recovered residual water samples and their respective visual observations are shown in Table 4.6. Based on the table, it can be observed that the visible suspended solid particles are effectively reduced and the clarity is significantly improved after each recovery process. The clear yellowish-brown colour of the solution after microfiltration is known to be the true colour as the particulate matter has been removed. Although the brown colour of residual water can be retained after both centrifugation and microfiltration process but generally microfiltration gives a better result compared to centrifugation as in term of clarity and the removal of suspended solid particles. However, the redistilled residual water was unable to retain its original colour as it has transformed into colourless liquid after the process but it does show best result in terms of clarity and the removal of suspended solid particles. The redistilled residual water has a similar visual appearance as the hydrosol as they are both the product of distillation. It can be said that the dissolved organic matters which give the brown colour of the residual water are non-volatile compounds as they do not evaporate during the distillation process and hence the products will appear to be colourless solution. By comparing the visual appearances of all recovered water samples, the recovery process which gives an optimum performance among others is

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microfiltration. This is because the microfiltration process is not only capable to clarify the residual water but it also retains the original appearance and character of the residual water. The comparison of physical appearances for all water samples can be observed from Figure 4.4 for a better illustration.

Table 4.6: Visual appearances of different types of recovered residual water samples

Types of Recovered Residual Water Samples	**Visual Observations**
Centrifuged RW	Light brown coloured solution with cloudy characteristic. Clumps of cloudy suspension were observed to be floating in the middle of the solution. Visible suspended solid particles were significantly reduced and the solution has a moderate clarity after centrifugation process.
Microfiltration RW	Clear and transparent yellowish-brown solution without any visible solid suspended particles or cloudy suspension. The solution has a better clarity compared to centrifuged solution.
Redistilled RW	Clear and transparent colourless solution without any visible suspended solid

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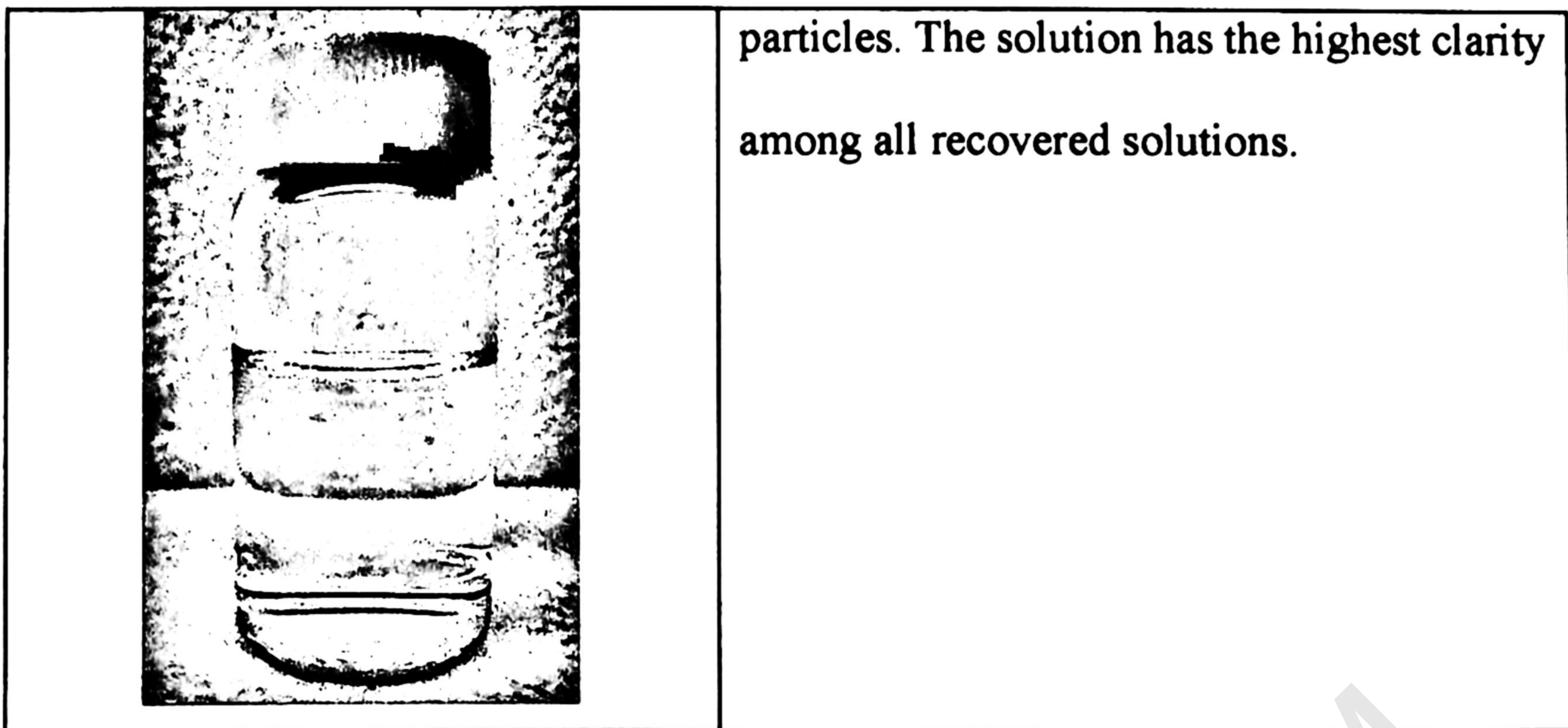

particles. The solution has the highest clarity among all recovered solutions.

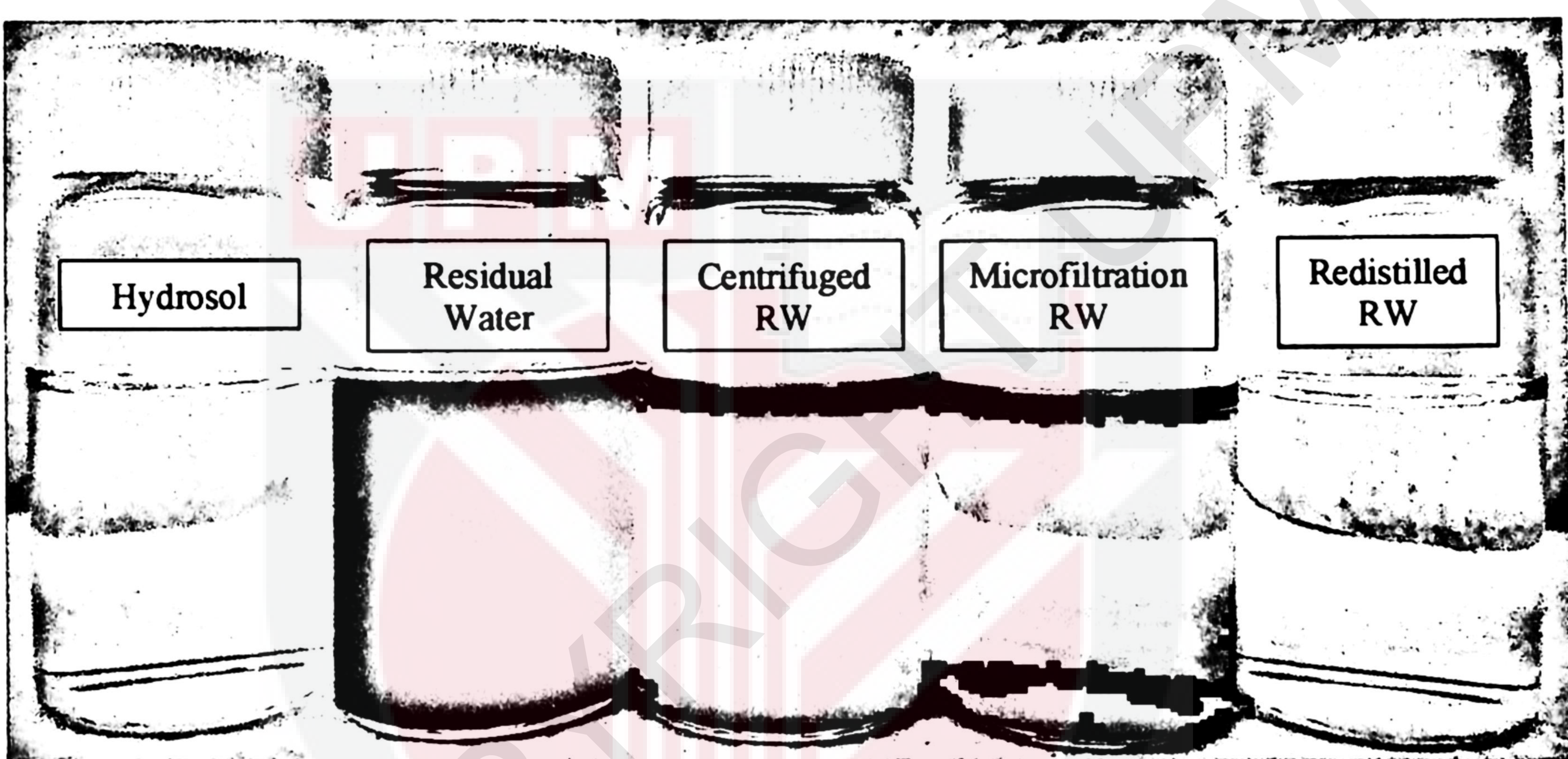

Figure 4.4: Physical appearances for different types of water samples

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4.2.2 Turbidity

Turbidity is the water 's capacity to disperse and absorb light, which prevents its transmission in depth. It involves the presence of fine suspended matter and dissolved matters. Such solids are often classified into three main classes: clays, organic particles resulting from decomposition of animals and plants debris decomposition, and fibrous particles (Favas et al., 2016). Turbidity is a variable measured in situ, and is specifically related to the visual appearance of water as it is a measure of the degree to which water loses its transparency caused by the presence of

suspended particles (Patel & Vashi, 2015). However, turbidity should not be confused with colour, nor colour with turbidity (Woodard, 2001). Nephelometric turbidity units (NTU) is the unit measurement for turbidity and can be initially observed by the naked eye above approximately 4.0 NTU (WHO, 2011).

The average turbidity values for different types of water samples are shown in Table 4.7. Based on the results obtained, it shows that the Spritzer distillation drinking water has given the lowest turbidity value which is 0.02 ± 0.02 NTU. As stated by WHO (2011), drinking water should have a turbidity of 5 NTU or less but it should be no more than 1 NTU and preferably much lower to ensure effectiveness of disinfection. Spritzer distillation drinking water is hence selected to be the control variable as it meets the drinking water quality set by WHO, therefore it is able to show a contrast when comparing to other water samples. Besides, it also functions to validate the accuracy and reliability of the turbidimeter and experimental data obtained.

