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DEVELOPMENT AND OPTIMIZATION OF ECO-FRIENDLY MARKER INK USING MANGOSTEEN LEAVES

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DEVELOPMENT AND OPTIMIZATION OF ECO-FRIENDLY MARKER INK USING MANGOSTEEN LEAVES

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A PROJECT REPORT SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE OF BACHELOR OF ENGINEERING (PROCESS AND FOOD)

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ABSTRACT

Based on the aspiration to protect the environment and reduce the use of non-renewable resources, this research work was conducted to demonstrate the mechanism of plant-based marker ink production using alternative raw materials which is mangosteen leaves. Glycerol, Ferrous Sulphate, Benzalkonium Chloride and Carboxymethyl Cellulose (CMC) were served as additives to enhance ink properties. Experimental designs using MINITAB software, screening process using Fractional Factorial Design (FrFD) and optimization process using Response Surface Methodology (RSM), were shown to be useful in conducting experimental work in identifying the significant factors and optimizing the responses. Based on Pareto Chart, Glycerol and CMC were identified to be the most significant factor. The optimum formulation of marker ink can be achieved when the amount of Glycerol, Benzalkonium Chloride, Ferrous Sulphate, and CMC is set as 5g, 5g, 1g, and 2.85g, respectively. The desirability of optimization was calculated as 0.82815, indicating that the optimization is accurate. The microstructure of Mangosteen leaves is analyzed using a Scanning Electron Microscope (SEM) and proved that Glycerol is significant during colour extraction. Based on TGA analysis, the optimized ink was proved to dry at the highest rate. Hence, the optimized ink exhibits desirable properties including low viscosity, high colour intensity and fast drying speed. This study is expected to commercially expand the local product with low production cost, unlimited resource and to be fully utilized as an alternative eco-friendly ink to replace the chemical-based marker ink in the market.

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ABSTRAK

Berdasarkan aspirasi untuk melindungi alam sekitar dan mengurangkan penggunaan sumber yang tidak boleh diperbaharui, kerja penyelidikan ini dilakukan untuk menunjukkan mekanisme pengeluaran dakwat penanda berasaskan tumbuhan menggunakan bahan mentah alternatif iaitu daun manggis. Gliserol, sulfat besi, benzalkonium klorida dan karboksimetil selulosa (CMC) digunakan sebagai bahan tambahan untuk meningkatkan sifat dakwat. Fractional Factorial Design (FrFD) yang menggunakan perisian MINITAB untuk proses penyaringan dan proses pengoptimuman menggunakan Response Surface Methodology (RSM), terbukti berguna dalam mengenal pasti faktor penting dan mengoptimumkan tindak balas. Berdasarkan Pareto Chart, Gliserol dan CMC dikenal pasti sebagai faktor yang paling signifikan. Rumusan dakwat yang optimum dapat dicapai apabila nisbah Gliserol, benzalkonium, klorida,sulfat besi dan CMC adalah 5g, 5g, 1g, dan 2.85g. Keperluan pengoptimuman dikira sebagai 0.82815, menunjukkan bahawa pengoptimuman tepat. Struktur mikro daun Manggis dianalisis menggunakan Mikroskop Elektron Pengimbasan (SEM) dan membuktikan bahawa Gliserol adalah signifikan semasa pengekstrakan warna. Berdasarkan analisis TGA, dakwat optimum terbukti kering pada kadar tertinggi. Oleh itu, dakwat optimum menunjukkan sifat yang baik termasuk kelikatan rendah, intensiti warna tinggi dan cepat kering. Kajian ini diharapkan dapat mengembangkan produk tempatan secara komersial dengan kos rendah dan sumber mesra alam.

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CHAPTER 1: INTRODUCTION

1.1 Research Background

Marker is known as a crucial tool to write and help in creating artwork (Alguzar, 2015). Other than writing, it is applied in poster creation, labelling and calligraphy. In the education field, marker is essential to both students and teachers because it is used to complete report and deliver information on the whiteboard.

Ink is regarded as a liquid or paste that contain pigments or dyes. It can be used by applying on a surface to create texts, images or designs by using a brush, pen or quill (H. Kipphan, 2001). Ink can present in a complex medium such as in fluid pattern or glutinous adhesive. They are usually made up of solvents, pigments or dyes, lubricants, resins, solubilizers, particulate matter, surfactants and fluoresces (P. Laden, 1999). The components of ink have various function such as act as ink carrier, colourants and additives which decide the thickness, flow and appearance when dried (G. J. Martin, 2006). The marker is preferable by communities, organizations and individuals instead of using chalk because of its convenience (Yuuko, 2003).

In the past five centuries, the knowledge and importance of ink has been widely expand. However, the negative impact of ink on human health has not been seriously known by consumers. A specific type of inks including those existed in printers and pens can cause

discomfort and health problems. Inappropriate contact can lead to adverse effects including severe headache, skin irritation or damage to nervous system. This is caused by solvents or pigment such as p-anisidine that is used to enhance colour and shiny properties of the ink. In order to protect the user's health, it is important to create ink from non-toxic material (W. J. Barrow, 1972). Thus, ink should be produced using a non-toxic and readily available plant-based raw material. This is the most suitable alternative to commercial marker ink and consequentially contributes to the development of an eco-friendly environment (D. N. Tewari, 1995).

Hence, the latest global trend emphasized on clean production opens a new chapter in exploring for more non-toxic material and renewable resources. This future aim can be extended to the development of plant-based marker ink. Typical inks used in the manufacturing of markers are made up of synthetic ingredients like petroleum and chemical-based solvents. These synthetic materials are hazardous and harmful to the health of users and the environment. Thus, economic and ecological restrictions were imposed by many countries on the usage of synthetic materials which caused toxic, allergic, carcinogenic and harmful reactions. The ink extraction from plant sources has been introduced and slowly gained popularity in various applications.

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During the early years of ink manufacturing, ink can be made from berries, barks and leaves extracted from plant. These natural sources are mixed with other substances to create a variety of colours for dye, paint and ink. Tea leaves are used to make ink due to its shades which can form colours like yellow, green, brown and black (Lopierre, 2011). In the middle of 1800, synthetic substitutes were created by chemists (Druding, 1982). A small amount of fabric dyes were successfully extracted from herbal sources by early 20th century. Because of ecological and environmental concern, interest in studying and creating natural ink has been increased (Balva, 1991).

Nowadays, natural ink is essential in the textile, leather, cosmetics, food or pharmaceutical industries because of its ideal biodegradable properties. Natural inks are more reliable when compared to pigment-based inks due to its ability to form higher colour intensity and dissolution in the liquid phase. The production of natural ink is a cheaper and efficient process.

Among natural colour sources, tannin from plants is an excellent alternative to replace or add into the synthetic dyes and increase its biodegradable and environmental-friendly properties. Tannins are naturally occurring compounds commonly found in parts of plant including leaves, roots, barks, fruits, flowers, skins and shells. Tannin is a good sources of phytoconstituents and colouring compounds (Mohammad, 2016).

Besides tannin, anthocyanin is a famous natural colour pigment that can be found in plant tissues. It can be used as an alternative natural colour extract to substitute synthetic colourants due to light colour and high solubility. Food scientists and horticulturists researched on anthocyanins for its effect on the colour quality of vegetables and fruits (Wrolstad R, 2004).

Mangosteen leaves have been studied to be an alternative raw material for ink, which can replace petroleum or chemical-based solvent. The pericarp of a mangosteen fruit has been used for centuries as a medicinal agent in Southeast Asia countries to treat inflammation, dysentery, diarrhea, cholera, skin infections and wounds (Mahabusarakam, 1987). Studies reported that the rind contains 7 to 15 percent tannin and is used to tan leather and dye fabric into black colour. It has been used to make soap, shampoo and conditioner.

Plant-based marker ink allows the establishment of long-term sustainability with the economy, ecological aesthetic and social benefits (Powar, 2014). This study on the development and optimization of plant-based marker ink using mangosteen leaves as eco-friendly ingredients has been conducted. Additives including Glycerol, Benzalkonium Chloride, Ferrous Sulphate and Carboxymethyl Cellulose (CMC) were incorporated in the formulation of ink to enhance its properties. Fractional Factorial Design (FrFD) and Response Surface Methodology (RSM) were implemented to optimize the formulation of the

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ink statistically. In order to study the properties of the ink, AR-G2 Rheometer and HunterLAB Ultrascan Spectrophotometer were used for viscosity and colour intensity tests, respectively. Thermogravimetric Analysis (TGA) was conducted to test the drying speed of ink while Scanning Electron Microscope (SEM) was used to investigate the surface morphology of the mangosteen leaves.

1.2 Problem Statement

Due to the limited studies on eco-friendly ink, the development of plant-based marker ink is conducted in this study. Although Mangosteen contributes to a wide range of applications in various fields, most studies have only discussed the utilization of Mangosteen in the medical field. Therefore, Mangosteen leaves are selected to be used as the raw material of plant-based marker ink due to its potential in the engineering field.

Inks are commonly made from non-renewable synthetic resources such as the petroleum and chemical-based solvents which harm the users and environment. Inappropriate contact with ink may cause severe headache, skin irritation, or nervous system damage. These effects may occur probably due to the solvents or pigment, such as p-anisidine.

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Other researchers have conducted the development of marker ink from leaves. However, the proper method in determining the optimum formulation using statistical optimization method was not applied. Besides, minimal research has been undertaken to identify the impact of a number of factors on the properties of the marker ink. In this study, a new and novel formulation of Mangosteen leaves-based marker ink was developed. By using a statistical approach, this innovative formulation is predicted to have significantly good viscosity, colour intensity, and drying properties. The new ink, based on Mangosteen leaves, is expected to have efficient and cost-effective properties with wide potential applications in the industry.

1.3 Research Objectives

1. To identify the significant effect of different additives on the viscosity, colour intensity, and drying speed of plant-based marker ink using Fractional Factorial Design (FrFD).

2. To determine the optimum composition of the plant-based marker ink using Response Surface Methodology (RSM).

3. To study the microstructure of Mangosteen leaves during colour extraction.

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1.4 Research Scope

The current research aimed to explore and develop plant-based marker ink from Mangosteen leaves with the incorporation of additives, including Glycerol, Benzalkonium Chloride, Ferrous Sulphate, and Carboxymethyl Cellulose (CMC). The viscosity, colour intensity, and drying properties of the ink are tested by using the AR-G2 Rheometer, HunterLAB Ultrascan Spectrophotometer, and Thermogravimetric Analysis (TGA) model, respectively. The significant effect of different additives on the ink properties is identified by using Fractional Factorial Design (FrFD). The optimal ink composition is optimized by the use of Response Surface Methodology (RSM). The microstructure of Mangosteen leaves during colour extraction is analyzed using a Scanning Electron Microscope (SEM).

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CHAPTER 2: LITERATURE REVIEW

2.1 Plant-based Marker Ink

For centuries, ink is produced from natural resources like leaves, berries and barks extract. Colour is extracted fro natural plant sources to produce ink, dye or paint when mixed with other substances. Early records proved that tea leaves are used to create ink due to its shades that can produce yellow, green, brown and black ink. (Lopierre,2011).

Alguzar *et al.* (2015) studied the usage of plant extract as an alternative to substitute marker ink. This study aimed to determine the effectiveness of Mayana and Alugbati extract as material to make marker ink. It has been proved that the amount of water and plant supply must be balanced when manufacture marker ink from plant extract. This can create good qualities of marker ink such as natural odour, optimum colour output, even distribution of ink on the cloth or paper. Although the Mayana and Alugbati extract did not produce considerable tanning of ink colour as compared to commercial ink, the ink did not have a strong odour, and its stain is easy to remove either on paper or fabric.

Caparas (2010) conducted a research to determine the effectiveness of the sawdust extract obtained from Narra tree as a dye. The results showed that 75 percent of the respondents agreed that sawdust extract could be made into a dye for clothing. Based on the findings and results, it can be proved that sawdust extract can be a source of natural dye, which is very useful and produce environmental-friendly dyes. Thomson (2006) stated that the heartwood of Narra (Pterocarpus indicus) contained various red compounds which can make a red dye. Narra bark extract can be used for tanning due to the brown ingredient known as tannin or tannic acid.

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Nwafulugo *et al.* (2019) studied the production of marker ink from berry ink extract. The liquid was extracted from the berry through cooking, mashing with addition of apple cider (vinegar) as a solvent extractor. The extracted liquid was mixed with gum arabic, methanol and dye at 108ml, 18ml, and two teaspoons, respectively. The physical properties of the sample were tested and compared to conventional ink. The results showed that the produced ink exhibits high quality. It has a pH of 9.3, drying time of 2.3 seconds and viscosity of 9.5×10^{-4} Ns/m^2.

Powar *et al.* (2014) explored the development of herbal ink as a safe, easy and environmentally friendly alternative. Four herbal inks were produced from different biological sources which are beet root, citrus peel, butterfly flow petals and butterfly tree. The herbal inks were evaluated according to several parameters, including colour, brightness, odour, taste, drying time, flowability, non-clogging property, viscosity, permanency of colour and stability. After one week of exposure in UV rays, there was no visible change of colour of the ink. The inks were free-flowing, non-clogging and contain correct colour concentration. All the synthesized inks showed permanent colour for one week, varying between three and eight days. All inks were shown to be edible, safe, easy to prepared and stable.

Noah *et al.* (2018) performed the preliminary study on the production of brown ink from gmelina arborea fruit extract. The objective is to determine the potential of producing brown ink from gmelina arborea fruit extract and to compare the ink produced with commercial ink using an ink flotation test. The Gmelina arborea fruit extract was produced by squeezing the fruit manually to extract the juice, then soaking the fruits in water for two hours. Five different concentration levels at 100, 80, 60, 40, and 20 percent of the ink were produced using ethanol as diluent and coconut vinegar as an additive. This is to preserve the ink from biodegradation and to enhance its stability and permanence on paper once dried.

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The ink produced was compared with the commercial ink (control) to determine the best concentration level for ideal ink penetration on specific paper surfaces. The results showed that the production of brown ink from Gmelina arborea fruit extract is desirable. The Gmelina fruits are effective as an alternative to cheap and environmentally safe raw materials for ink production.

Dagde (2019) studied the formulation of whiteboard marker ink using locally sourced raw materials like natural wood from mango, rubber and ugba (oil bean) trees. The pyrolysis process carried out on the different woods resulting in a feasible result. The pH, viscosity, volumetric flow rate, and density test conducted revealed a range of values for the ink produced. In comparison, a more suitable match in oil bean charcoal ink was recorded as compared to the international standard ink, but higher values were reported for Mango and Rubber charcoal ink. Finally, the comparison proved that oil bean charcoal ink is better. The model developed showed that the final concentration of the ink has great potential to be estimated in the future.