As mentioned earlier, the visual appearance of the water sample is dependent on the turbidity value. By comparing the turbidity value for both agarwood hydrodistillation by-products, it shows that the residual water has an enormously high value which is 586 ± 5 NTU when compared to hydrosol with the value of 0.53 ± 0.02 NTU. This could provide the explanation for the huge contrast between their visual appearance as "crystal-clear" water has a turbidity of <1 NTU; at 4 NTU and above, water becomes visibly cloudy; at 500 NTU and above, water becomes completely opaque (World Health Organization, 2011). The reason of high turbidity value in residual water is probably due to the presence of high amount of suspended solids, microorganisms and coloured dissolved organic matter (CDOM). CDOM is also known as humic stain which can be referred to the tea colour produced from decaying

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plants and leaves in water due to the release of tannins and other molecules (Kemker, 2014). Besides, the more total suspended solids presence in the water, the murkier and chalkier it appears and the greater the turbidity (Patel & Vashi, 2015). On the contrary, it shows that hydrosol could be classified as drinking water as it meets the turbidity level for drinking water quality stated by WHO which is less than 1 NTU for filtered and disinfected water.

Table 4.7: Average turbidity values for different types of water samples

Types of Water Samples	**Average Turbidity (NTU)**
Spritzer Distillation Drinking Water (control)	0.02 ± 0.02
Hydrosol	0.53 ± 0.02
Residual water	586 ± 5
Centrifuged RW	34.0 ± 0.9
Microfiltration RW	6.56 ± 0.02
Redistilled RW	0.63 ± 0.04

As from the information provided by an employee from industry X, the residual water is commonly discharged as effluent into the drain. However, it is important to note that the agarwood hydrodistillation residual water with high turbidity level (> 500 NTU) should not be discharged directly to the streams as effluent because high concentrations of suspended solid particles will affect light penetration, ecological productivity, recreational values, and habitat quality in the rivers, lakes or oceans. In terms of water quality, the presence of high levels of total suspended solids will increase water temperatures and reduce dissolved oxygen (DO) levels. This is due to that the heat from solar radiation is mostly absorbed by the suspended particles and the heat is then transferred to the surrounding water. Higher temperature water cannot hold as much dissolved oxygen as lower temperature water, therefore DO levels will be reduced in this situation. In addition, stratification or layering (lower and upper

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water layers are formed) of a body of water might occurred as the surface temperature of water increases. The lower layer can turn out to be too hypoxic (low DO levels) for organisms to survive since decomposition and respiration often occur in the lower layer. Turbidity can also obstruct photosynthesis process of aquatic plants by hindering the sunlight as the higher the turbidity level, the lesser the light that can penetrate to the lower level of water. Reduced photosynthesis will cause a decrease in plant survival and decreased dissolved oxygen output (Kemker, 2014).

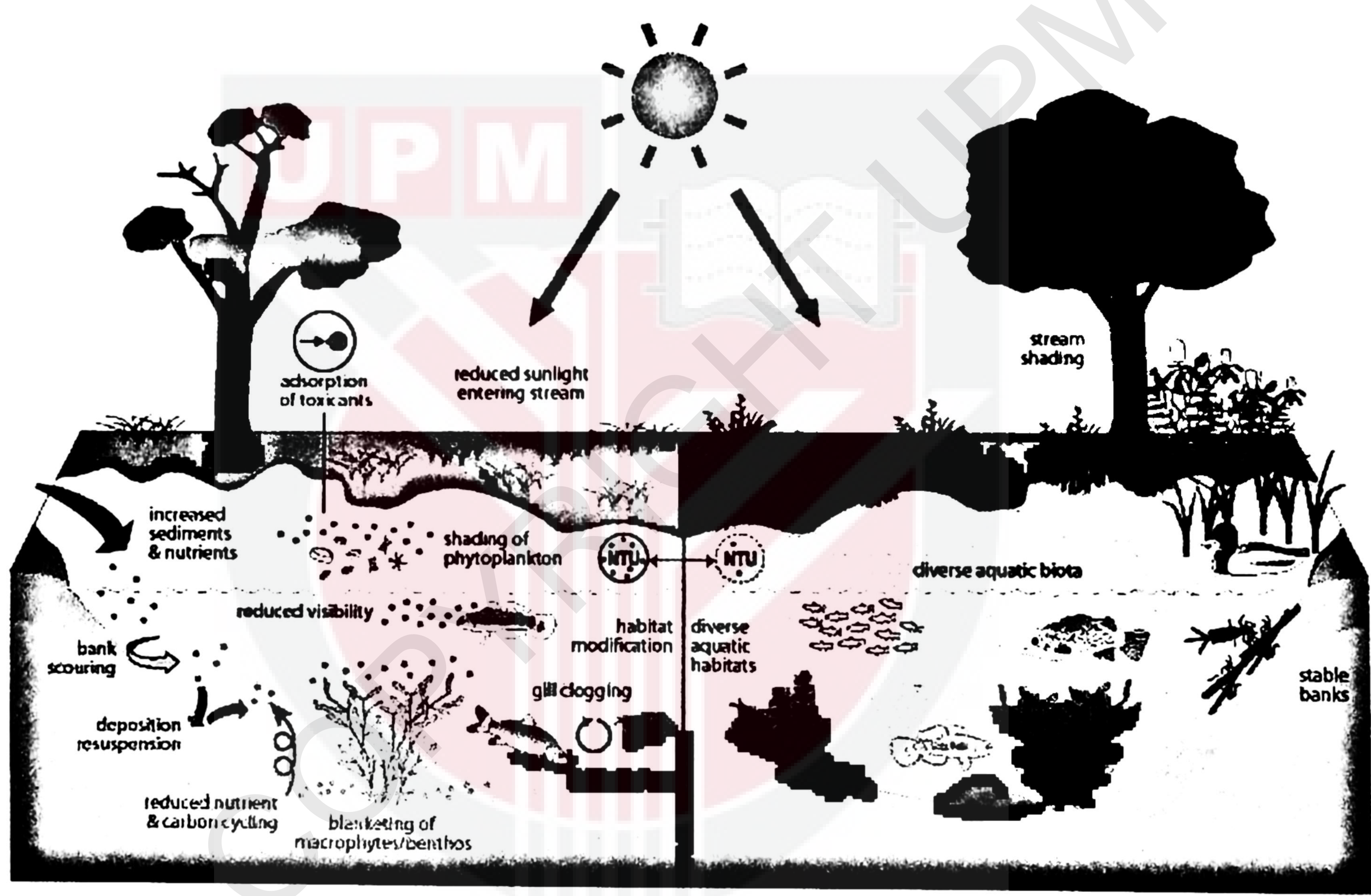

Figure 4.5: Effects of high water turbidity to the ecology (Queensland Goverment WetlandInfo, 2013)

Besides, pollutants such as pathogens and dissolved metals could attach to suspended particles and blend with the water. This is the reason why an increase in turbidity will not just causing a decrease in water quality but often indicate as potential water pollution. The addition of nutrients from the source of turbid water will promote the growth of harmful algal blooms. The presence of bacteria, protozoa and viruses in

water are more likely to be occurred when the suspended solids concentration is due to organic materials, particularly decaying organic matter. These organic suspended solids will probably decrease dissolved oxygen levels when they are decomposed. CDOM can cause water to appear as red or brown colour, depending on the type of plants or leaves presence in the water. These dissolved substances may be too small to be counted in a suspended solids concentration, but they are still part of a turbidity measurement as they affect water clarity (Kemker, 2014). The overall effects of high turbidity in water to the ecology are summarised and illustrated in Figure 4.5. Hence, agarwood hydrodistillation residual water must undergo certain water treatment techniques to comply with the standard value of discharge that has been set by the Malaysia's Environmental Law, Environmental Quality Act 1974 (Legislation Federal Subsidiary, 1979). Alternatively, it could be further processed and utilized in fragrance products only after a proper recovery or clarification process.

The percentage of turbidity reduction for each recovery process is calculated and tabulated in Table 4.8. Based on the results, it can be observed that there is a significant reduction for the turbidity levels after each recovery process. The recovery process which gives the lowest turbidity value is redistillation (0.63 ± 0.04 NTU), followed by microfiltration (6.56 ± 0.02 NTU) and lastly centrifugation (34.0 ± 0.9 NTU). The effectiveness of each recovery process can be clearly studied based on the percentage of turbidity reduction. It shows that redistillation process has the highest effectiveness as it reduces the turbidity level of residual water up to 99.89 %, followed by microfiltration with 98.88 % of turbidity reduction and the process which give the least effectiveness is the centrifugation process with only 94.20 % of turbidity reduction.

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Table 4.8: Percentage of turbidity reduction for different types of recovery processes

Types of Recovery Processes	Percentage of Turbidity Reduction (%)
Centrifugation	94.20
Microfiltration	98.88
Redistillation	99.89

Centrifugation is a technique that separates solids suspended in a liquid by rotating the sample at high speed. Rotation at very high speeds (strong centrifugal force) ensures that the denser components of the mixture settle to the bottom of the tube (known as pellet), leaving the less dense components on the top (known as supernatant) (Isac-García, Dobado et al., 2016). Centrifugation process of residual water was operated at speed of 9000 g and duration of 25 minutes as a research showed that the parameters are sufficient to remove the residual plant materials and debris from the aqueous extract of rosemary, sage and thyme before further analysis (Mielnik et al., 2008). By comparing with other recovery processes, centrifugation process gives the least effectiveness as the turbidity level might be due to the presence of remaining lighter or lower density particles and microorganisms (further discussed in section 4.3.1) which cannot be completely removed.

Membrane filtration, is an effective water treatment process which provide a physical barrier against turbidity-causing particles and microorganisms (White, 2016). Microfiltration process is commonly applied in herbal pharmaceutical wastewater treatment by using ceramic microfiltration membrane integrated with an adsorptive treatment which has resulted in almost 99 % removal of turbidity and total suspended solids (Affam & Ezechi, 2019). Preliminary filtration carried out before microfiltration not only remove larger suspended solid particles that might cause blockage to the membrane of syringe filter and reduce spikes in the feedwater turbidity but it also

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enhances the membrane operation (e.g., allowing operation at lower pressures) and extend useful life of the membrane (White, 2016). Although microfiltration process in this study shows a moderate effectiveness among the other recovery processes but it does show a considerably high percentage of turbidity reduction which is almost achieving 99 %. The reason why it gives a higher turbidity value than redistilled residual water is probably due to the presence of CDOM that cannot be removed as it can be seen from the colour retained after microfiltration. Since the microfiltration is performed manually using syringe filter therefore some of the operating parameters such as the flux and operating pressure are difficult to be considered. Even though microfiltration can effectively reduce the turbidity level, but the efficiency of the process for example the time taken that can be studied from flux of membrane are yet to be determined due to insufficient of available data.

Distillation is the truly effective way to eliminate bacteria, viruses, dirt, salt, rust, chemical and other minerals. Other methods of water purification only remove certain contaminants, but only distillation can almost eliminates all impurities (Posner, 1969). Based on the result obtained, redistilled residual water has the nearest turbidity value as the hydrosol since they are both products of distillation. However, the slightly higher turbidity value in redistilled residual water is due to the higher ppm of total dissolved solid when compared to the hydrosol. This analysis supports the relationship that the higher the total dissolved solids value, the higher the turbidity of the solution. Redistillation gives the highest effectiveness among all recovery processes since it is capable to reduce the turbidity level up to almost 99.9 %. Therefore, it can be assumed that all the turbidity causing matter in residual water such as suspended solid particles, microorganisms and coloured dissolved organic matter (CDOM) have been effectively removed.

4.2.3 Particle Size Analysis

Dynamic light scattering is a well-established technique for determining the size and size distribution of molecules and particles that are dispersed or dissolved in a liquid, typically within the submicron range. DLS is a fast, accurate, and repeatable sizing method that is highly flexible and it only requires small sample volumes for analysis, and is non-destructive (Malvern Panalytical, 2018). One of the important parameters for measuring water treatment's effectiveness is by particle size analysis as it can indicate the level of contaminant removal in the water. The size of the suspended solids in the wastewater will significantly influences the separation process (Khanam et al., 2016). The wastewater load can be characterized according to size as; dissolved matter (< 1 nm), colloidal (0.001 μm – 1 μm), supracolloidal (1 – 100 μm) or settleable (> 100 μm) (Arimi, 2018). Polydispersity index (PI) describes the degree of non-uniformity of a size distribution of particles. International standards organizations (ISOs) have established that PI values < 0.05 are more common to monodisperse samples, whereas values > 0.7 are common to a broad size (polydisperse) distribution of particles (Mudalige et al., 2019).

Particle size analysis is more effective than turbidity in water treatment decision making, as it provides more information about processes to help remove particles of the same size as most of the dangerous pathogens. Generally, particle size distribution more precisely characterizes the quality of treated water than turbidity (Zielina, 2007). Based on the research done by He and Nan (2012), it shows that particles larger than 5 μm were strongly related to water turbidity, but a poor correlation between the number of particles and turbidity was found for particles smaller than 5 μm. Therefore, the number of particles smaller than 5 μm should be

specifically measured and controlled during water treatment processes in order to achieve maximum particle removal from the water.

The mean particle diameter and average polydispersity index for different types of water samples are tabulated in Table 4.9. Based on the result, it shows that residual water has the largest average particle size and polydispersity index which are 814.4 ± 15.3 nm and 0.419 ± 0.039 respectively; followed by centrifuged residual water with the values of 231.9 ± 8.6 nm and 0.188 ± 0.023 respectively; and lastly microfiltration residual water with the values of 178.5 ± 6.5 nm and 0.169 ± 0.012 respectively. The results obtained show that they can be classified as colloidal. By referring to the turbidity values in previous section, it shows a relationship that the larger the average particle diameter, the higher the turbidity value. The dispersing state of the water samples are difficult to be determined as they have data that fall between two extreme values of PDI (i.e., 0.05 – 0.7). In contrast, hydrosol and redistilled residual water have a zero value for both of the parameters which are similar to the Spritzer distillation drinking water (controlling variable). The results indicate that there are either absence of any detectable particle or the particles have extremely small size which is below 0.1 nm as the instrument only can measure the size of particles suspended in liquids in the range of 0.1 nm to 12.3 μm (Chemical Technology, 2013). Although hydrosol and redistilled residual water do give a reading on the total dissolved solids, but the particle analysis shows that the total dissolved solids detected could probably have a size smaller than 0.1 nm.

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Table 4.9: Average particle diameter and average polydispersity index for different types of water samples

Types of Water Samples	Average Particle Diameter (nm)	Average Polydispersity Index
Spritzer Distillation Drinking Water (control)	0	0
Hydrosol	0	0
Residual Water	814.4 ± 15.3	0.419 ± 0.039
Centrifuged RW	231.9 ± 8.6	0.188 ± 0.023
Microfiltration RW	178.5 ± 6.5	0.169 ± 0.012
Redistilled RW	0	0

By comparing the effectiveness of recovery process in removing larger size suspended particles, redistillation gives the best outcome, followed by microfiltration and centrifugation. The results also further enhance the reliability of the data obtained for recovery processes as it shows a strong linkage between each physical parameter in such that they are interdependent on each other. The microfiltration process with a pore size of 0.2 μm (200 nm) has proven its effectiveness as the average particle diameter obtained is smaller than the pore size. This means that the particles with size larger than the pore size are retained on the membrane whereas the particles with size smaller than the pore size are allowed to pass through. Besides, centrifugation has the least effectiveness as it is only able to settle the denser suspended particles or microorganisms with the size larger than the average particle diameter obtained. However, the effectiveness of each recovery process in removing the suspended solids are yet to be determined by measuring the total suspended solids for each water samples since the values obtained for turbidity and particle size analysis are unable to represent the actual content of total suspended solid.

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4.3 Microbiological Properties

4.3.1 Microbial Load

One of the ways that cosmetics products may become contaminated with bacteria or fungi is probably due to the nature of the product whether it is a pure compound, an extract, raw material or its combination (ASEAN, 2017). Therefore, it is important to study the microbial load of agarwood hydrodistillation by-products and the effectiveness of the proposed recovery processes to reduce the microbial load before considering to apply them in the manufacturing of cosmetics products. Microbial contamination of cosmetics products is a matter of great importance to the industry and it can become a major cause of both product and economic losses. Moreover, the contamination of cosmetics can result in being converted into products hazardous for consumers. Methods to detect microbial contamination in cosmetics and their raw materials are usually based on traditional plate counts (Orus & Leranoz, 2005). According to the ASEAN limits of contaminants for cosmetics, it shows that total aerobic mesophilic microorganisms (bacteria, yeast & molds) for the products of children under 3 years, eye area and mucous membranes must be $\leq$ 500 CFU/ml whereas it must be $\leq$ 1000 CFU/ml for other types of products (ASEAN, 2017). However, if the products are used for drinking purposes, it must contain no more than 100 CFU/ml of heterotrophic bacteria (referred to as total aerobic bacteria within the Food and Drug Act) for all bottled water not designated as mineral water or spring water (Bartram et al., 2003).

Microbiological data are inherently variable and the "plate count" is at best an interpretation of an approximation of the number of cells present. Experience has shown that different researchers (each skilled) can and frequently do observe different

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counts on the same sample therefore it is important to take note that CFU is only an estimate of the number of cells present. It is a skewed estimate at best as the only cells able to form colonies are those that can grow under the conditions of the test (e.g., incubation media, temperature, time, oxygen conditions) (Sutton, 2011). One of the major limitations for the plate count method will be the relatively narrow countable range. Since ASTM has provided the countable ranges for spread plates method to be 20 - 200 CFU bacteria on a standard petri dish (ASTM D5465-93 (1998), 2012), therefore the plates with CFU values that fall in between the range are selected and hence the average total plate count (CFU/ml) are calculated based on volume of culture plated (0.1 ml) and their respective dilution factor. The average total plate count (CFU/ml) for different types of water samples are then tabulated as shown in Table 4.10. Spritzer distillation drinking water is selected to be the control variable and dilution solvent in this experiment as it shows an absence of microbial load. This could indicate that the results obtained are reliable and accurate as they are truly based on the microbial characteristics of each sample and not affected by the sterile dilution solvent.

By comparing the average total plate count for agarwood hydrodistillation by-products, it shows that residual water has an enormously high value which is $4.60 \times 10^6 \pm 0.28 \times 10^6$ CFU/ml whereas hydrosol gives a relatively low value which is 20 ± 10 CFU/ml. The presence of high CFU/ml in agarwood residual water was probably due to the microbiological properties of the agarwood resin itself as the formation of fragrance resin is the outcome of complex biotic, abiotic, or physical stress on the *Aquilaria* trees (Naziz et al., 2019). Based on the GC-MS chemical profile for agarwood residual water, it shows that acetic acid is presence in high quantity and hence acetic acid bacteria (AAB) are suspected to give arise to the result of high

microbial load. Even though the residual water was heated up to 100 °C for 3 days during hydrodistillation process, but it is still susceptible to the growth of microorganisms when it is cooled down as due to the presence of organic matter in the water. The presence of high amount of organic matter especially from the decaying of agarwood chips will tends to provide a "nutrient broth" to promote the growth of microorganisms. Another possible explanation can be related to the presence of thermophile bacteria in the agarwood itself during the formation of resin or during soaking fermentation process. Boiling process during hydrodistillation does not kill the thermophile bacteria but it is only able to temporary inactivate the thermophile bacteria that thrives at relatively high temperatures, between 41 to 122 °C (Madigan et al., 1997). Further research could be carried out in order to have a better understanding on the types and sources of microorganisms that presence in the residual water.

From the view of microbiological properties, the low average total plate count of hydrosol indicates that it has a character similar to drinking water with a value below 100 CFU/ml as mentioned above. Based on the research done by Abdullah et al. (2010), fungi were found absence while bacteria were found presence in very low number for some agarwood hydrosol samples. The research paper also stated that the presence of bacteria was probably influenced by the technique used during sampling, either aseptic technique being applied or not (Abdullah et al., 2010). In this study, low value of CFU/ml presence in the hydrosol sample was probably due to that the hydrosol sample used in this study was collected non-aseptically and not filtered by factory worker before kept in a non-sterile plastic container. In order to obtain an accurate and valid result, it is suggested that the sampling process of agarwood hydrosol must be carried out aseptically and collected in a sterile glass container so as

to reduce the risk of microbe contamination from external sources. The hydrosols after sterile bottling should be stored in a cool or cold and constant temperature, away from heat and light. In some of the European Union countries, hydrosols marketed for cosmetic/aesthetic purposes must now legally contain alcohol with at least 12 % by volume. The added alcohol has been effective in preventing contamination and prolonging the shelf life, even allowing safe storage of hydrosols at room temperature in retail stores without any worry of spoilage (Catty, 2001).