2.2 Mangosteen Leaves

Mangosteen is categorised as a superfruit that is produced by Garcinia mangostana L. The genus Garcinia is native to Asia and Africa and consists more than 300 distinct species from several families of bioactive compounds (Chin *et al.*, 2008). Demand for Mangosteen is increasing in the current market due to its potential health benefits (Yapwattanaphun, 2002).

Mangosteen is planted in majority of the countries of Southeast Asia, such as Malaysia, Indonesia, Philippines and Thailand. The primary active components of the Mangosteen fruit are known as xanthones, which have several benefits, including anti-

inflammatory, anti-allergic and anti-convulants properties. The pulp and seed possess a delicious nutty flavour that can be boiled with sugar to produce topping for ice cream or sherbet. The wood can be used to make cabinets, furniture, fence and building materials. In the Philippines, people used to make a decoction of mangosteen leaves, bark, and rind that can reduce body temperatures. The rind contains 7 to 15 percent tannin and is used for tanning leather and dyeing in black colour. It is used to make soap, shampoo and conditioner (Araño, 2004).

In the material science and engineering field, different parts of the plants have been utilised and converted into useful components of fabric and solar cells, carbon dots (C-dots), activated carbons (ACs) and advanced biomedical materials. One of the major uses of Mangosteen in this field is a natural dye for fabric and solar cells due to its prominent colour. Fully ripened Mangosteen pericarp contains anthocyanins which contribute to the dark purple or red colour (Abdul-Rahman, 2017).

Tannin can be extracted from Mangosteen for brown colouring property (Kusumawati, 2017). These natural dyes exhibits great potential in the textile industry. Because of their biodegradable and nontoxic properties, they are safe for the environment compared with synthetic dyes, such as Ru complexes. For instance, researchers have successfully dyed cotton fabrics by using mangosteen extract.

The use of fixing chemicals such as iron sulfate, aluminum, and lime add together with Mangosteen extract, produced different colours for the fabric, such as green, light brown, and dark brown. Additionally, Faiz *et al.* showed that vitamin C treatment improves the colour retention of silk fabric dyed with Mangosteen husk (Faiz, 2016). These studies proved that mangosteen waste extract can be used as a cheap natural dye in the fabric industry.

In general, Mangosteen has been utilized for various purposes, ranging from industrial products to applications in advanced technologies and biomedical field. Nowadays, the use of Mangosteen is studied in postharvest biology areas, food science areas and engineering fields for fabric and solar cell dyes, activated carbon and advanced biomedical materials.

2.3 Colour Pigments and its Function

2.3.1 Tannin

Tannins are defined as polyphenolic secondary metabolites of higher plants. They are either galloyl esters and their derivatives that are attached to a variety of polyol-, catechin- and triterpenoid cores or they are oligomeric and polymeric proanthocyanidins that can possess various interflavanyl coupling and substitution patterns (Khanbabaee & Ree, 2014).

The name 'tannin' is derived from the French language 'tanin' which means tanning substance (Römpp, 1997). Since prehistoric era, it is known that certain organic substances contain tanning properties and can be used to tan animal skins for leather production. The traditional tanning of animal skins using plant tannins has been replaced slowly by mineral tanning, as known as alum tanning. Chromium tanning has been used more frequently since the end of the 19th century (H. Ottiger, 1991).

Naturally, the tannins are founded in many different families of higher plants such as in chestnut wood, oak wood and plant galls. Their composition varies according to their sources and has a molar mass of up to 20,000 D. Almost every part of the plant possess high concentrations of tannin, such as in the leaves, bark, wood, fruit, roots and seed. An increase in tannin production is generally linked to several diseases of the plant (L. J. Porter, 1989).

Hence, it was assumed that the biological role of tannin in many plants is associated with protection against infection, insects or animal herbivory (E. Haslam, 1989).

The tannins appeared as light yellow or white amorphous powders or shiny, nearly colourless, loose masses, with a specific strange odour and strong taste (J. Falbe, 1995). The uses of tannins ranging from tanning since 1500 BC, to medicinal applications and food industry. In the medical field, tannin-containing plant extracts are used as astringents to heal stomach and duodenal tumors, and act as anti-inflammatory, antiseptic and hemostatic drugs especially in Asian countries like Japan and China (R. Saijo, 1989). Thus, it is proved that tannins are the active ingredients of plant-based medicines (E. Haslam, 1996).

In the dye industry, tannins act as caustics for cationic dyes as known as tannin dyes. Tannins also used in processing of inks called iron gallate ink. In the food industry, tannins are used in the clarification of beverages like wine, beer and fruit juices (G. Würdig, 1989). Other industrial applications of tannins include manufacturing of textile dyes, as antioxidants in the beverage industries, and as coagulants to produce rubber (J. Falbe, 1995).

2.3.2 Anthocyanin

Anthocyanins are essential colour pigments present in all plant tissues (Kong, 2003). The name anthocyanin derived from the combination of two Greek words whhich are Anthos (flower) and Kyanos (blue). Anthocyanins are naturally occurring compounds consist of a member of the flavonoid class of phytochemicals, which can be found in cocoa, cereals, fruits, honey, olive oil, nuts, wines, tea, vegetables, red cabbage, red radish, blackcurrant and black carrot (Grotewold, 2006). These pigments are discovered widely in various parts of a plant

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such as leaves, stem, fruits, flower, root and tubers and give different colours such as red, orange, purple and blue (Holton, 1995).

Anthocyanin is vital and essential in the predation of carnivorous plants and pollination to attract insects for crop plantation (Gould, 2004). The pigments also eliminate cell multiplication and other pivotal cellular mechanisms like protein synthesis (Reddy, 2005).

Besides serving as colouring agent in plants as the primary role of the anthocyanin, they have other health-related benefits. Studies reported that anthocyanins in fruit juices were effective in solving age-related problems related to the human body (Joseph, 2003). The metabolites produced during digestion could be responsible for the health advantages related with anthocyanins (Kamiloglu, 2015). It aids to reduce the problems associated with cancers, oxidative stress and cardiac disorders (Xie, 2014). In addition, anthocyanins are important for human health because of their marked dietary use of US 180-215 mg/day (Lo Piero, 2005). It was reported with positive results in the treatment of various diseases (Cao, 2001).

Due to the light colour and higher solubility of anthocyanin, it can be considered as a potential natural colour extract to substitute synthetic colourants. Food scientists and horticulturists examined anthocyanins for its high effect on the colouring quality of vegetables and fruits (Wrolstad R, 2004). Anthocyanins are grouped under the family of flavonoids compound. These colour pigments are found in fruits and flowers and able to attract insects and animals (Rasmussen, 2005).

Chaovanalikit *et al.* (2012) studied the anthocyanin content of Mangosteen. It was found that most anthocyanin content was found in the outer pericarp. This research recognized anthocyanin for its antioxidant properties.

2.4 Additives and its Function in Marker Ink Production

2.4.1 Glycerol

Glycerol (1,2,3-propanetriol) is a colourless, odourless, viscous liquid with a sweet taste. It is made from both natural and petrochemical substances (Pagliaro, 2008). Glycerol is one of the most versatile and valuable chemical materials (J. Bonnardeaux, 2006).

In 1779 of the modern era, Glycerol was discovered by Swedish chemist Carl W Scheele, who founded a new transparent liquid when heating olive oil with litharge. It is completely soluble in water and alcohol while slightly soluble in most of the accessible solvents. However, it is insoluble in hydrocarbons. At a pure anhydrous state, glycerol has a specific gravity of 1.261 gmL^{-1}, a melting point of 18.2 °C and a boiling point of 290 °C at normal atmospheric pressure. Generally, glycerol has a ideal combination of physical and chemical properties, so it can be used in many commercial products (CRC, 2006).

Glycerol has more than 1500 known end uses, which include applications as a material or processing substance (M. A. David,1996). Glycerol has high stability under standard storage condition as it is consistent with different chemical substances. It is practically non-irritating and does not cause negative environmental impacts. Traditional commercial applications use glycerol as an additive or raw material. It acts as additive for food, tobacco and drugs. It also used to synthesis various substances like trinitroglycerine, alkyd resins and polyurethanes (Frost, 2006).

Today, the amount of glycerol accounted for approximately 160,000 tonnes for technical applications yearly and is expected to grow at an annual rate of 2.8 percent (J. Bonnardeaux, 2006). Around 28% of glycerol market used for pharmaceuticals, toothpaste, and cosmetics, 15% for tobacco and 13% for food products, and the synthesis of urethanes

accounted at 11%. The rest is used for industrial uses such as inks, varnishes, adhesives. Glycerol is typically used as a softener and plasticizer in alkyd resins and regenerated cellulose to determine properties in surface coatings and paints including flexibility, pliability, and toughness. It serves as binders in paints and inks. In short, glycerol is useful as a reactant or additive because of its non-toxicity and overall safety in general applications.

2.4.2 Ferrous Sulphate

Ferrous sulphate could be the only ferrous salt used in the production of ink. Ferrous sulphate is not hygroscopic. Thus, it does not absorb moisture or oxidize quickly in the air. These properties show the potential of using this material for the preparation of ink. A study has been conducted to determine the effect of substituting an equivalent amount of ferric sulphate for ferrous sulphate during the preparation of ink and compared their corrosiveness of ink. It is shown that the dye has no corrosive effect.

In the preparation of ink, less water required by material to prevent the prepared ink powder from forming a cake layer is preferred. In order to determine the iron salts that meet this requirement, three iron salts are tested using ferrous sulphate crystals, ferric sulphate and ferric chlorosulphate. These powders were placed in open test tubes and left them in a condition with 50 percent relative humidity. The powder containing ferric chlorosulphate started to form a cake after one month, whereas other iron salts were still loose after two months. It proved that ferrous sulphate crystals and anhydrous ferric sulphate are suitable for the production of ink with moderate protection from atmospheric moisture (Zimmerman, 1935). In this study, ferrous sulphate is responsible for the black colouration of ink and can be used as a disinfectant (Fallis, 2013).

2.4.3 Benzalkonium Chloride

Benzalkonium Chloride refers to a quaternary ammonium antiseptic and disinfectant with actions and functions similar to other cationic surfactants. Besides that, it is used as an antimicrobial preservative for pharmaceutical products. Benzalkonium chloride is selected as a preservative for many multidose aqueous nasal, ophthalmic, and otic products. Since the 1950s, it has been used as a preservative in eye drops, and it is still the most frequently used preservative in ophthalmic solutions at a concentration of 0.01 to 0.02 percent. It is an efficient bactericidal and fungicidal agent that helps to reduce the growth of organisms in multidose containers (European Medicines Agency, 2017).

Based on the survey on preservatives in ophthalmic preparations performed in 2009, which involved 17 member states, the result shows that benzalkonium chloride is the main preservative in ophthalmic preparations on the EU market, nearly 74 percent of ophthalmic preparations contain benzalkonium chloride as a preservative (EMEA, 2009). Benzalkonium chloride is further utilized as a preservative in more than 200 medicinal products for the nasal route of administration. In this study, benzalkonium chloride is used as an additive that prevents plant-based marker ink from solidifying (Fallis, 2013).

2.4.4 Carboxymethyl Cellulose (CMC)

Carboxymethyl Cellulose (CMC), also known as cellulose gum is a cellulose derivative with carboxymethyl groups (-CH_2-COOH) incorporated to some of the hydroxyl groups of the glucopyranose monomers that contribute to the cellulose backbone. It is often used as its sodium salt, sodium carboxymethyl cellulose (Codex, 2016).

CMC is used as a viscosity modifier or thickener. It is used to stabilize emulsions in various products. It is also a constituent of many non-food products. For example, toothpaste, diet pills, detergents, laxatives, textile sizing, water-based paints, reusable heat packs, and a variety of paper products. It is mainly used because it has a high viscosity, is non-toxic, and is generally considered to be hypoallergenic as the major source fiber is either softwood pulp or cotton linter (Dow, 2015). CMC is used extensively in gluten-free and reduced-fat food products (Stanford, 2012). For laundry detergents, it is used as a soil suspension polymer designated to deposit onto cotton and other cellulosic fabrics, producing a negatively charged barrier to soils in the wash solution. CMC served as a lubricant in artificial tears.

Moreover, CMC is used as a thickening agent. For instance, CMC is used as an ingredient of drilling mud in the oil-drilling industry, where it acts as a viscosity modifier and water retention agent.

Habib and Khoda (2018) studied the development of clay-based novel bio-ink by optimizing the composition of materials, which are montmorillonite (MMT), carboxymethylcellulose (CMC), and sodium alginate. CMC is a high molecular-weighted water-soluble polysaccharide used for viscosity modifier or thickener. It is reported that the binding of CMC's matrix protein assists in cell migration and cell attachment. Moreover, the alginate-CMC hybrid hydrogel is utilized to fabricate beads for various drug delivery experiments. The incremental addition of CMC into alginate and MMT mixture makes the bio-ink more viscous. It is expected that an improvement in the viscosity of bio-ink would help to improve printability and to achieve good shape consistency. Bio-ink with too much viscosity requires high air pressure to suspend the filament that may limit the use of available bio-printer. In fact, the encapsulated cells in higher viscous bio-ink undergo too much shear stress during suspension and inevitably be impaired.

With increasing the percentage of CMC, the materials get proper gelation and show a smooth filament surface. The microstructure of fabricated filament is analyzed by scanning electron microscopy. The cross-section diagram of the filament shows that the number of micro-pores increases with an increase in the amount of CMC. Therefore, those micro-pores potentially improve the nutrients and growth factor supply in the case of cell-laden scaffold fabrication. The qualitative observation of the collapse test for various compositions of material identifies that with increasing the percentage of CMC up to two, the bio-ink gets from under gelation to proper gelation state, which leads to a minimal deflection of the suspended filament.

2.5 Ink Properties

2.5.1 Viscosity

Viscosity is a measure of the ability of a liquid to resist flow. A thick liquid that does not flow easily has high-viscosity, while a thin liquid that readily flows has low-viscosity. Ink viscosity dictates many aspects of a job. To control the viscosity of ink, understanding the factor resulting in high or low viscosity of the ink is essential. The viscosity of ink has a strong effect on the way it behaves and eventually displayed on the surface, such as the paper. Low viscosity inks have several benefits, such as the need to add less pressure to the pen to ensure that it is written cleanly. It makes it possible to keep the pen with less tension on the palm, conserve energy, and improve comfort. It can also be converted into higher writing speeds. Inks typically have a broader range of colours and tend to write fine lines and more simply (ZebraPen, 2016).

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Typically, the ink should flow easily and not close the end of the marker. Ideally, the maximum colour intensity of the ink should be at a minimum speed. Liquids such as printing inks have thixotropic properties. Inks become highly viscous under normal conditions but slowly become less viscous when force or stress is applied (Cindy Thai, 2011). The higher the effort and the resulting measurement values, the thicker the ink, the more difficult for the ink to flow, in contrary, the lower the value, the thinner the ink. The higher the viscosity of ink, the longer the drying time. At the same ambient conditions, the thinner ink can flow faster.