Table 4.10: Average total plate count (CFU/ml) for different types of water samples

Types of Water Samples	**Average Colony Forming Unit (CFU)**	**Dilution Factor**	**Average Total Plate Count (CFU/ml)**
Spritzer Distillation Drinking Water (control)	0	10^0	n.d.
Hydrosol	2 ± 1	10^0	20 ± 10
Residual Water	46 ± 3	10^4	$4.60\times10^6 \pm 0.30\times10^6$
Centrifuged RW	24 ± 3	10^4	$2.40\times10^6 \pm 0.30\times10^6$
Microfiltration RW	0	10^0	n.d.
Redistilled RW	0	10^0	n.d.

n.d. = Not detected

Based on results for recovered residual water samples, it shows that only centrifuged residual water still contains high microbial load with an average total plate count value of $2.40\times10^6 \pm 0.30\times10^6$ CFU/ml whereas microfiltration and redistilled residual water show an absence of detectable colony on the agar plates. The effectiveness of each recovery process can be studied from Table 4.11 which the percentage of microbial load reduction and log reduction values for each recovery process were calculated. The table shows a 100 % of microbial load reduction or 6 log

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reduction for both microfiltration and redistillation process whereas only 47.83 % or 0.28 log reduction for centrifugation process as the average total plate count of residual water has been reduced to almost half after centrifugation process.

Table 4.11: Microbial log reduction for different types of recovery processes

Types of Recovery Processes	Percentage of Microbial Load Reduction (%)	Log Reduction, LRV
Centrifugation	47.83	0.28
Microfiltration	100	6
Redistilled	100	6

Centrifugation is commonly utilized in laboratory practice for collecting suspended bacteria (pellet) such as E. coli in suspended cultures. However, there are only few studies focused on the remaining bacteria levels in the supernatant after high speed centrifugation. As reported by Liu et al. (2017), centrifugation at the speed higher than 5000 g will cause a loss of viability in some bacterial strains. The research also found that higher centrifugation speed may achieve greater results in liquid phase microbial reduction although centrifugal separator with speed higher than 10,000 g might not be feasible in industrial-scale practice (Liu et al., 2017; Records & Sutherland, 2001). In this study, the effect of microbial load reduction in centrifugation process might due to the presence of suspended solids. The bacteria cells are possibly entrapped within the centrifuge pellets (concentrated with suspended solids), therefore the microbial densities would be reduced in the liquid layer. Moreover, shear damage from shear forces and collisions with suspended particles may suffered by bacterial cells when centrifuged at high speed (Liu et al., 2017). When compared to other recovery processes, centrifugation has the lowest effectiveness in microbial load reduction as it is probably due to that the average density of bacteria cell (1.1 g/cm^3) (Loferer-Krößbacher et al., 1998) is close to the density of water (1 g/cm^3). In this

case, it is assumed that the density of residual water is approximately equal to the density of water. Hence, the centrifugation process that separates the medium based on differences in density might cannot be carried out effectively especially in the situation where the difference of density is insignificant.

Liquid filtration through 0.2 µm filters is a common and frequently used technique (known to be microfiltration) for removing microorganisms from heat-sensitive solutions. Such 0.2 µm filtrations are often referred to as 'sterile filtration' because this filtration is generally believed to remove all microorganisms (except viruses) from the filtered solutions (Atlas, 1997; Hahn, 2004; Madigan et al., 1997). Based on WHO Guidelines for Drinking-water Quality (WHO, 2011), it shows that the membrane filtration treatment process of water namely microfiltration is able to achieve a baseline removal of bacteria, viruses and protozoa up to 2 log reduction and a maximum removal up to 4 to 6 log reduction. The values vary with the pore size of membrane integrity of filter medium and filter seals, and resistance to chemical and biological degradation. In this study, it shows that the syringe filter with 0.2 µm is able to effectively remove the microbial load of residual water by at least 6 log reduction since the initial microbial load is up to 10^6 CFU/ml. Based on a review article published by Aryal (2015), he stated that the average diameter of spherical bacteria is 0.5 - 2.0 µm whereas for rod-shaped or filamentous bacteria, length is 1 - 10 µm and diameter is 0.25 - 1.0 µm. The average size range for bacteria as well as other cellular components can be clearly identified from the illustration shown in Figure 4.6. It can be said that microfiltration by using pore size of 0.2 µm is sufficient to remove all the microbial load contained in the residual water since the reported average size of the bacteria cells is larger than the 0.2 µm, therefore all the bacteria cells can be retained and a sterile liquid is obtained.

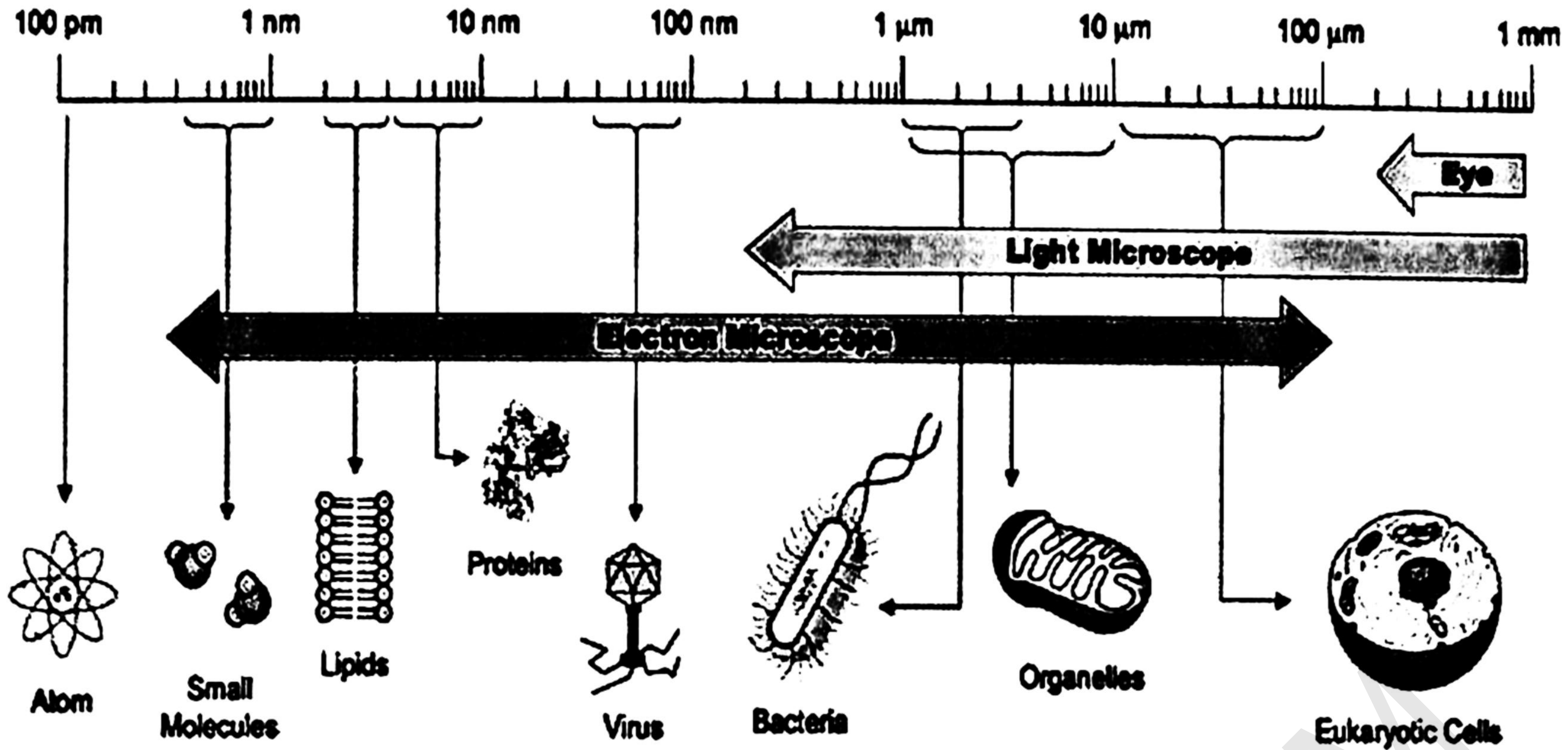

Figure 4.6: Size range for different kinds of cells and cellular components

Distillation is a traditional process of vaporizing water so that the pure water molecules are separated as steam from microorganisms and contaminants which having higher boiling point (Bartholomew, 2010). The boiling process of the distillation kills viruses, bacteria, protozoa and other pathogens by using heat to damage structural components and disrupt essential life processes (e.g. denature proteins) (Reynolds, 2013). Ideally, the distilled water should only contain pure water molecules and trace of volatile organic compounds (VOCs) which has a boiling point lower than water. In this study, redistillation of residual water carried out at 100 °C is capable to remove all the microbial load up to 6 log reduction. This result can be validate based on WHO Guidelines for Drinking-water Quality (WHO, 2011) as it shows that the thermal treatment process of water for example boiling is able to achieve a baseline removal of bacteria, viruses and protozoa up to 6 log reduction and a maximum removal up to 9 log reduction.

CHAPTER 5

CONCLUSION AND RECOMMENDATION

In this study, agarwood hydrodistillation by-products namely residual water and hydrosol were characterised by chemical, physical and microbiological properties. Three recovery processes such as centrifugation, microfiltration and redistillation were proposed in order to clarify the residual water and retain its quality at the same time. The effectiveness of the recovery processes is compared based on the three properties mentioned above. The parameters set for each of the recovery process were: centrifugation (9000 g, 25 minutes); microfiltration (0.2 μm syringe filter); and redistillation (100 °C).

Chemical properties such as chemical profile by GC-MS analysis, pH value and total dissolved solids were studied for each water samples. The results of GC-MS analysis show none of the agarwood significant compounds (sesquiterpene or terpenoid) were detected but benzylacetone, a common major chemical constituent found in agarwood oil is detected for both of the by-products. Acetic acid (mainly produced by bacteria) is found to give the highest peak in residual water chromatogram whereas benzylacetone (sweet flowery smelling liquid) is found to occupy the highest peak in hydrosol chromatogram. The results of pH show that residual water with pH of 6.40 ± 0.08 is slightly acidic whereas hydrosol pH of 4.26 ± 0.04 is considered to be acidic. Centrifugation and microfiltration processes were able to retain the pH of residual water but redistillation has reduced the pH to more acidic. The results of TDS show that residual water with value of 435 ± 1 ppm contain more dissolved solids compared to hydrosol with value of 4 ± 1 ppm. Microfiltration process is able to retain

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the TDS of residual water whereas centrifugation and redistillation process show a reduction of TDS.

Physical properties such as visual observation, turbidity and particle size analysis were studied. By comparing the visual appearance for both by-products, residual water appeared as brown coloured murky and opaque solution concentrated with large quantity of visible suspended solid particles whereas hydrosol is a clear and transparent colourless solution. The results of turbidity show that residual water has an enormously high value which is 586 ± 5 NTU compared to hydrosol with value of 0.53 ± 0.02 NTU. The results of particle size analysis also show that residual water has a very large average particle diameter (814.4 ± 15.3 nm) and high polydispersity index (0.419 ± 0.039) whereas hydrosol shows a zero value for both parameters. By comparing the effectiveness of recovery processes in removing turbidity and larger size particles, redistillation gives the best outcome, followed by microfiltration and centrifugation.

Microbial load is one of the microbiological properties determined in this study. The results show that residual water with the average total plate count of 4.60×106 ± 0.30×106 CFU/ml has an enormously high microbial load compared to hydrosol with value of 20 ± 10 CFU/ml. Microfiltration and redistillation processes were able to effectively remove all the microbial load whereas centrifugation only partially removes the microbial load in residual water.

Based on all the properties studied, it can be concluded that the agarwood hydrosol is suitable to be applied for further usage such as manufacturing of cosmetics products providing that it must be collected aseptically and stored in good conditions to prevent any contamination that will reduce its shelf-life. However, the properties of

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recovered agarwood residual water have to be studied in more detail especially for those with direct impact to the safety and health issues before really considering to apply it for further usage. Among all proposed recovery processes, microfiltration is known to give an optimum performance as it is not only effective in removing all the contaminants but also retain the quality and appearance of the residual water.

Some of the future recommendations for this study are:

- Reduce the holding time of the agarwood hydrodistillation by-products. They should be analysed immediately after the hydrodistillation process in order to obtain more accurate and reliable results.
- The by-products should be collected aseptically and stored in a sterile glass container to prevent any contamination due to external source.
- The parameters for centrifugation process such as the speed and time should be varied in order to obtain a set of parameters that gives the best performance.
- Microfiltration should be carried out by using appropriate apparatus and membrane instead of using syringe filter with manual pushing force. Parameters such as flux and pressure should be studied to determine the efficiency of the whole process. Ultrafiltration along with ultraviolet process with a higher effectiveness is recommended for further research.
- The exact amount of secondary oil remaining in the by-products and recovered residual water samples should be quantified and qualified. The appropriate method for solvent extraction has to be figured out to prevent the formation an emulsion layer that has a cloudy or milky appearance between the boundary of solvent and sample. According to U.S. EPA Method 1664, "if the emulsion is greater than one-

third the volume of the solvent layer, the laboratory must employ emulsion-breaking techniques to complete the phase separation."

- The colour of the water samples should be measured by colorimeter instead using subjective measurement for example human observation.
- Total suspended solid (TSS) should be measured as turbidity and particle size analysis are not a direct measurement of the total suspended materials in water.
- Other than microbial activity, fungi activity of the water samples should be studied as the by-products are also prone to fungi growth.
- Microbial and fungi load of the water samples should be studied for consecutive weeks in order to observe the effect of storage period upon the microbial and fungi activities.

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```
                    INSTRUMENT CONTROL PARAMETERS:    GCMSD
                    --------------------------------------

   C:\MSDCHEM\1\METHODS\HP5MS.M
      Tue Jul 21 11:49:21 2020

Control Information
------- -----------

Sample Inlet      :  GC
Injection Source  :  GC ALS
Mass Spectrometer :  Enabled


 No Sample Prep method has been assigned to this method.

Oven
Equilibration Time                          0.5 min
Max Temperature                             325 degrees C
Slow Fan                                    Disabled
Oven Program                                On
    60 °C for 10 min
    then 3 °C/min to 210 °C for 1 min
Run Time                                    61 min
1 min (Post Run)                            320 °C
Cryo                                        Off

Front Injector
Syringe Size                                10 µL
Injection Volume                            1 µL
Solvent A Washes (PreInj)                   3
Solvent A Washes (PostInj)                  3
Solvent A Volume                            8 µL
Solvent B Washes (PreInj)                   3
Solvent B Washes (PostInj)                  3
Solvent B Volume                            8 µL
Sample Washes                               3
Sample Wash Volume                          8 µL
Sample Pumps                                6
Dwell Time (PreInj)                         0 min
Dwell Time (PostInj)                        0 min
Solvent Wash Draw Speed                     300 µL/min
Solvent Wash Dispense Speed                 6000 µL/min
Sample Wash Draw Speed                      300 µL/min
Sample Wash Dispense Speed                  6000 µL/min
Injection Dispense Speed                    6000 µL/min
Viscosity Delay                             0 sec
Sample Depth                                Disabled
Injection Type                              Standard
L1 Airgap                                   0.2 µL

Back Injector
Syringe Size                                500 µL
Injection Volume                            50 µL
Solvent A Washes (PreInj)                   0
Solvent A Washes (PostInj)                  0
Solvent A Volume                            400 µL
Solvent B Washes (PreInj)                   0
Solvent B Washes (PostInj)                  0
Solvent B Volume                            400 µL
Sample Washes                               0
Sample Wash Volume                          400 µL
Sample Pumps                                6
Dwell Time (PreInj)                         0 min
Dwell Time (PostInj)                        0 min
Solvent Wash Draw Speed                     15000 µL/min
Solvent Wash Dispense Speed                 300000 µL/min
Sample Wash Draw Speed                      15000 µL/min
Sample Wash Dispense Speed                  300000 µL/min
Injection Dispense Speed                    300000 µL/min
Viscosity Delay                             0 sec
Sample Depth                                Disabled

HP5MS.M Tue Jul 21 11:49:21 2020                                   Page:  2
```

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(cont.)

Injection Type	Standard
Barcode Reader	
Barcode heater	Disabled
Barcode mixer	Disabled
Sample Overlap	
Sample overlap is not enabled	
Front SS Inlet He	
Mode	Splitless
Heater	On 250 °C
Pressure	On 17.729 psi
Total Flow	On 3 mL/min
Septum Purge Flow	On 1 mL/min
Gas Saver	Off
Purge Flow to Split Vent	1 mL/min at 0 min
Thermal Aux 2 (MSD Transfer Line)	
Heater	On
Temperature Program	On
280 °C for 0 min	
Run Time	61 min
Column #1	
Agilent 19091S-436HP-5MS	
325 °C: 60 m x 250 µm x 0.25 µm	
In: Front SS Inlet He	
Out: Vacuum	
(Initial)	60 °C
Pressure	17.729 psi
Flow	1 mL/min
Average Velocity	25.896 cm/sec
Holdup Time	3.8616 min
Flow Program	Off
1 mL/min for 2.5 min	
Run Time	61 min
1 min (Post Run)	0.9636 mL/min
Signals	
Signal #1: Test Plot	Save Off 50 Hz
Signal #2: Test Plot	Save Off 50 Hz
Signal #3: Test Plot	Save Off 50 Hz
Signal #4: Test Plot	Save Off 50 Hz

MS ACQUISITION PARAMETERS

General Information
------- -----------

Tune File : atune.u
Acquistion Mode : Scan/SIM

MS Information
-- -----------

Solvent Delay : 5.00 min

EMV Mode : Gain Factor
Gain Factor : 1.00
Resulting EM Voltage : 2129

HP5MS.M Tue Jul 21 11:49:21 2020 Page: 3

(cont.)

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```
[Scan Parameters]

Low Mass                    : 40.0
High Mass                   : 1000.0
Threshold                   : 150
Sample #                    : 2       A/D Samples    4
[Sim Parameters]

GROUP 1
Group ID                    : 1
Resolution                  : Low
Plot 1 Ion                  : 74.10
Ions/Dwell In Group         (  Mass,  Dwell)
                            ( 74.10,    100)

[MSZones]

MS Source                   : 230 C   maximum 250 C
MS Quad                     : 150 C   maximum 200 C

                  END OF MS ACQUISITION PARAMETERS


                  TUNE PARAMETERS for SN: US10372602
              -----------------------------

 Trace Ion Detection is OFF.

 EMISSION    :       34.610
 ENERGY      :       69.922
 REPELLER    :       34.814
 IONFOCUS    :       90.157
 ENTRANCE_LE :       22.000
 EMVOLTS     :     2282.353
                               Actual EMV  :     2282.35
                               GAIN FACTOR :        2.33
 AMUGAIN     :     1672.000
 AMUOFFSET   :      121.000
 FILAMENT    :        1.000
 DCPOLARITY  :        0.000
 ENTLENSOFFS :       18.071
 MASSGAIN    :     -856.000
 MASSOFFSET  :      -31.000

                 END OF TUNE PARAMETERS
            ------------------------------------


                       END OF INSTRUMENT CONTROL PARAMETERS
                       ------------------------------------

HP5MS.M Tue Jul 21 11:49:21 2020                                    Page:  4
```