Yusuf and Sardar (2018) studied the rheological behaviour of xanthan gum solutions in the presence of additives. The viscosity of all samples decreases with an increasing shear rate showing the shear-thinning behaviour of xanthan gum solution at all concentrations. This is because the polymer chains are uncoiled and partly aligned. Xanthan gum solutions are highly pseudoplastic, which means that the resistance to flow decreases as the shear rate increases. Although the shear-thinning behaviour is shown under the applied shear rate, the original viscosity is restored nearly instantaneously when the shear rate is removed (Zhong, 2013).

Syafiq, Amir, and Sharon (2014) studied the effects of additives on the rheological properties of dark chocolate. The impacts of xanthan gum, corn starch, and glycerin blends on both rheological parameters, which are casson yield stress and casson viscosity, were determined. As the inclusion of blends (XG / CS / GL) in chocolate production increased from 5 to 15 percent, all parameters, including viscosity and chocolate yield stress, were gradually increased. William and Phillips (2003) have shown that the application of a thickening agent affects the rheological properties of the fluid. They stated that higher yield stress and viscosity of solution could result from xanthan gum-glycerin affinity.

2.5.2 Colour Intensity

The properties of the ink representing the good quality of the ink are a smooth, filterable solution but not a suspension, it flows smoothly from the pen and does not disperse to the paper. Since the ink is natural, the mold must not be seen on the solution and free from pronounced unpleasant odour. Likewise, the ink should possess an intense colour that does not become paler nor bleach out entirely as it is used in writing (Lindquist, 2002).

The technology of ink's colour is based on the Young-Helmholtz theory of three colour vision. The theory says that white light is composed of light from a continuous spectrum of wavelengths in which humans perceive only three broad bands of this light, which are blue, green and red light. Any other colour of light is assisted by a correct combination of these three primary colours.' Subtractive colours' are created by' subtracting' one of these three primary colours from white light, such as red and blue light. When white light strikes an object, some light is absorbed, and the rest is reflected. The colour that is viewed as the colour of the object is the colour of the reflected light. Four different colours of ink are used in printing inks, which are cyan, magenta, yellow and black (Ahmed, 2007).

2.5.3 Drying Speed

In the ink industry, shortening the ink drying time is an important factor towards achieving shorter lead times. It is difficult for the printing industry to deliver printed products in a shorter time because it takes time to make the proof and wait for inks to dry. For example, in duplex printing, materials printed on one face need to be left for several hours or overnight before printing on the other face in order to prevent offset. A high drying speed also contributes to benefits such as low substrate damage and superior image quality.

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In this study, Thermogravimetric analysis (TGA) is used to conduct the drying test. It is a method of thermal analysis in which the mass of a sample is measured over time as the temperature changes. A thermogravimetric analyzer continuously measures mass while the temperature of a sample is changed over time, as low substrate damage and superior image quality.

Gu et al. (2017) studied the comparison of thermal decomposition and chemical reduction of particle-free silver ink for inkjet printing. TGA analysis is carried out to study the thermal decomposition and chemical reduction processes. The result indicates that the silver film is formed at a very low temperature compared with thermal decomposition.

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CHAPTER 3: Methodology

3.1 Overview

This chapter presented the materials used in developing marker ink from Mangosteen leaves. The detail on sample preparation is discussed as well. Testing conducted and their procedures are presented. Experimental design for Fractional Factorial Design (FrFD) and Response Surface Methodology (RSM) are thoroughly explained at the end of this chapter. This study used the experimental method of research. Figure 3.1 shows the flowchart for this study.

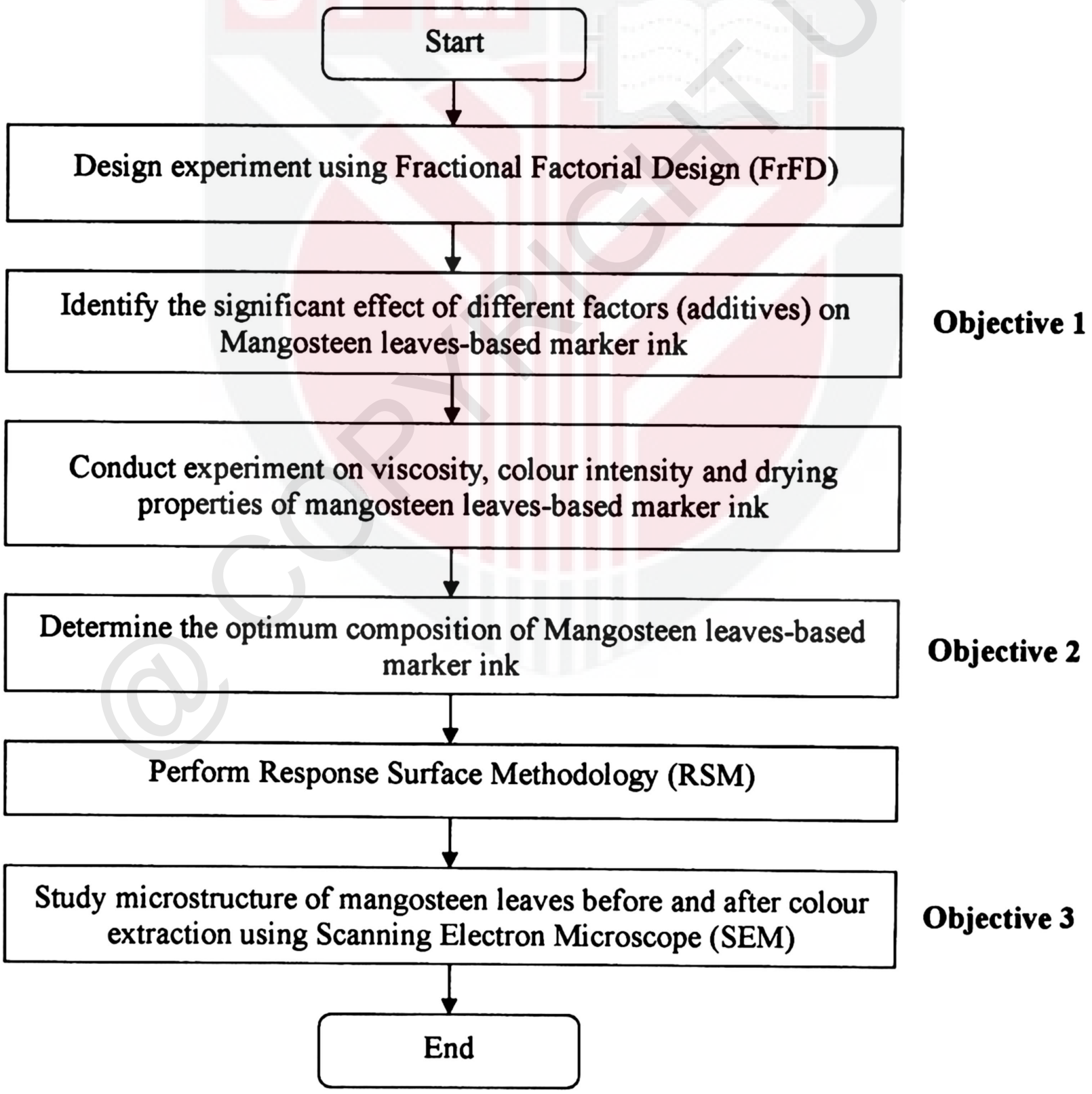

Figure 3.1: Flowchart of the study.

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3.2 Sample Preparation

Preliminary experiments showed that the steps in preparing the Mangosteen leaves-based marker ink were significantly important in obtaining a homogeneous and proper colour and texture of the ink. The marker ink was prepared by following the steps, as shown in Figure 3.2.

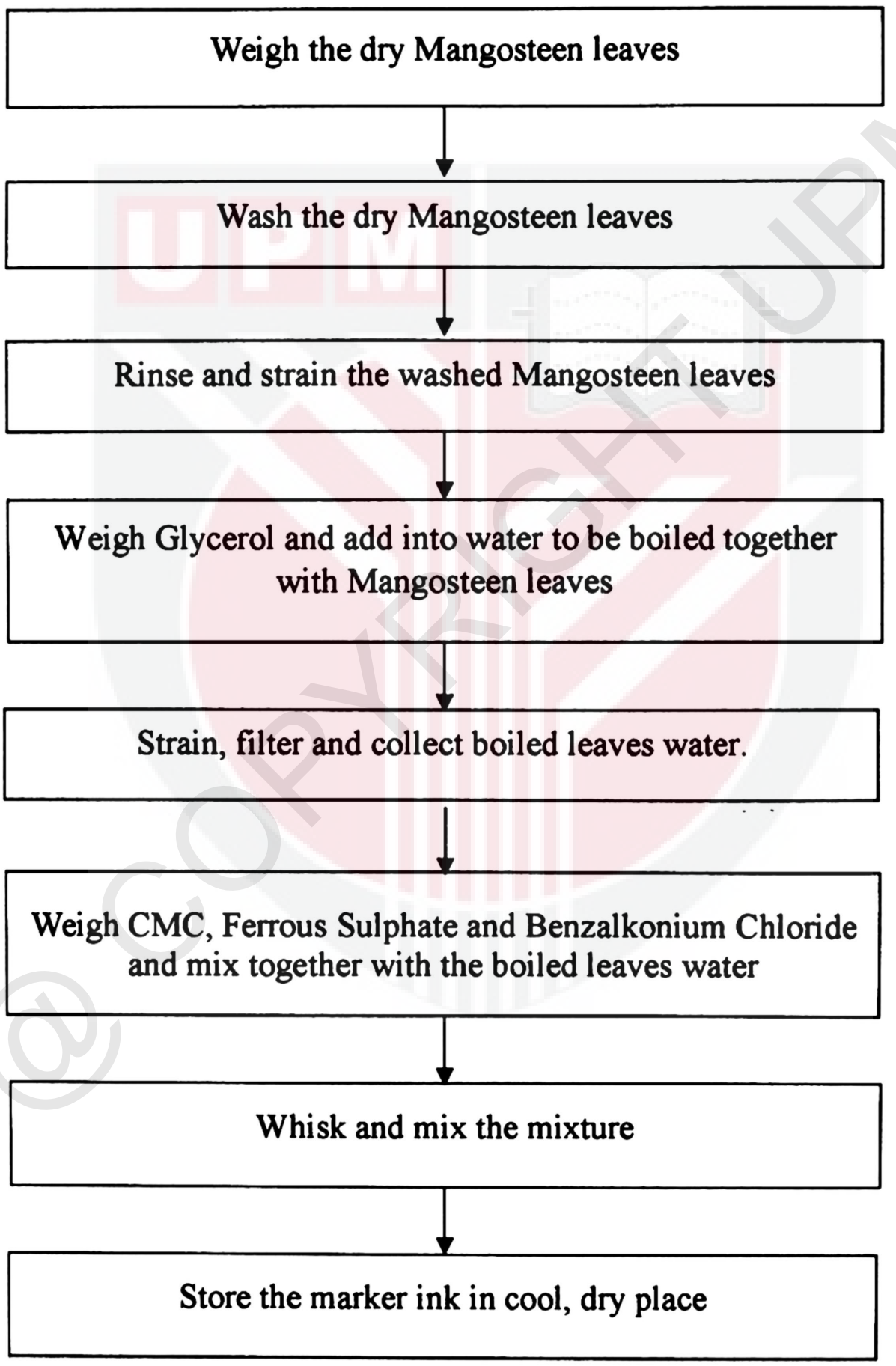

Figure 3.2: Flowchart for mixture and fabrication process of Mangosteen leaves-based marker ink

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The steps from weighing the dry leaves until the mixture of leaves, glycerol, and water were boiled is shown in Figure 3.3(a-d). The dry Mangosteen leaves were first weighed by using a digital weighing scale (Figure 3.3(a)). Then, the dry Mangosteen leaves were washed thoroughly and rinsed (Figure 3.3(b)). Glycerol was weighed by using a digital weighing scale whereas water is measured by using measuring cylinder, then added into the beaker and thoroughly stirred for 30 seconds (Figure 3.3(c)). Glycerol was boiled together with water to increase the extraction rate of colour from the leaves. A beaker containing water and Glycerol, which were first measured at designated weight, was boiled using a hot plate at a heating degree of 8. Once the mixture boiled, the heating degree was reduced to 1. The washed Mangosteen leaves were weighed and added into the boiled water. The beaker was covered with plastic foil, to reduce vapour evaporation, and left to boil for 30 minutes (Figure 3.3(d)). After 30 minutes, the hot plate was switched off, and the boiled leaves water was collected into a container using a strainer.