Figure A-1: Report of GC-MS operating parameters

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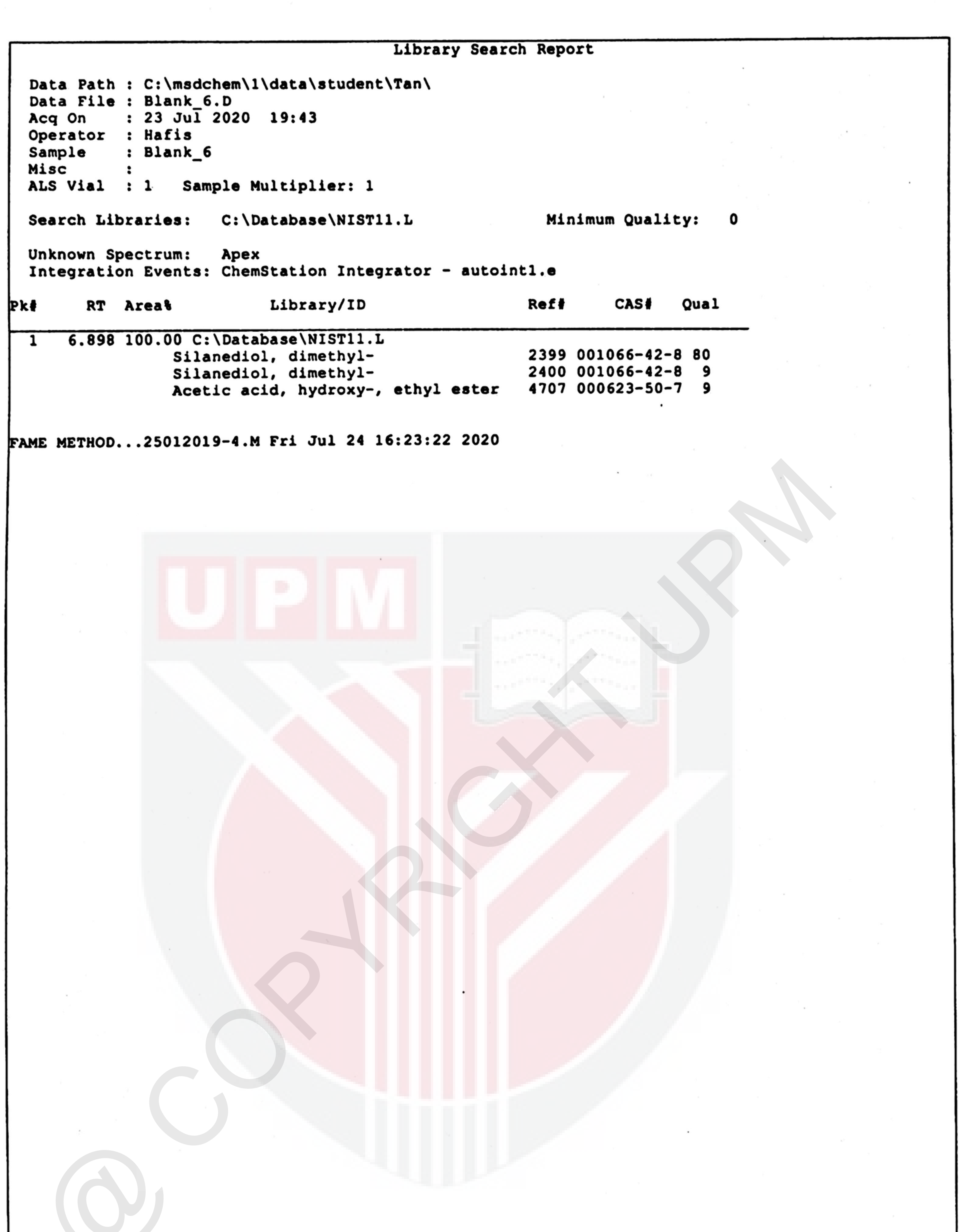

Library Search Report

```
Data Path : C:\msdchem\1\data\student\Tan\
Data File : Blank_6.D
Acq On    : 23 Jul 2020  19:43
Operator  : Hafis
Sample    : Blank_6
Misc      :
ALS Vial  : 1   Sample Multiplier: 1

Search Libraries:   C:\Database\NIST11.L          Minimum Quality:   0

Unknown Spectrum:   Apex
Integration Events: ChemStation Integrator - autoint1.e
```

Pk#	RT	Area%	Library/ID	Ref#	CAS#	Qual
1	6.898	100.00	C:\Database\NIST11.L			
			Silanediol, dimethyl-	2399	001066-42-8	80
			Silanediol, dimethyl-	2400	001066-42-8	9
			Acetic acid, hydroxy-, ethyl ester	4707	000623-50-7	9

FAME METHOD...25012019-4.M Fri Jul 24 16:23:22 2020

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Figure A-2: GC-MS analysis report for blank

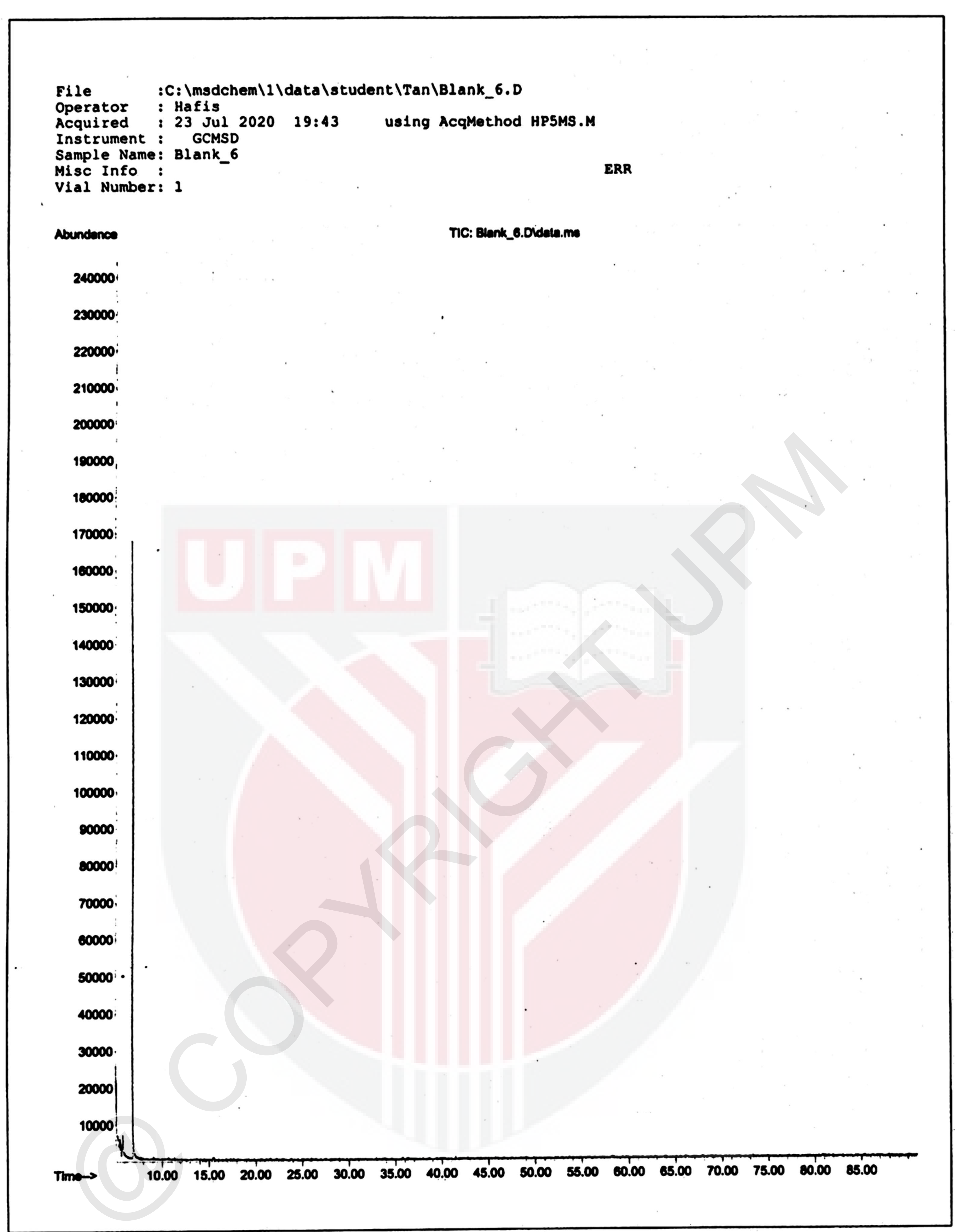

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Figure A-3: GC-MS chromatogram report for blank

Library Search Report

Data Path : C:\msdchem\1\data\student\Tan\
Data File : RW_6.D
Acq On : 17 Jul 2020 9:23
Operator : AFNAN
Sample : RW_6
Misc :
ALS Vial : 1 Sample Multiplier: 1

Search Libraries: C:\Database\NIST11.L Minimum Quality: 0

Unknown Spectrum: Apex
Integration Events: ChemStation Integrator - autoint1.e

Pk#	RT	Area%	Library/ID	Ref#	CAS#	Qual
1	5.863	20.75	C:\Database\NIST11.L			
			Acetic acid	262	000064-19-7	90
			Acetic acid	264	000064-19-7	86
			Acetic acid	263	000064-19-7	86
2	6.923	28.42	C:\Database\NIST11.L			
			Silanediol, dimethyl-	2399	001066-42-8	74
			Silanediol, dimethyl-	2400	001066-42-8	9
			(S)-(-)-2-Chloropropionic acid	5225	029617-66-1	9
3	9.250	13.42	C:\Database\NIST11.L			
			Butanoic acid	2020	000107-92-6	59
			Butanoic acid	2016	000107-92-6	59
			Hexanoic acid	8069	000142-62-1	9
4	14.952	17.37	C:\Database\NIST11.L			
			Oxime-, methoxy-phenyl-_	24837	1000222-86-6	90
			2-Amino-5-methylbenzoic acid	24857	002941-78-8	64
			4-Ethylbenzoic acid, 2-butyl ester	65946	1000293-31-8	50
5	34.076	12.77	C:\Database\NIST11.L			
			2-Butanone, 4-phenyl-	22635	002550-26-7	83
			3-Buten-2-ol, 4-phenyl-	22646	017488-65-2	76
			Benzene, (1-methylbutyl)-	22788	002719-52-0	52
6	59.999	7.28	C:\Database\NIST11.L			
			Tricyclo[4.3.0.0(7,9)]non-3-ene, 2,2,5,5,8,8-hexamethyl-, (1.alpha.,6.beta.,7.alpha.,9.alpha.)-	64513	054832-80-3	32
			Furan, 2-(4-nitrophenyl)-	52245	028123-72-0	27
			6S-2,3,8,8-Tetramethyltricyclo[5.2.2.0(1,6)]undec-2-ene	64413	137235-48-4	22