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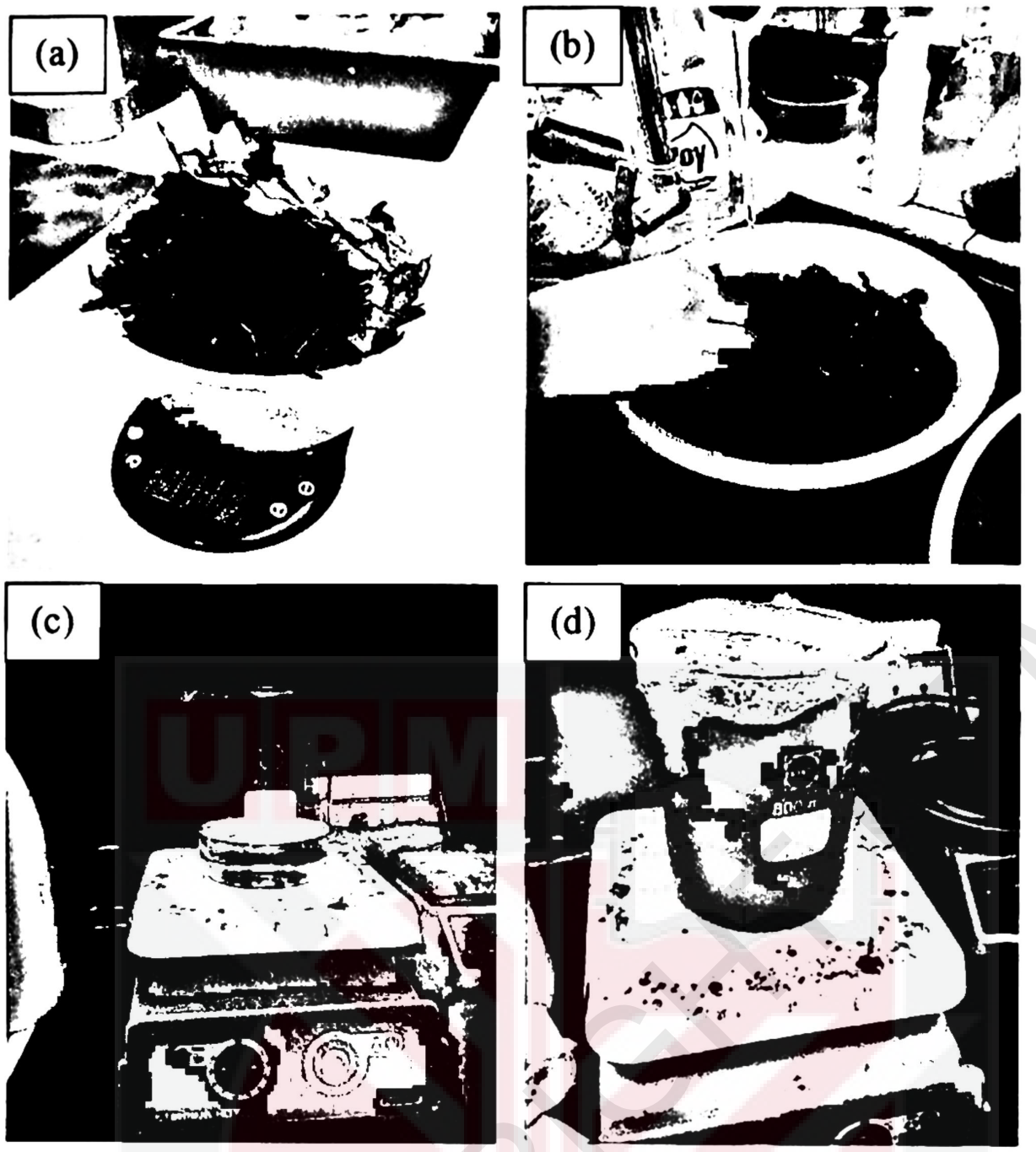

Figure 3.3: Steps in the ink preparation - (a) weigh dry leaves, (b) wash dry leaves, (c) weigh Glycerol and water, and (d) boil the leaves, Glycerol and water

Carboxymethyl cellulose (CMC), Ferrous Sulphate and Benzalkonium Chloride were weighed using a digital scale and added into the boiled leaves water as shown in Figure 3.4(a-c). The mixture was stirred thoroughly by using a whisker (Figure 3.4(d)). Whisker was used to prevent the mixture from clumping, thus producing a homogeneous ink solution. The produced ink was stored in a glass container and covered with a cap (Figure 3.4(e)). The ink was kept in a cool, dry place for 24 hours prior to testing.

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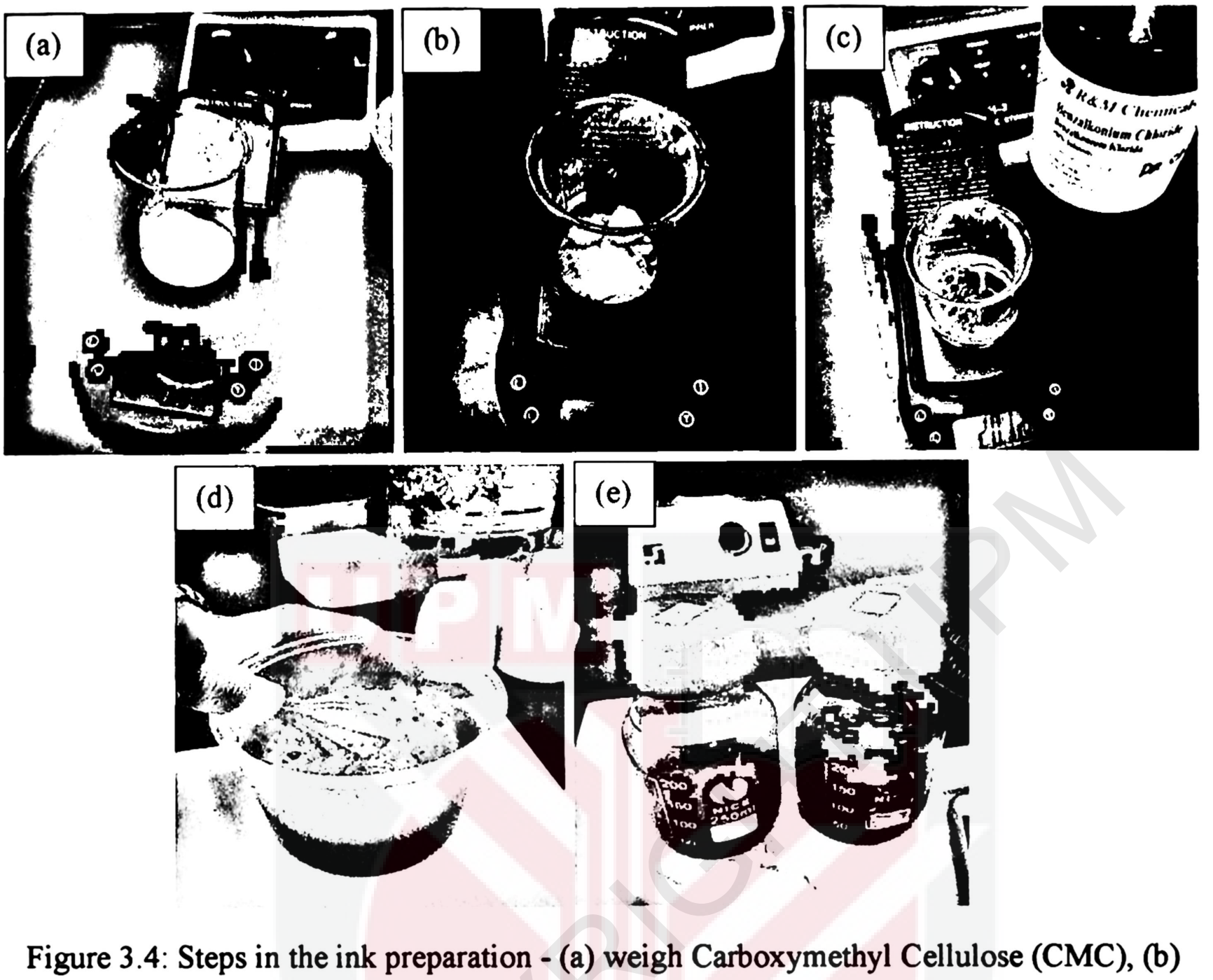

Figure 3.4: Steps in the ink preparation - (a) weigh Carboxymethyl Cellulose (CMC), (b) weigh Ferrous Sulphate, (c) weigh Benzalkonium Chloride, (d) mix with boiled leaves water, and (e) store the ink

3.3 Experimental Procedure

3.3.1 Viscosity Test

The viscosity of the produced inks was investigated to develop a low viscous ink solution with high drying rate. The samples were tested 24 hours after the ink samples were prepared. As shown in Figure 3.5, the advanced AR-G2 rheometer (TA Instruments, USA), which equipped with zero gap geometry and measurement, was used to conduct the viscosity test following the ASTM D-445 standard at 23°C (Standard, A. S. T. M., 2006).

The running procedure is as follows; (1) the air compressor was switched on, (2) the rheometer power supply was switched on and beep sound continues until it stopped, (3) the geometry cover at the rheometer was opened, (4) an AR instrument software was opened

from the desktop, (5) room temperature of 23°C for rheometer was set in the software, (6) a geometry with properties of 60mm cone diameter, 1:0:0 cone angle ratio (deg:min:sec) and 30 μm truncation was installed to the rheometer, (7) zero-gap was set for 30 micrometres and the 5g of ink sample was placed on the Peltier plate by using a dropper, and (8) the experiment was started by clicking 'Run' icon and the results were recorded.

Figure 3.5: AR-G2 Rheometer

3.3.2 Colour Intensity Test

Colour intensity is important in the quality of ink. The colour intensity was evaluated using a HunterLAB Ultrascan PRO d/8 Spectrophotometer (Figure 3.6) operated in accordance with ASTM E1348 (ASTM, 2015). The samples were tested based on the following steps; (1) software EasyMatchQC was selected from the desktop and beep sound have waited for connection, (2) 50ml of ink sample was placed in front of the porthole, (3) 'Read Sample' was clicked on the software, and (4) the colour of ink samples were recorded. The colourimetric values were reported in terms of CIELAB (L*, a*, b* colour space) for CIE Illuminant D65 and CIE 10-degree Standard Observer.

Figure 3.6: HunterLAB Ultrascan PRO d/8 Spectrophotometer

3.3.3 Thermogravimetric Analysis (TGA)

Thermogravimetric analysis (TGA) was performed on plant-based marker ink samples in the Material Characterization Laboratory at Faculty of Engineering, Universiti Putra Malaysia. Four ink samples which exhibited good, moderate, poor and optimised properties were tested to investigate and differentiate their drying rates. The composition of the samples is shown in Table 3.1.

Table 3.1: The composition of ink samples tested for TGA

Ink Properties	Glycerol	CMC	Benzalkonium Chloride	Ferrous Sulphate
Optimised	25	3.14	5	1
Good	25	3	5	1
Moderate	20	4	5	1
Poor	10	4	5	1

The schematic diagram and working mechanism of this equipment are shown in Figure 3.7. As drying proceeded, the weight of coating decreases due to the solvent evaporation. The amount of solvent loss with time is monitored by the balance (Kim, 2001).

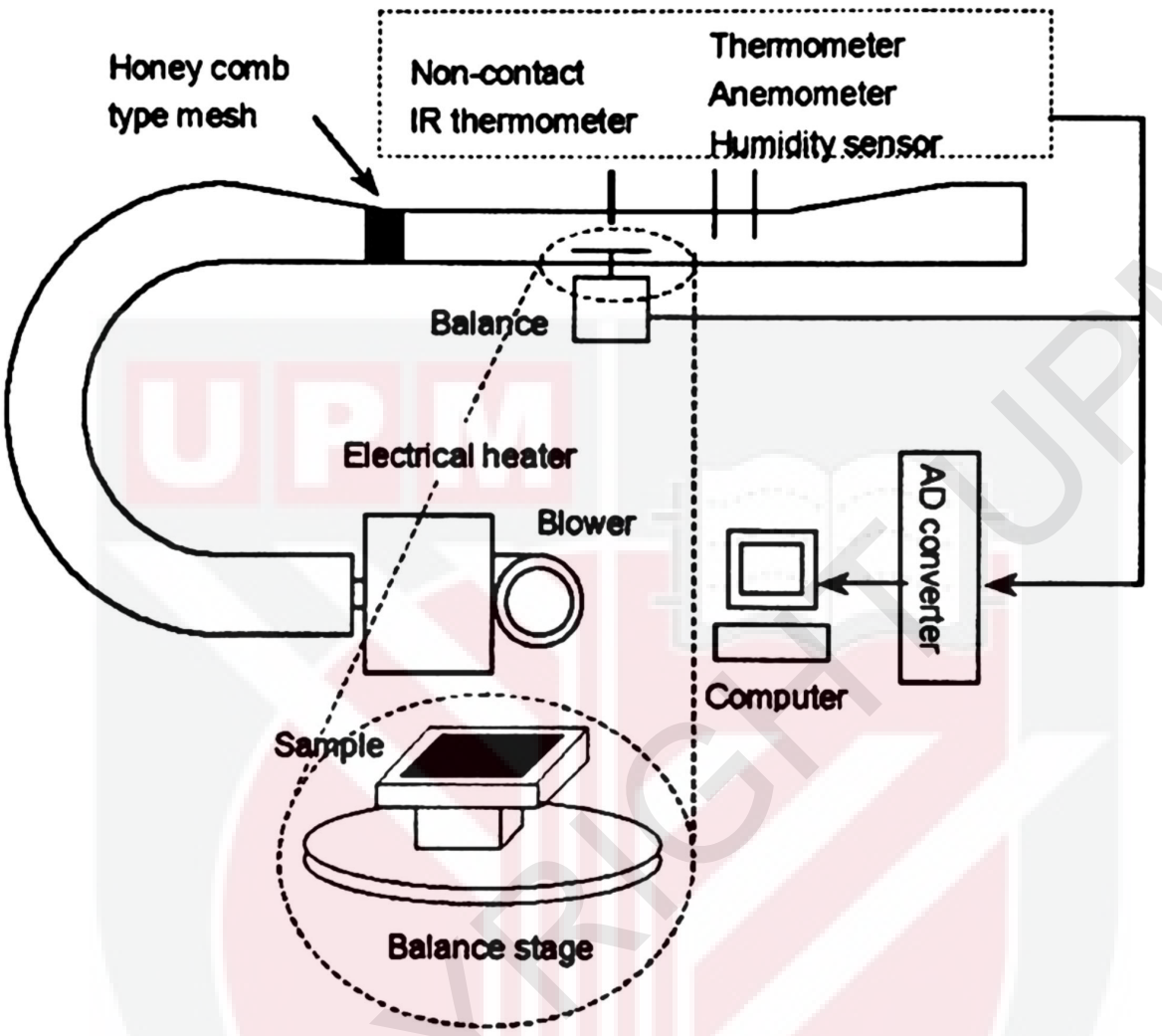

Figure 3.7: Schematic diagram of drying experiment apparatus (Kim, 2001)

The drying rate of coating is the weight of solvent loss per time divided by the area of evaporation and can be calculated using Equation 1 where R_{mass} is the evaporation rate, W is the weight of sample at a specific time, t is the time, Δt is the time interval between measurements, and A is the evaporation area.

$$R_{mass} = -\frac{dW}{Adt} \approx -\frac{W_{t+\Delta t} - W_t}{A\Delta t} \qquad \text{(Eq. 1)}$$

The equipment used is the Mettle Toledo AG-TGA/SDTA 851e model. For specimen preparation, 20mg of ink is required for each test. The tests used nitrogen as a medium while the specimens were heated from 20°C to 200 °C with a rate of 25 °C/min. Figure 3.8 shows the equipment.

Figure 3.8: Mettle Toledo AG-TGA/SDTA 851e model

3.4 Morphology Study

3.4.1 Microstructural Analysis

The Scanning Electron Microscope (SEM) is one of the most versatile instruments available for the examination and analysis of the microstructure morphology (Zhou, Apkarian, & Wang, 1971). Since the discovery that electrons can be deflected by the magnetic, electron microscopy has been developed by replacing the light source with a high-energy electron beam (O. C. Wells, 1974).

SEM analysis was carried out in the Bio-Science Institute, Universiti Putra Malaysia. The equipment used was the Hitachi S-3400N variable scanning electron microscope, as

shown in Figure 3.9. The microstructure images of mangosteen leaves including the raw leave, boiled leave without glycerol and boiled leaves with glycerol which exhibited good, moderate, and poor properties. Samples were prepared by placing on aluminium sample stubs with carbon conductive tape and were then sputter-coated with gold-palladium using Baltec SCD-005 Sputter Coater to produce high quality and brighter image. The stub with coated samples was inserted into the sample chamber of the SEM for viewing.

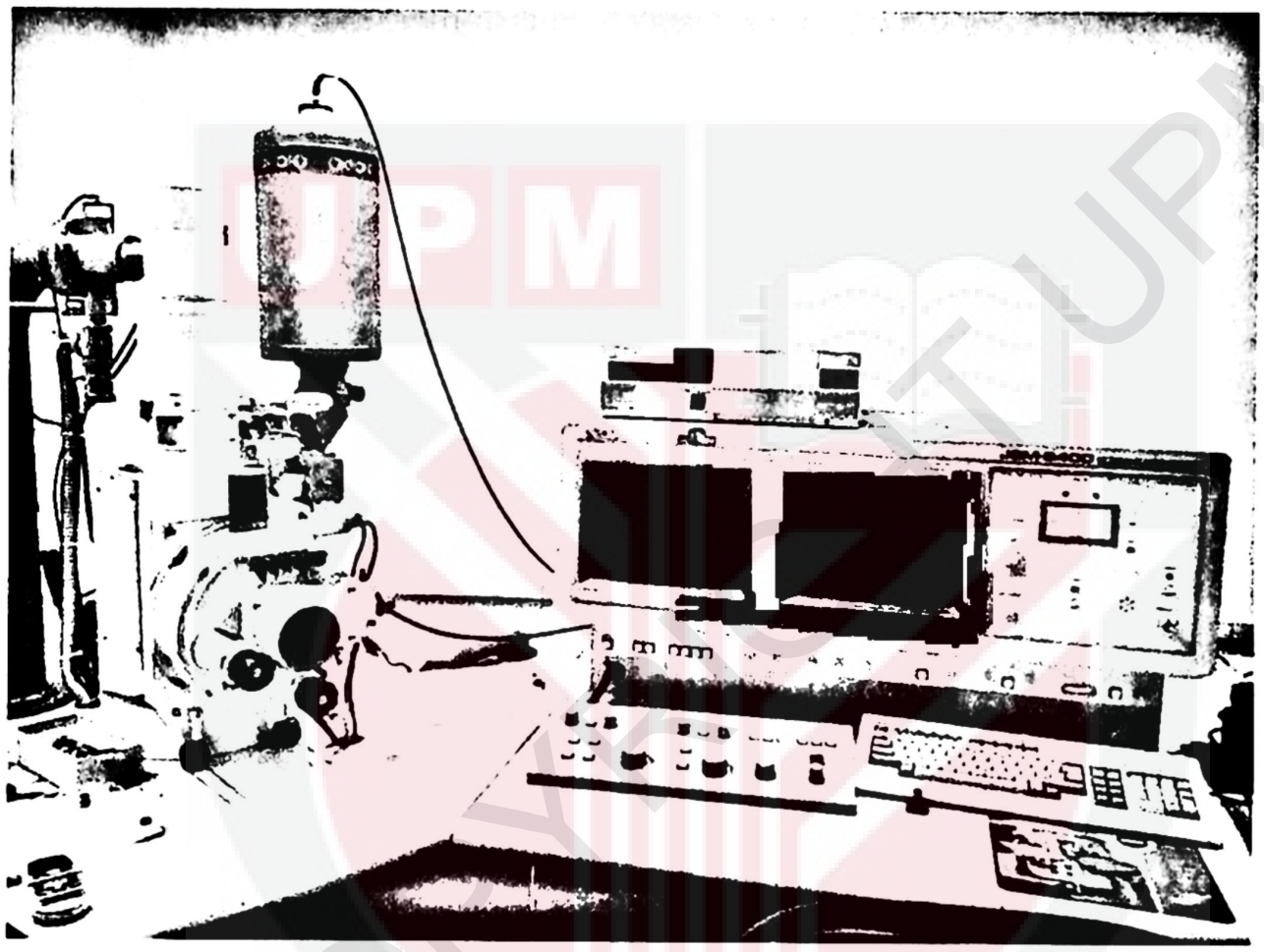

Figure 3.9: Hitachi S-3400N variable scanning electron microscope

3.5 Experimental Design and Analysis

This research was performed by a two-experimental method to determine the optimized formulation for plant-based marker ink. The first phase of experiments was called the screening process, which involved Fractional Factorial Design (FrFD) to minimize the number of factors by determining few significant main effects and grouping them from other less important ones. Following the identification of significant factors, the second phase, called the optimization process, was performed using the Response Surface Methodology (RSM). RSM has been applied to optimize the response and quantify the correlation between

factors. It was concluded by a validation process, which is optional, to assess the reliability of the response prediction model. The mean difference in percentage between the experimental and the estimated outcomes for each response defines the accuracy of the prediction model.

3.5.1 Fractional Factorial Design (FrFD)

This statistical approach began with determining the significant factors including Glycerol, Ferrous Sulphate, Benzalkonium Chloride, and Carboxymethyl Cellulose (CMC), and their levels which were based on preliminary laboratory work or results from previous literature. MINITAB, a statistical software, was used to design the experiments that were conducted. Data obtained from experiments were inserted in the MINITAB software under response column and analysed. Pareto chart, main effect and its interactions were generated. The steps conducted for FrFD are summarized as follows; (1) four factors including CMC, Glycerol, Ferrous Sulphate and Benzalkonium Chloride were considered, and the level for each factor was identified, (2) FrFD with half fraction, two levels and three replications were designed, (3) the total number of samples was 24, and the experiments were conducted in two phases which involved 12 samples per phase, (4) the results were analyzed at two orders which are the main factors and its second-degree interactions, (5) Pareto charts were generated which identified the most significant factors, and (6) two significant factors were considered for optimization process in Response Surface Methodology (RSM). Low and high level for each factor is as shown in Table 3.2. Value of each level is the percentage of the factor to the boiled leaves water.

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Table 3.2: Low and high level for the factors

Factors	Low level	High level
Carboxymethyl Cellulose	2	10
Glycerol	2	10
Ferrous Sulphate	2	10
Benzalkonium Chloride	10	50

3.5.2 Response Surface Methodology (RSM)

Box and collaborators developed response surface methodology (RSM) in the 50s (R.E. Bruns, 2006). RSM consists of a group of mathematical and statistical techniques that are based on the fit of empirical models to the experimental data obtained concerning experimental design (Bezerra, 2008). Toward this objective, linear or square polynomial functions are employed to describe the system studied and, consequently, to explore (modelling and displacing) experimental conditions until its optimization (R.F. Te ´ofilo, 2006).

RSM only requires 13 runs to complete the same experiment considering one replication. RSM is relatively more beneficial and advantageous in design with two or more factors. There are three types of the regression model to consider namely; linear, square and interaction. The full regression model equation has a vital role in providing a significant relationship between factors and estimation of the optimum value of the predicted target. Since the optimum point is unknown at the beginning of RSM experiments, it is thus reasonable to have a rotatable design which able to provide consistent precision in estimation at all points equidistant from the design centre (Box, 2005).

Before eliminating insignificant terms, all regression models were selected and analyzed. This was followed by software generation of ANOVA table. Except for main terms, other terms which were not significant were eliminated, and data were analyzed again.

According to Meyers at al. (2009), a reduced model consisting of only significant terms can increase the precision of the predictors when the goal was to obtain the optimal settings of important factors. Since this study aimed at optimizing the synthesizing factors, only insignificant terms were considered to elicit the best fit models by eliminating insignificant terms.

Contour plots were generated, and responses were optimized. In the modelling process, experimental validation is the final step to verify the accuracy and the reproducibility of the developed regression model and the RSM model. The levels for each factor are shown in Table 3.3. Value of each level is the percentage of the factor to the boiled leaves water.

Table 3.3: Analysed results from FrFD with 5 levels

Factors	**Level**				
	Lowest	**Low**	**Mid**	**High**	**Highest**
Carboxymethyl cellulose	1	2	3	4	5
Glycerol	5	10	15	20	25

The steps to conduct RSM are as follows; (1) two significant factors were considered in designing the experiment based on their significance from the FrFD, (2) full design with 13 samples, five levels and three replications were chosen, (3) a total number of samples were 39 considering 3 replications and the experiment were conducted in three phases involving 13 samples per phase, (4) the experimental results obtained were analyzed and ANOVA table was generated, (5) contour plots were generated to study the relationship between both factors and their effect on the outputs, and obtain the optimum composition of the ink.

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CHAPTER 4: Results and Discussion

4.1 Overview

A recent global trend towards sustainable production opens a new window in searching for more renewable sources and toxic-free ingredients. This future goal can be extended to the development of eco-friendly marker ink. In this study, plant-based marker ink is developed by using mangosteen leaves as raw materials with the incorporation of additives including Glycerol, Benzalkonium Chloride, Ferrous Sulphate and Carboxymethyl Cellulose (CMC) to enhance the quality. Ideally, ink should have maximum colour strength, minimum velocity and fast drying speed. The viscosity, colour intensity and drying properties of the ink are tested by using AR-G2 Rheometer, HunterLAB Ultrascan Spectrophotometer and Thermogravimetric Analysis (TGA) model respectively. The significant effect of different additives on the ink properties is identified by using Fractional Factorial Design (FrFD). The optimum formulation of the ink is optimized by using Response Surface Methodology (RSM). The microstructure of Mangosteen leaves is analysed using a Scanning Electron Microscope (SEM). The purpose of the microstructure test is to study the surface morphology and cell structure of Mangosteen leaves during colour extraction.

4.2 Fractional Factorial Design (FrFD)

The objective of FrFD was to screen and identify the significant factors (additives) that can give maximum influence on the properties of marker ink. The statistical software package (MINITAB Release 16.2) was used to analyze the experimental design. The uncoded (actual value) design matrix for the factors and both responses, which are colour intensity and viscosity for 24 experimental runs, including replication are given in Appendix 1. The response obtained was statistically evaluated, and the model was developed based on the variables with confidence levels of more than 95.00%.

4.2.1 Statistical Analysis

A reduced linear regression model was fitted with the experimental data using the least square technique. The standardized effect, P of the effects and model coefficients for colour intensity and viscosity are shown in Table 4.1 and Table 4.2, respectively. Complete statistical results for this study are shown in Appendix 3 and Appendix 4.

The P in those tables were used to determine the significance of the effects in the model. Since the confidence level was set to be 95.00% ($P \leq 0.050$), the effect was considered statistically significant. Based on Table 4.1 and Table 4.2, P equals to 0 for all factors, such condition is known as overfitted condition. Overfitting model is expected due to the low number of experimental runs and including second-order terms which are the interaction between two factors. However, since the sole purpose of the FrFD is to screen the significant factors and their interactions, the overfitted model has no effect on screening the factors. In addition, the overfitted model can be found when the difference between r-square and r-square(pred) is substantial.

Table 4.1 shows that all individual and two-way interaction effects were significant at $P \leq 0.050$. The interaction between V_1 and V_2, V_1 and V_3, V_1 and V_4 were highly significant at $P < 0.000$. All individual factors, which are V_1, V_2, V_3 and V_4, were significant at $P < 0.000$. V_1 (Glycerol) was the most significant factor because it has the highest value of net effect and standardized effect. The value for R^2, which is 0.9974 and R^2 (adjusted) of 0.9962 were very high, indicating a very good fit for the model with 99.74% of the results that can be explained. The model also showed high dependence and correlation between the observed and the predicted response values.

Table 4.1: Estimated effects and coefficient for colour intensity

Term	Notation	Net Effect	Coeff.	Std. error of coefficient	Standardi-zed Effect	P
Constant			28.122	0.02203	1276.62	0.000
Glycerol	V_1	1.578	0.789	0.02203	35.83	0.000
Benzalkonium Chloride	V_2	-2.128	-1.064	0.02203	-48.31	0.000
Ferrous Sulphate	V_3	0.565	0.282	0.02203	12.82	0.000
CMC	V_4	1.132	0.566	0.02203	25.69	0.000
Glycerol*Benzalkonium Chloride	V_1*V_2	-0.813	-0.407	0.02203	-18.46	0.000
Glycerol*Ferrous Sulphate	V_1*V_3	-1.517	-0.758	0.02203	-34.43	0.000
Glycerol*CMC	V_1*V_4	0.513	0.257	0.02203	11.65	0.000

$R^2 = 0.9974$ R^2 (adj) = 0.9962

In Table 4.2, all factors were highly significant ($P \leq 0.000$) at 95.00% confidence level. V_4 (CMC) was the most significant factor because it has the highest value of net effect and standardized effect, followed by V_3 and V_1. The value for R^2 (0.9993) and R^2 (adjusted) (0.9990) were very high as well. The value of the effects in Table 4.1 and Table 4.2 determined either the factor had a higher or lower effect on the response. For Table 4.1, the interaction between V_1 and V_4 had the greatest influence on the response with 0.513 followed by "V_1*V_3" and "V_1*V_2" with -1.517 and -0.813, respectively. For Table 4.2, the interaction between V_1 and V_2 had the greatest influence on the response with 0.2579 followed by "V_1*V_3" and "V_1*V_4" with -0.2523 and -0.1242, respectively. The positive and negative sign of the net effect indicates either the individual factors or interactions were positively or negatively affect the responses.