FAME METHOD...25012019-4.M Mon Jul 20 09:33:52 2020 Page: 1

Figure A-4: GC-MS analysis report for residual water

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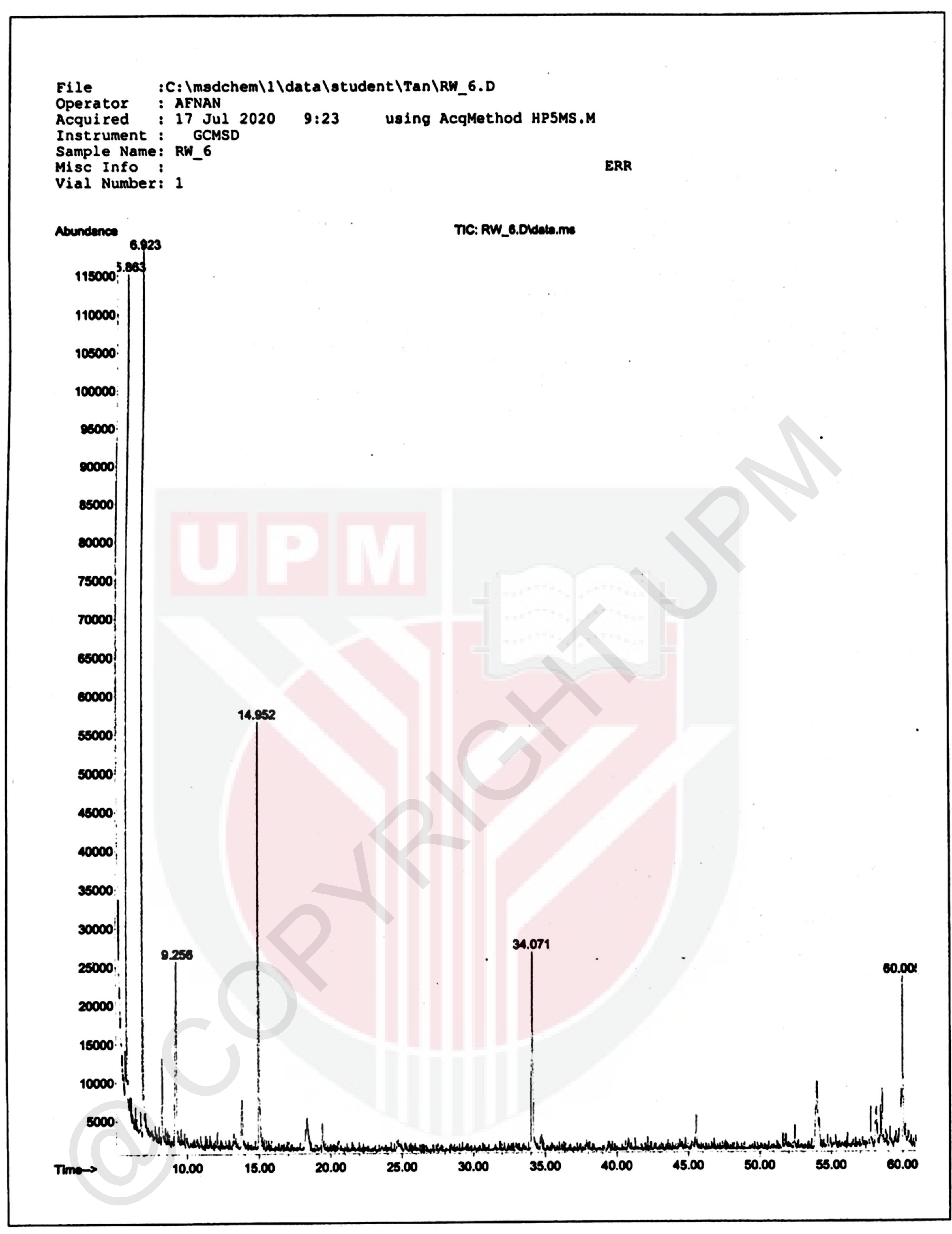

Figure A-5: GC-MS chromatogram report for residual water

Library Search Report

```
Data Path : C:\msdchem\1\data\student\Tan\
Data File : Hydrolysol.D
Acq On    : 23 Jul 2020  18:04
Operator  : Hafis
Sample    : Hydrolysol
Misc      :
ALS Vial  : 2   Sample Multiplier: 1

Search Libraries:   C:\Database\NIST11.L          Minimum Quality:   0

Unknown Spectrum:   Apex
Integration Events: ChemStation Integrator - autoint1.e
```

Pk#	RT	Area%	Library/ID	Ref#	CAS#	Qual
1	7.181	41.04	C:\Database\NIST11.L			
			Silanediol, dimethyl-	2399	001066-42-8	80
			Silanediol, dimethyl-	2400	001066-42-8	40
			(S)-(-)-2-Chloropropionic acid	5225	029617-66-1	4
2	15.506	5.16	C:\Database\NIST11.L			
			Oxime-, methoxy-phenyl-_	24837	1000222-86-6	64
			4-Ethylbenzoic acid, nonyl ester	124504	1000292-19-7	50
			4-Ethylbenzoic acid, cyclohexyl es ter	87676	1000293-32-1	45
3	40.640	23.51	C:\Database\NIST11.L			
			2-Butanone, 4-phenyl-	22635	002550-26-7	55
			Benzene, (1-methylbutyl)-	22787	002719-52-0	42
			2-Butanone, 4-phenyl-	22631	002550-26-7	42
4	41.613	23.19	C:\Database\NIST11.L			
			Benzenepropanol, .beta.-methyl-	23775	007384-80-7	87
			4-Phenyl-2-butanol	23705	002344-70-9	72
			Benzenepropanol, .alpha.-methyl-, acetate	54466	010415-88-0	64
5	67.547	7.11	C:\Database\NIST11.L			
			1(3H)-Isobenzofuranone, 3a,4,5,7a-tetrahydro-4-hydroxy-3a,7a-dimethy l-, (3a.alpha.,4.beta.,7a.alpha.)-(.+/-.)-	46907	054346-06-4	36
			3-Pyridinol, 6-methyl-	5566	001121-78-4	27
			Bicyclo[2.2.2]octane, 1-bromo-	51950	007697-09-8	25

FAME METHOD...25012019-4.M Fri Jul 24 16:20:49 2020

Figure A-6: GC-MS analysis report for hydrosol

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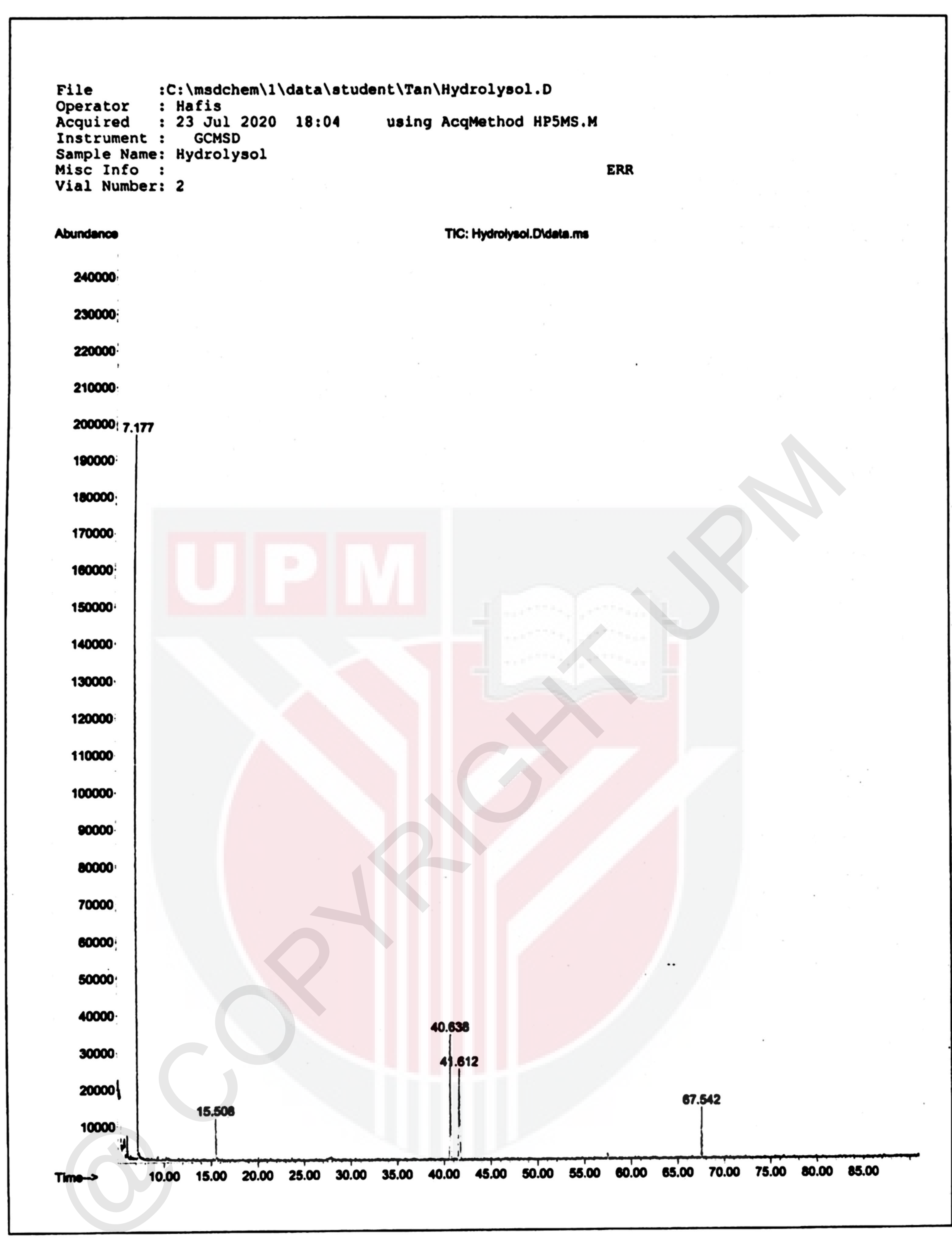

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Figure A-7: GC-MS chromatogram report for hydrosol

Table A-1: Raw data for pH analysis

Types of Water Samples	pH			
	1	2	3	Average
Spritzer Distillation Drinking Water (control)	7.05	6.98	7.03	7.02 ± 0.04
Residual water	6.32	6.45	6.38	6.40 ± 0.08
Hydrosol	4.26	4.28	4.32	4.26 ± 0.04
Centrifuged RW	6.38	6.40	6.45	6.41 ± 0.03
Microfiltration RW	6.45	6.40	6.44	6.43 ± 0.03
Redistilled RW	4.36	4.40	4.42	4.39 ± 0.03

Table A-2: Raw data for total dissolved solid analysis

Types of Water Samples	Total Dissolved Solid (ppm)			
	1	2	3	Average
Spritzer Distillation Drinking Water (control)	0	0	0	0
Residual water	436	435	434	435 ± 1
Hydrosol	4	4	5	4 ± 1
Centrifuged RW	403	402	404	403 ± 1
Microfiltration RW	435	431	433	433 ± 2
Redistilled RW	11	12	12	12 ± 1

Table A-3: Raw data for turbidity analysis

Types of Water Samples	Turbidity (NTU)			
	1	2	3	Average
Spritzer Distillation Drinking Water (control)	0.02	0	0.04	0.02 ± 0.02
Residual water	583	592	584	586 ± 5
Hydrosol	0.55	0.53	0.52	0.53 ± 0.02
Centrifuged RW	35.0	33.3	33.7	34.0 ± 0.9
Microfiltration RW	6.58	6.56	6.55	6.56 ± 0.02
Redistilled RW	0.60	0.68	0.62	0.63 ± 0.04

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Table A-4: Raw data for particle size analysis (particle diameter)

Types of Water Samples	Particle Diameter (nm)						
	A1	**A2**	**B1**	**B2**	**C1**	**C2**	**Average**
Spritzer Distillation Drinking Water (control)	0	0	0	0	0	0	0
Residual water	795.3	801.2	835.0	828.5	810.6	815.7	814.4 ± 15.3
Hydrosol	0	0	0	0	0	0	0
Centrifuged RW	231.4	240.5	217.1	235.1	239.2	228.2	231.9 ± 8.6
Microfiltration RW	182.3	179.2	187.7	178.7	168.8	174.0	178.5 ± 6.5
Redistilled RW	0	0	0	0	0	0	0