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Table 4.2: Estimated effects and coefficient for viscosity

Term	Notation	Net Effect	Coeff.	Std. error of coefficient	Standardi-zed Effect	P
Constant			0.2592	0.002122	122.17	0.000
Glycerol	V_1	-0.0801	-0.0400	0.002122	-18.87	0.000
Benzalkonium Chloride	V_2	-0.2484	-0.1242	0.002122	-58.53	0.000
Ferrous Sulphate	V_3	0.2447	0.1223	0.002122	57.66	0.000
CMC	V_4	0.3622	0.1811	0.002122	85.35	0.000
Glycerol*Benzalkonium Chloride	V_1*V_2	0.2579	0.1290	0.002122	60.79	0.000
Glycerol*Ferrous Sulphate	V_1*V_3	-0.2523	-0.1261	0.002122	-59.45	0.000
Glycerol*CMC	V_1*V_4	-0.1242	-0.0621	0.002122	-29.27	0.000

$R^2 = 0.9993$ R^2 (adj) = 0.9990

4.2.2 Regression Model

Using coefficient and the terms from Table 4.1 and Table 4.2, the regression models can be formulated as shown in Equation 4.1 and 4.2 where Y_{COL} is the response of colour intensity, Y_{VIS} is the response of viscosity, V_1 is glycerol, V_2 is Benzalkonium Chloride, V_3 is Ferrous Sulphate, and V_4 is CMC.

$$Y_{COL} = 28.122 + 0.789\,(V_1) - 1.064\,(V_2) + 0.282\,(V_3) + 0.566\,(V_4) - 0.407\,(V_1V_2) - 0.758\,(V_1V_3) + 0.257\,(V_1V_4) \quad \text{(Eq. 4.2)}$$

$$Y_{VIS} = 0.2592 - 0.0400\,(V_1) - 0.1242\,(V_2) + 0.1223\,(V_3) + 0.1811\,(V_4) + 0.1290\,(V_1V_2) - 0.1261\,(V_1V_3) - 0.0621\,(V_1V_4) \quad \text{(Eq. 4.3)}$$

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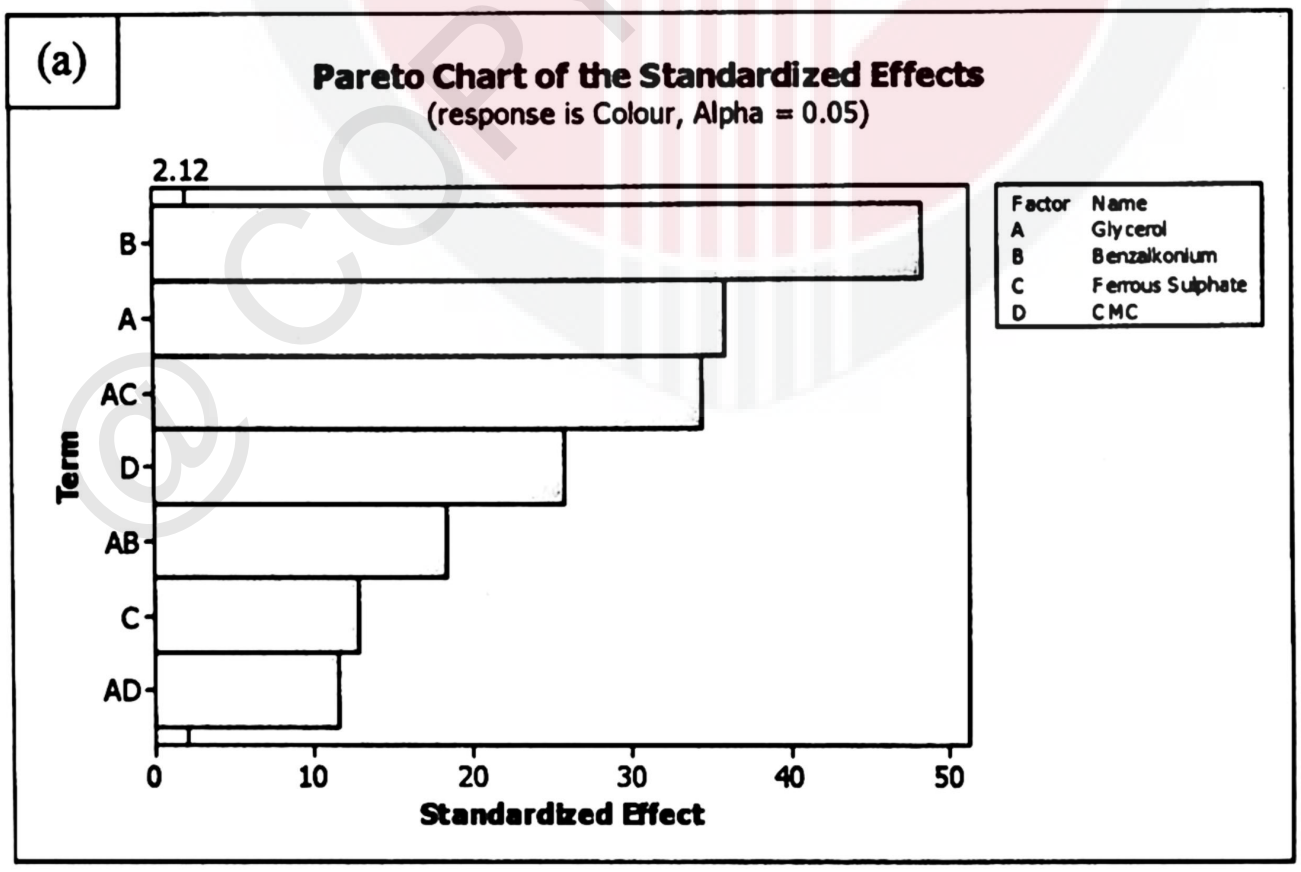
4.2.3 Pareto Chart

Figure 4.1 shows the Pareto chart on the effects of the main factors and their interactions. Based on the Pareto chart for colour intensity as shown in Figure 4.1(a), factor B (Benzalkonium Chloride) extended the most beyond the reference line, followed by factor A (Glycerol). For interactions between two additives, those occurring between factor A (Glycerol) and C (Ferrous Sulphate) collectively had the strongest effect on responses occurred during colour intensity test. Therefore, Glycerol is chosen as the most significant additive for colour intensity because it has extended beyond reference line and can react well with other additives. Based on Figure 4.1(b), factor D (CMC) was found to be the most influential since it extended the most beyond the reference line. All factors were statistically significant at 95% confidence level. On this basis, factors A (Glycerol) and D (CMC) were thus identified for subsequent optimization process using response surface methodology. It is because factor A (Glycerol) is the most significant on colour intensity while factor D (CMC) is the most significant factor on viscosity.



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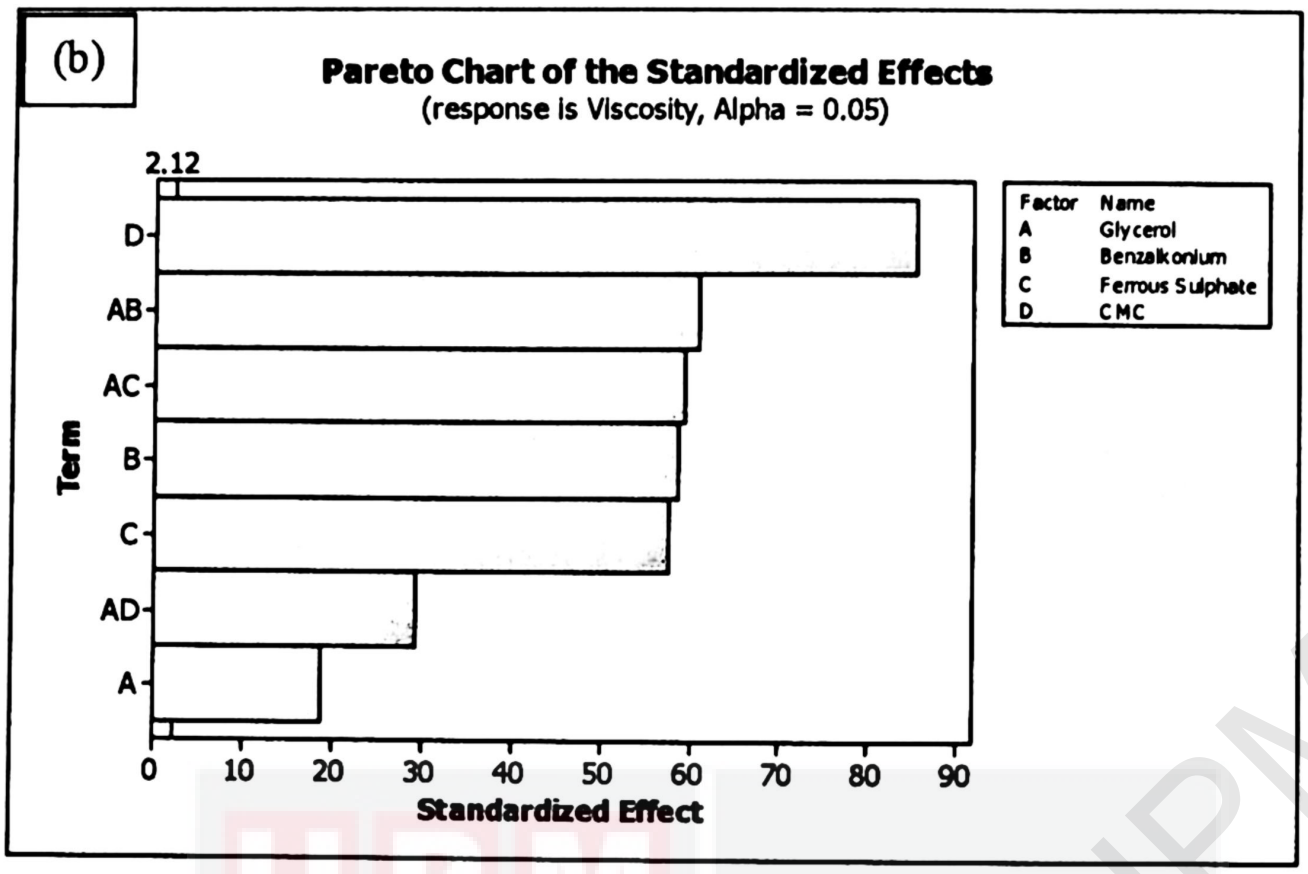

Figure 4.1: Pareto plot of the standardized effects of (a) colour intensity, and (b) viscosity.

4.2.4 Main Effect Plot

It can be seen from Figure 4.2 that Glycerol had the greatest positive effect on the colour intensity as shown in Figure 4.2(a) while CMC has the greatest positive effect on the viscosity as shown in Figure 4.2(b) as indicated by the largest angle of inclination to the horizontal. All other factors showed smaller changes and Benzalkonium Chloride shows negative effects on both responses. This pattern proved to be statistically significant, as referred to Table 4.1 and Table 4.2.

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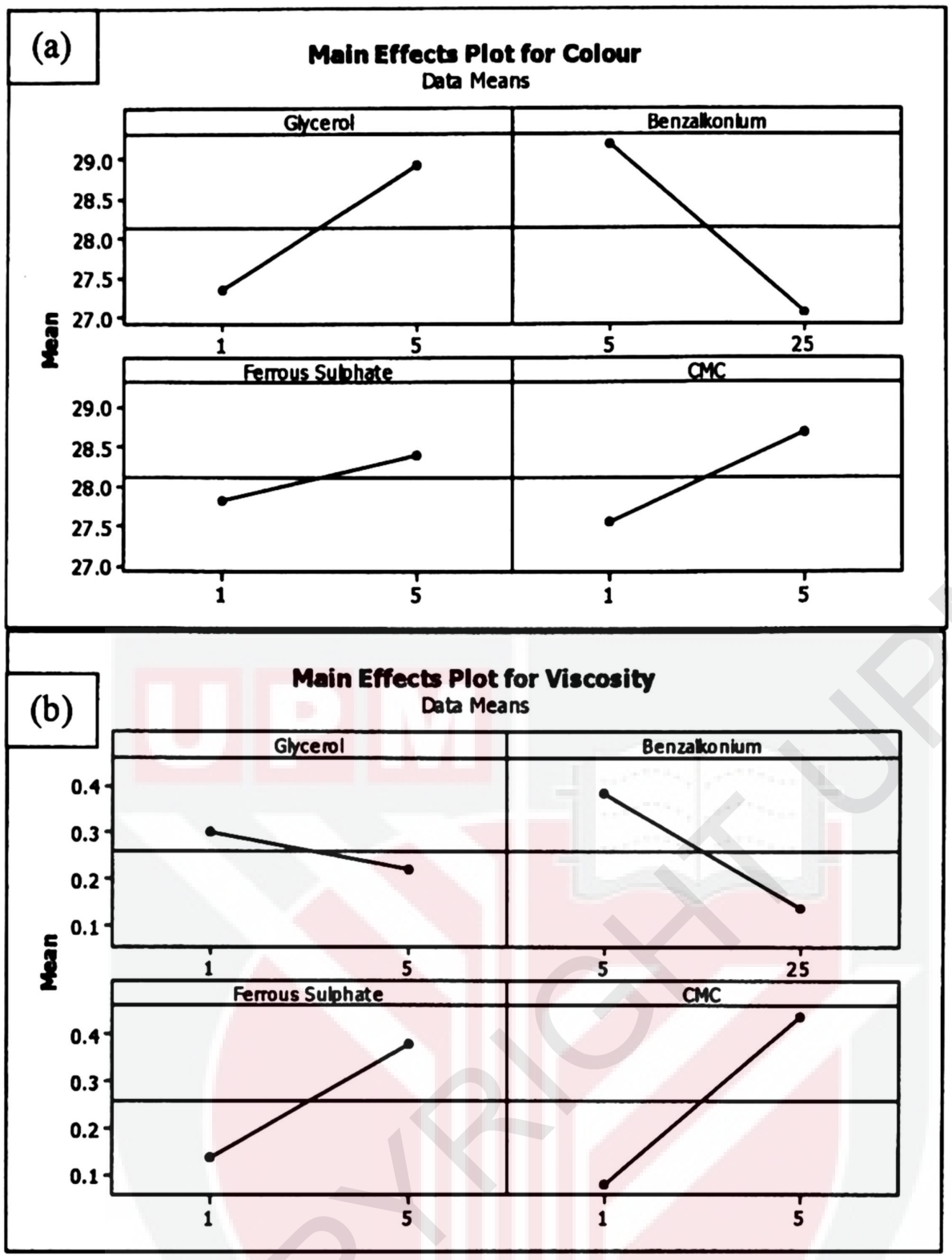

Figure 4.2: Main effect plot for (a) colour intensity and (b) viscosity.

4.2.5 Interaction Plot

Contour plots were used to analyze the effect of various factors on the marker ink's properties by providing an easy-to-understand illustration. Figure 4.3 and Figure 4.4 illustrate the effect of Glycerol and Benzalkonium chloride on the colour intensity and viscosity. According to Nwosibe *et al.* (2018), a good ink should flow easily and does not clog the marker tip. Ideally, ink should have maximum colour intensity at a low viscosity. Based on Figure 4.3, the value of colour intensity exceeds 30 L* colour units when the percentage of Benzalkonium Chloride and Glycerol is 5% and 5%, respectively. It indicates that decrease

the amount of Benzalkonium Chloride and increases the amount of Glycerol able to produce a marker ink with an increase in colour intensity.

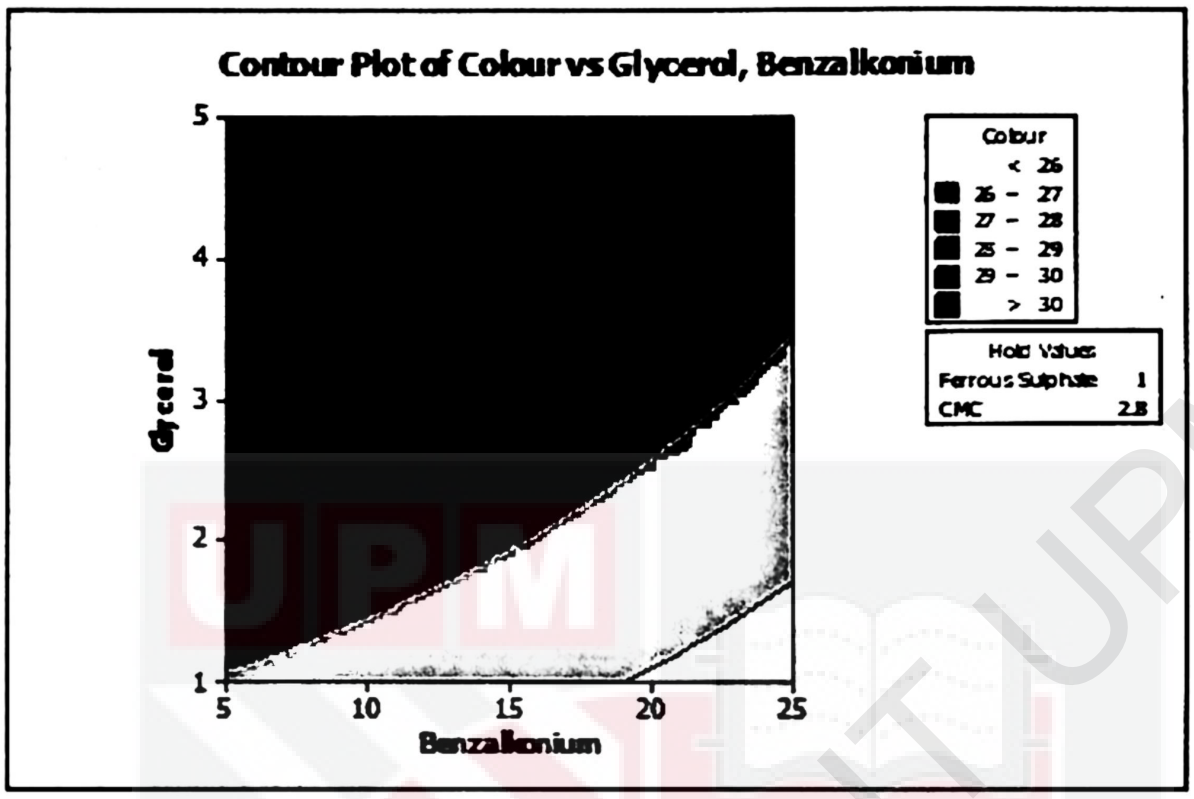

Figure 4.3: Contour plot for colour intensity.

Based on Figure 4.4, the viscosity of marker ink is below 0.02 Pa.s when the percentage of benzalkonium chloride is between 20% and 25% and the glycerol percentage is between 1% and 2%. High benzalkonium chloride content and low glycerol content was found to produce marker ink that has a low viscosity. Also, both contour plots proved high reactivity between glycerol and other additives in developing an ideal ink formulation.

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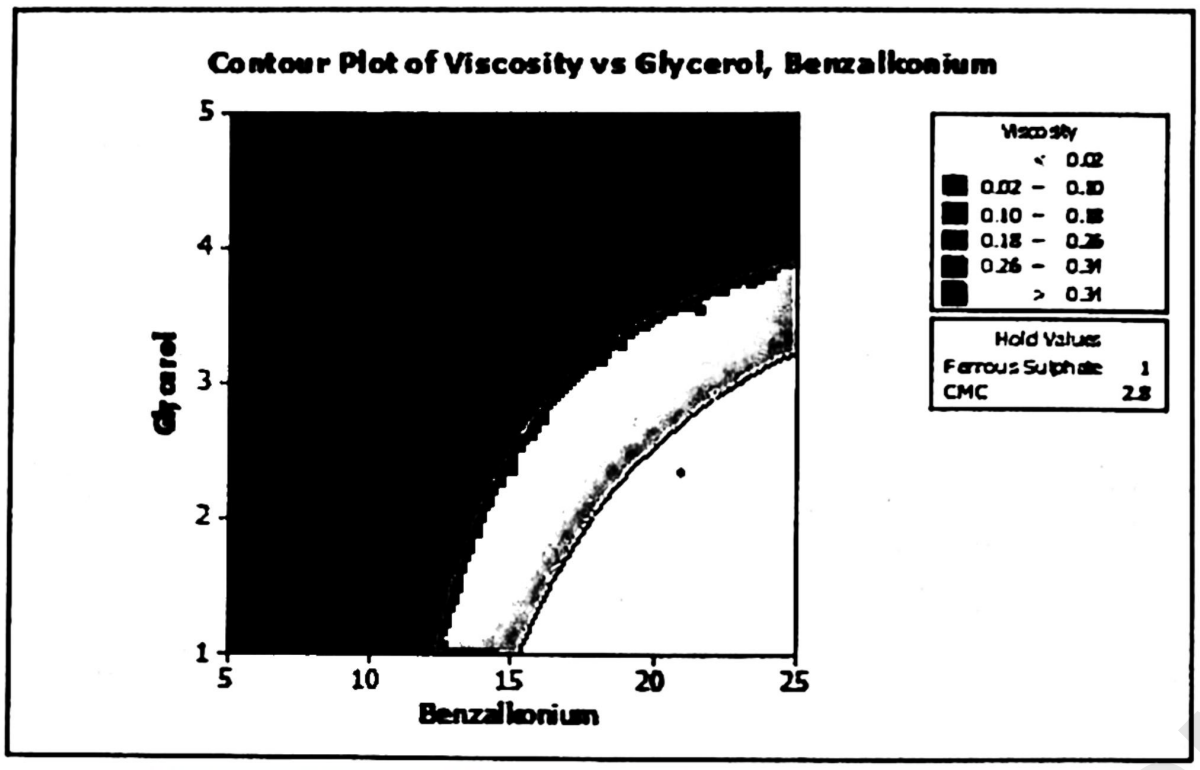

Figure 4.4: Contour plot for viscosity.

4.3 Response Surface Methodology (RSM)

The objective of this study is to determine the significance of each factor and their interaction, in particular; to elucidate the relationship between the factors and response under the condition of colour intensity and viscosity; to provide an optimum formulation of plant-based marker ink through optimization and validation process; and to elucidate the behaviour and properties of plant-based marker ink. Benzalkonium Chloride and ferrous sulphate were fixed at the percentage of 5% and 1%, respectively. Design matrix and responses values for sample S35, S19 and S26 were only shown in Table 4.3 since these samples showed good, moderate, and poor colour intensity and viscosity properties. Figure 4.5 shows ink samples with different colour intensity.

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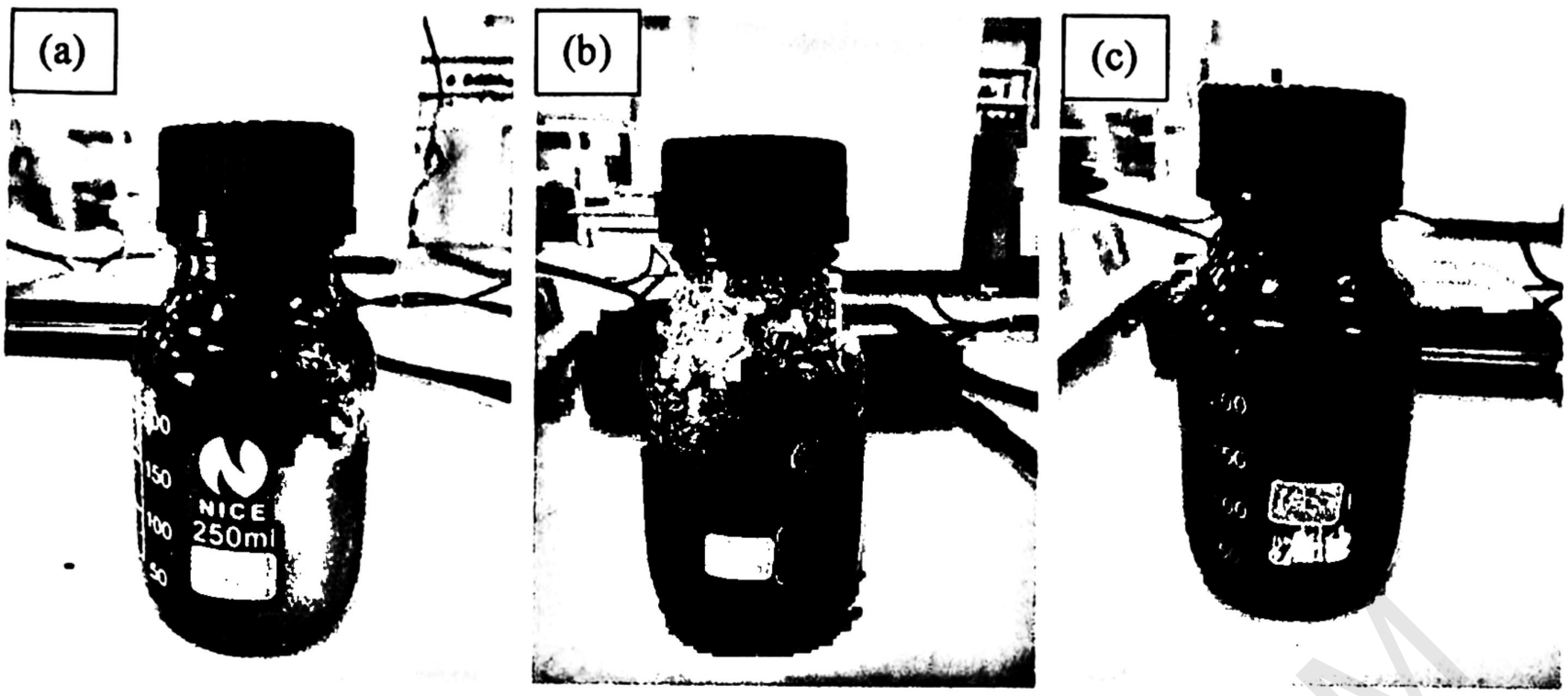

Figure 4.5: Ink samples with (a) good, (b) moderate and (c) poor colour intensity and viscosity properties.

They were discussed further in material characterization and microstructural analysis section. Complete design matrix and responses values are given in Appendix 2, and data were analyzed using MINITAB.

Table 4.3: Design matrix and response values for sample S31, S30 and S28

Run	Sample	Coded factor		Uncoded factor		Responses	
		Glycerol (V_1)	CMC (V_4)	Glycerol (V_1)	CMC (V_4)	Colour intensity	Viscosity
35	S35	2	0	25	3	28.78	0.0100
19	S19	1	1	20	4	27.40	0.1595
26	S26	-1	1	10	4	25.09	0.2245

4.3.1 Statistical Analysis of Colour Intensity and Viscosity Properties

A linear regression model was fitted to the experimental data using the least square technique. Several main parameters were considered in evaluating the statistical results, namely the coefficients of regression, the standardized error of coefficient, and the P-value of each factor and its interaction for both responses which are colour intensity and viscosity. Complete statistical results for this study are shown in Appendix 5 and Appendix 6. The

results in Table 4.4 indicated that all factors and interaction effects were highly significant (P < 0.000) except for V_4 with P < 0.113 and 'V_4* V_4' with P < 0.529. Values for R^2 = 0.9873 and R^2 (adjusted) = 0.9858 were considerably high, which indicated that 98.73% of the sample variation in the response was attributed to the factors.

Table 4.4: Estimated effects and coefficient for Glycerol and CMC on the colour intensity.

Term	Notation	Coefficient	Std. error of coefficient	T	P
Constant		25.7739	0.03071	839.238	0.000
Glycerol	V_1	1.0331	0.02135	48.384	0.000
CMC	V_4	0.0347	0.02135	1.626	0.113
Glycerol*Glycerol	V_1* V_1	0.2607	0.01545	16.870	0.000
CMC*CMC	V_4* V_4	0.0098	0.01545	0.636	0.529
R^2 = 0.9873	R^2 (adj) = 0.9858				

For viscosity, the P-value for both factors and their interactions were considered significant as well due to the value of P, which is below the confidence level at 95% (P of 0.050). The results shown in Table 4.5 indicated that all factors and interaction effects were significant. The P of all factors and their interactions were highly significant (P < 0.000) except for V_1 with P < 0.382. Value for R^2 = 0.9292 and R^2 (adjusted) = 0.9209 were considered very high, which indicated that 92.92% of the sample variation in the response was attributed to the independent variables.

Table 4.5: Estimated effects and coefficient for Glycerol and CMC in viscosity test.

Term	Notation	Coefficient	Std. error of coefficient	T	P
Constant		0.0938	0.01191	7.871	0.000
Glycerol	V_1	-0.0073	0.00828	-0.886	0.382
CMC	V_4	0.1431	0.00828	17.273	0.000
Glycerol*Glycerol	V_1* V_1	-0.0235	0.00599	-3.925	0.000
CMC*CMC	V_4* V_4	0.0593	0.00599	9.897	0.000
R^2 = 0.9292	R^2 (adj) = 0.9209				

Equation 4.3 and 4.4 represent the regression models for the colour intensity and viscosity, respectively.

$$Y_{COL} = 25.7739 + 1.0331\,(V_1) + 0.0347\,(V_4) + 0.2607\,(V_1^2) + 0.0098\,(V_4^2)$$

(Eq. 4.3)

$$Y_{VIS} = 0.0938 - 0.0073\,(V_1) + 0.1431\,(V_4) - 0.0235\,(V_1^2) - 0.0593\,(V_4^2)$$

(Eq. 4.4)

Where Y_{COL} and Y_{VIS} represent the responses which are colour intensity and viscosity, respectively. V_1 and V_4 are the decoded values of glycerol and CMC, respectively. The regression models can be used to calculate and analyze the effect of factors on the properties of plant-based marker ink.

4.3.2 Effect of Factors on Plant-based Marker Ink Properties

ANOVA and the regression models were used to analyze the effect of various factors on the properties of the plant-based marker ink. Contour plots were used for better illustration. Figure 4.6 and Figure 4.7 illustrate the effect of glycerol (V_1) and CMC (V_4) on the responses, respectively. From the figures, it shows that higher V_1 resulted in higher colour intensity, and lower V_4 resulted in lower viscosity. Based on Figure 4.6, the colour intensity of plant-based marker ink exceeds 28.8 L* units when the percentage of glycerol concentration in the ink formulation is approximately 25%. It indicates that a high concentration of glycerol increased the colour intensity.