A: 1st run, B: 2nd run, C: 3rd run

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Table A-5: Raw data for particle size analysis (polydispersity index)

Types of Water Samples	Polydispersity Index						
	A1	A2	B1	B2	C1	C2	Average
Spritzer Distillation Drinking Water (control)	0	0	0	0	0	0	0
Residual water	0.373	0.385	0.468	0.461	0.417	0.412	0.419 ± 0.039
Hydrosol	0	0	0	0	0	0	0
Centrifuged RW	0.193	0.170	0.161	0.162	0.164	0.163	0.188 ± 0.023
Microfiltration RW	0.171	0.203	0.129	0.213	0.216	0.198	0.169 ± 0.012
Redistilled RW	0	0	0	0	0	0	0

A: 1st run, B: 2nd run, C: 3rd run

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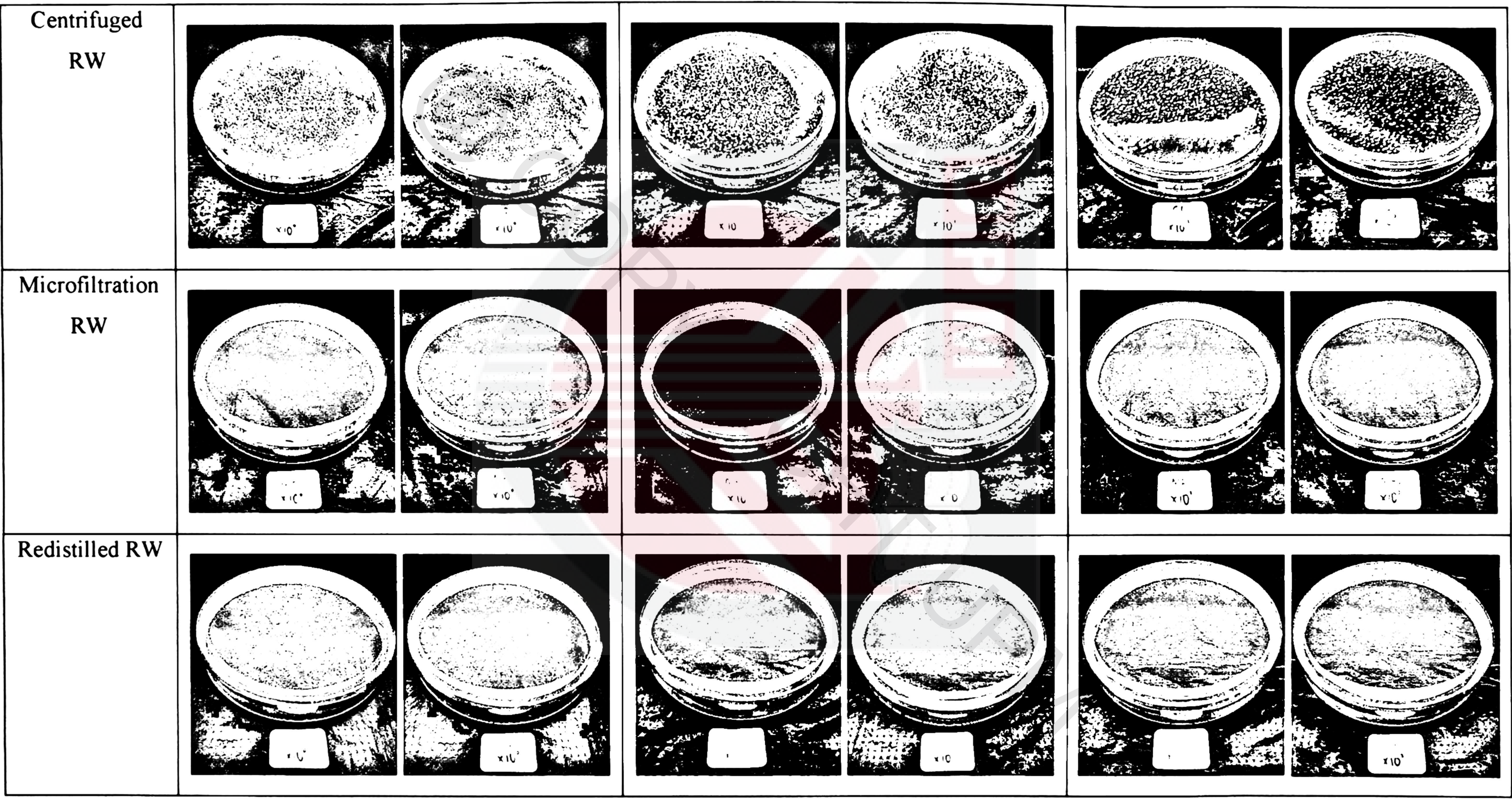
Table A-6: Images of plate according to dilution factor

Types of Water Samples	Dilution Factor		
	1:1 **10^0**	**1:10** **10^1**	**1:100** **10^2**
Residual Water			
Hydrosol			


Centrifuged RW
Microfiltration RW
Redistilled RW

(cont.

Types of Water Samples	Serial Dilution		
	1:1000 10^3	1:10000 10^4	1:100000 10^5
Residual Water			
Hydrosol			

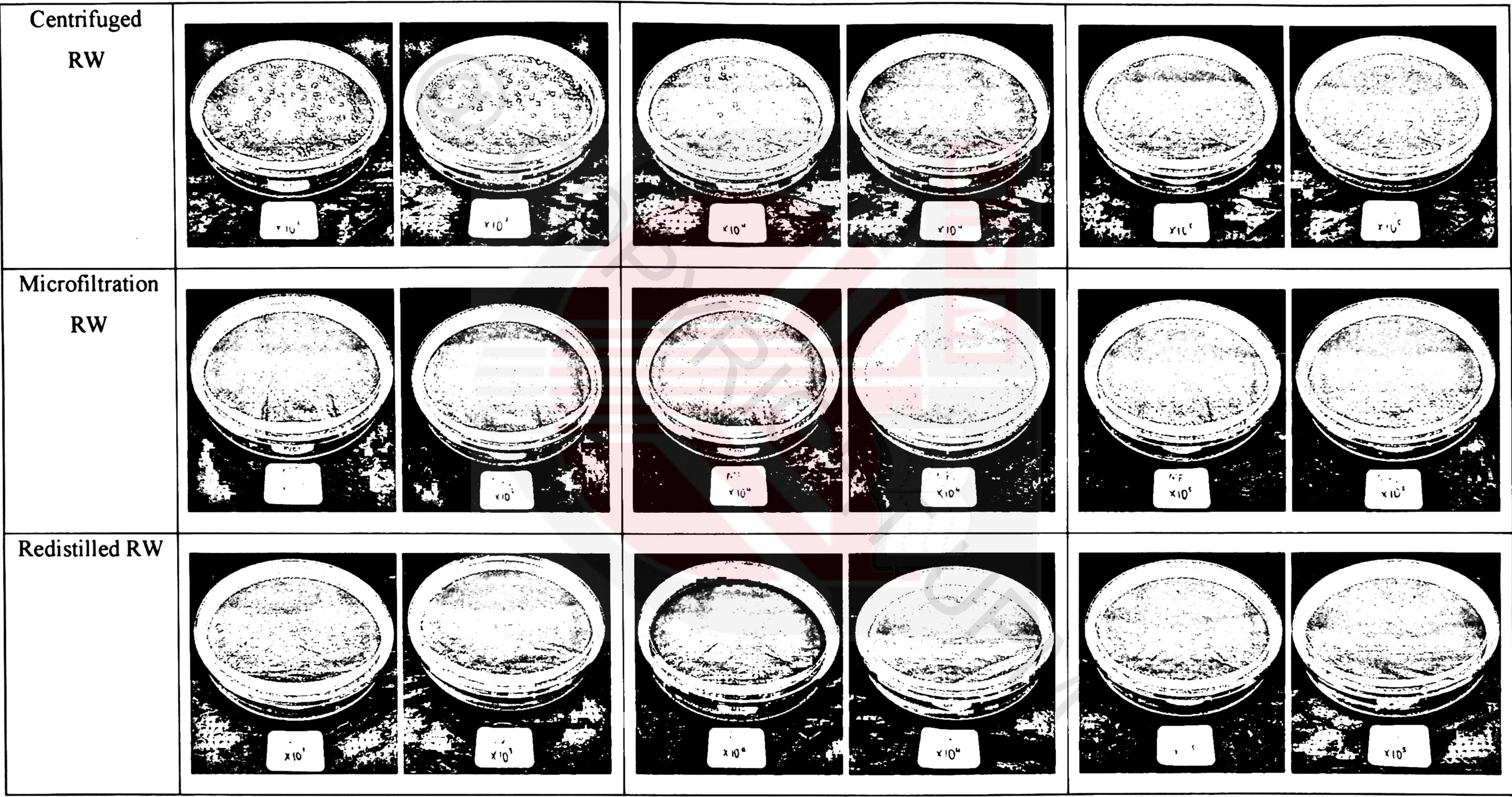

Centrifuged RW
Microfiltration RW
Redistilled RW

Table A-7: Raw data for microbial load analysis according to serial dilution

Types of Water Samples	Colony Forming Unit (CFU)					
	1:1 10^0		1:10 10^1		1:100 10^2	
	1	2	1	2	1	2
Spritzer Distillation Drinking Water (control)	0	0	0	0	0	0
Residual water	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
Hydrosol	2	1	0	0	0	0
Centrifuged RW	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
Microfiltration RW	0	0	0	0	0	0
Redistilled RW	0	0	0	0	0	0

TNTC: Too numerous to count

(cont.)

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Types of Water Samples	Colony Forming Unit (CFU)					
	1:1000 10^3		1:10000 10^4		1:100000 10^5	
	1	2	1	2	1	2
Spritzer Distillation Drinking Water (control)	0	0	0	0	0	0
Residual water	475	453	48	44	9	8
Hydrosol	0	0	0	0	0	0
Centrifuged RW	200	210	26	22	7	5
Microfiltration RW	0	0	0	0	0	0
Redistilled RW	0	0	0	0	0	0

Table A-8: Raw data for colony forming unit

Types of Water Samples	Average Colony Forming Unit (CFU)		
	1	2	Average
Residual water (DF: 10^4)	48	44	46 ± 3
Hydrosol (DF: 10^0)	2	1	2 ± 1
Centrifuged RW (DF: 10^4)	26	22	24 ± 3

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