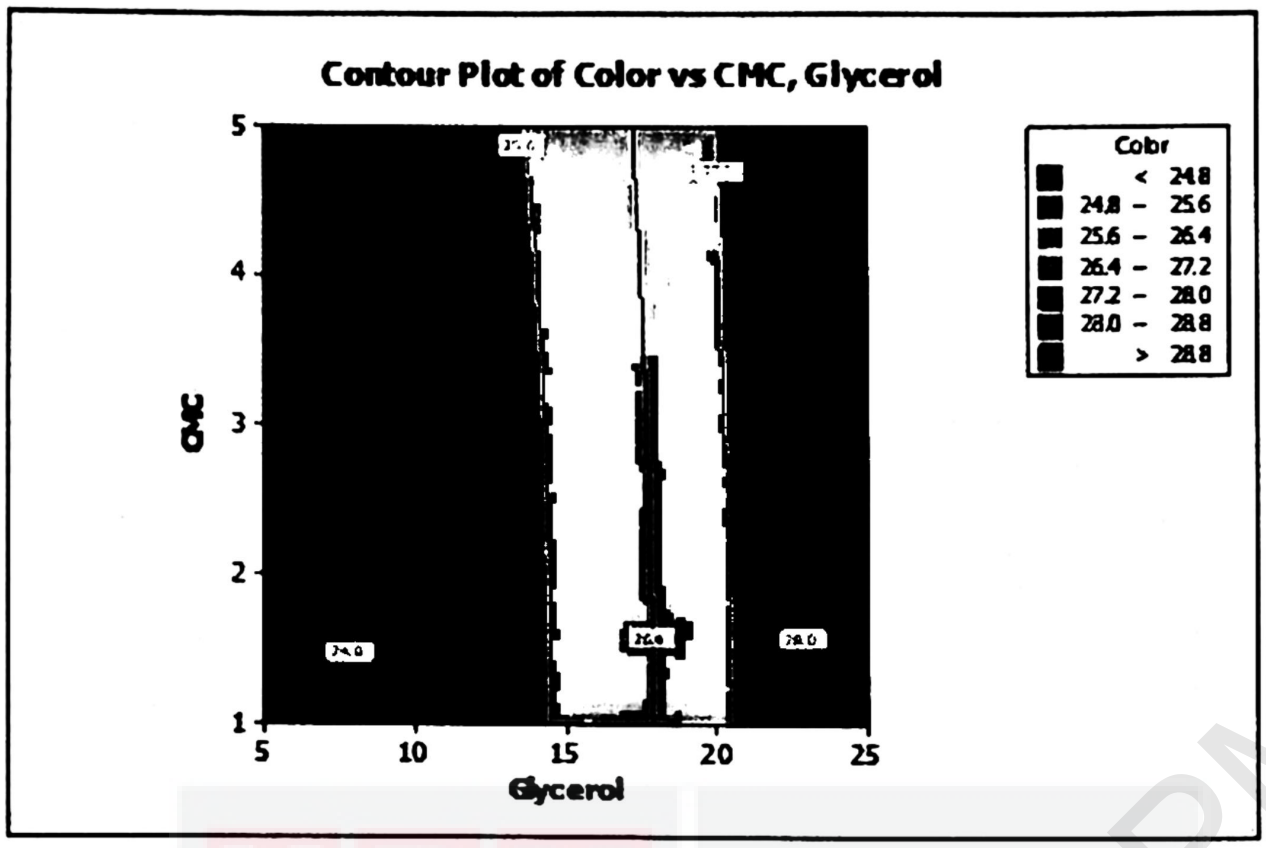

Figure 4.6: Contour plot for the effect of glycerol and CMC concentration on the colour intensity of plant-based marker ink.

An increase in the amount of glycerol in a liquid may increase the boiling temperature of the liquid (Medeiros, 2010). Glycerol is alcohol with low vapour pressure. When it enters the water, it alters the colligative properties of water, increasing its boiling point and lowering the melting point in the solution (Dow Chemical, 2010). According to Nabli *et al.* (2013), an increase in the boiling temperature may consequently increase the total anthocyanin extraction yield, which significantly contributes to the colour of plant-based marker ink. Anthocyanin pigments are known as natural colourants due to their wide range of colours and high solubility in aqueous media (Teszlák *et al.*, 2015). Solvents such as glycerol are generally used in water for the extraction of the anthocyanin pigments (Nabli et al., 2012).

Besides, it was found that glycerol concentration provides a positive effect on the amount of extracted anthocyanin due to low viscosity. As an increase in boiling temperature resulted in a low viscosity solution, the diffusion of the anthocyanin molecules in glycerol solvent increase significantly (Corrales *et al.*, 2009).

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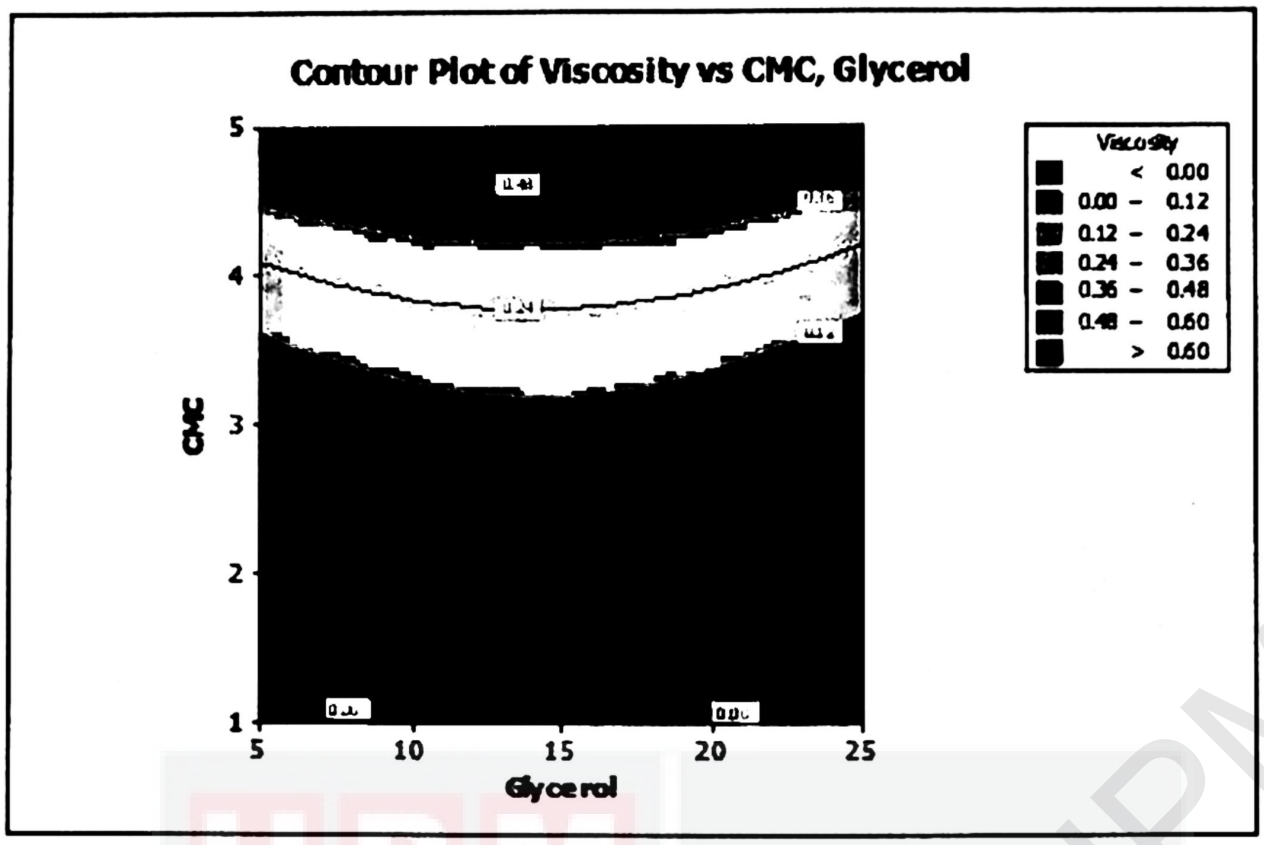

Figure 4.7: Contour plot for the effect of glycerol and CMC concentration on the viscosity of plant-based marker ink.

Contour plot in Figure 4.7 shows the effect of glycerol and CMC concentration on the viscosity of plant-based marker ink. At glycerol concentration of 5%, the viscosity decrease as the CMC concentration decreases. Ghannam and Esmail (1997) have investigated the rheological properties of CMC solutions in a concentration ranging from 1 to 5 percent. They reported that Newtonian behaviour was observed when the concentration is at the lowest, and non-Newtonian behaviour, including pseudoplastic, thixotropic, and viscoelastic was determined at the high concentrations. Similar results were obtained by Edali *et al.* (2001), who investigated the rheological behaviour of CMC solutions at higher concentrations. They found that the solution possessed both non-Newtonian and viscoelastic properties. Other research concluded that the apparent viscosity of CMC solution increases with increasing concentrations. The results were obtained from the flow curves of the CMC solutions at different concentrations plotted over a log-log scale. This rise in the apparent viscosity is due to the increase in the intermolecular interactions between the CMC molecules (Benchabane, 2008).

4.3.3 Optimization of the Responses

The optimum formulation of marker ink can be achieved with the combination of Glycerol percentage = 25 and CMC percentage = 3.14, as shown in Figure 4.8. The desirability of optimization was calculated as 0.98318, indicating that all parameters were within the target, which was to obtain the desirable ink properties.

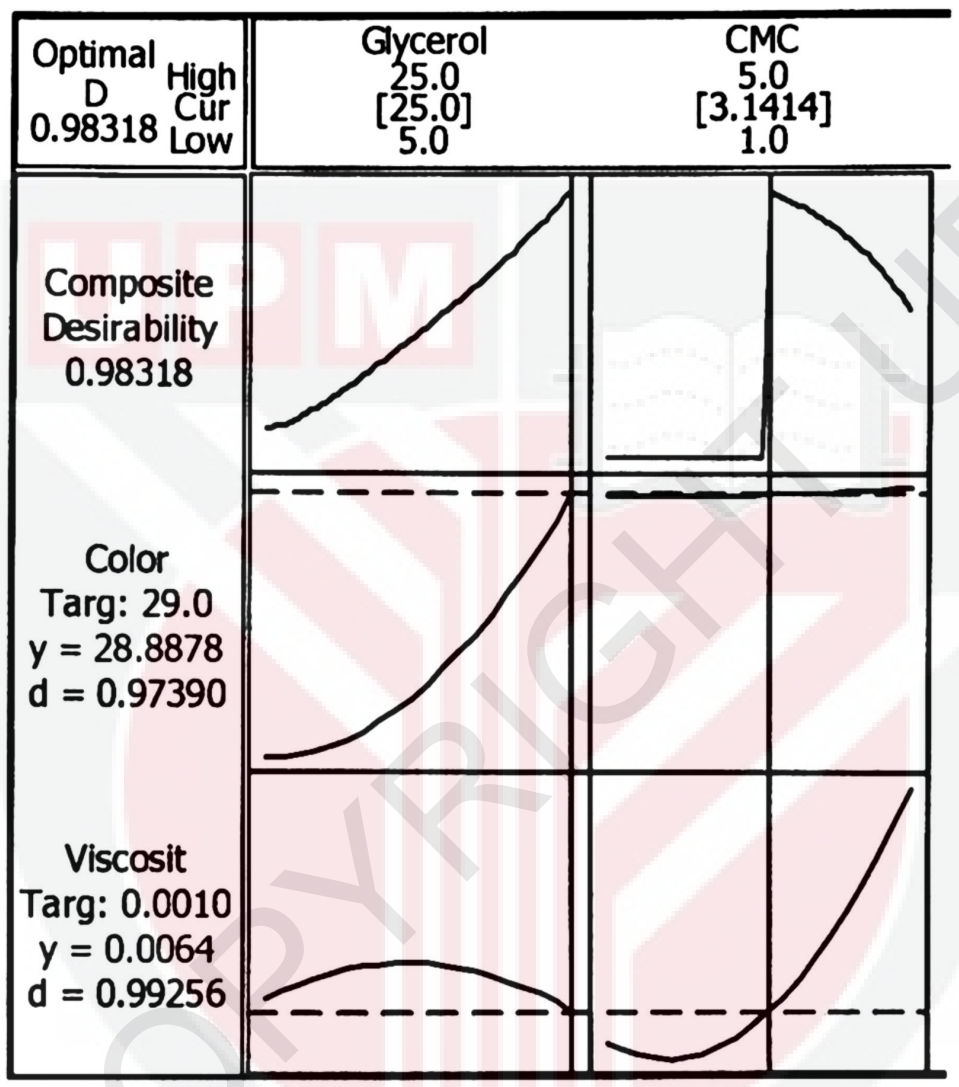

Figure 4.8: Optimization plot for Glycerol and CMC

The value of composite desirability was calculated as 0.98318, which is high and proved that the prediction is accurate. This also indicates that all parameters were within the target, which can obtain the desirable ink properties. The optimization was performed under designated parameters. For the colour properties, the lower, target and the upper value was set at 24.7 L* units, 29.0 L* units, and 29.2L* units, respectively. The values for lower, target and upper boundaries for viscosity properties were set at 0Pa.s, 0.001Pa.s, and 0.7278Pa.s, respectively.

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4.3.4 Experimental Validation

From Table 4.6, it was found that the average error for the colour intensity and viscosity were well below 15% at only 0.52% and 2.08% respectively. It was concluded that the developed regression model established using this method was able to optimize value for the responses accurately._

Table 4.6: Experimental validation for plant-based marker ink properties

Sample no.	Colour Intensity (L*)			Viscosity (Pa.s)		
	Experimental value	Predicted value	Error (%)	Experimental value	Predicted value	Error (%)
SV1	28.99	28.89	0.35	0.0065	0.0064	1.56
SV2	28.74	28.89	0.52	0.0066	0.0064	3.13
SV3	28.69	28.89	0.69	0.0063	0.0064	1.56
	x̄ Error		0.52	x̄ Error		2.08

4.4 Material Characterization and Microstructural Analysis

Five samples were selected to determine the microstructural and drying properties of the dry mangosteen leaves. The samples were dry mangosteen leaves, boiled mangosteen leaves without glycerol, mangosteen leaves boiled with glycerol with poor (sample S26), moderate (sample S19), and good (sample S35) colour intensity properties. The sample was analysed using a scanning electron microscope (SEM). Furthermore, thermogravimetric analysis (TGA) is conducted on four samples of ink to determine the drying speed. The samples are determined based on the good (sample S35), moderate (sample S19), poor (sample S26), and optimized (sample SV1) colour intensity and viscosity.

4.4.1 Surface Morphology

Figure 4.9 illustrates the SEM micrographs of dry Mangosteen leaves at 100x and 500x magnifications. Former magnification shows a relative corrugated surface, in the form of wrinkles, developed on leave. The amount of corrugated surface formed is due to the drying process of the leave at ambient temperature. Water evaporated from the leaves

resulted in a small degree of shrinkage of the cell structure. Latter magnification shows no pores on the smooth surface of the dry Mangosteen leaves. It confirmed that the leave dried due to evaporation of water at ambient temperature and without the influence of elevated temperature. In this stage, the drying shrinkage is closely related to the total volume of evaporated water (Lewicki, 1998).

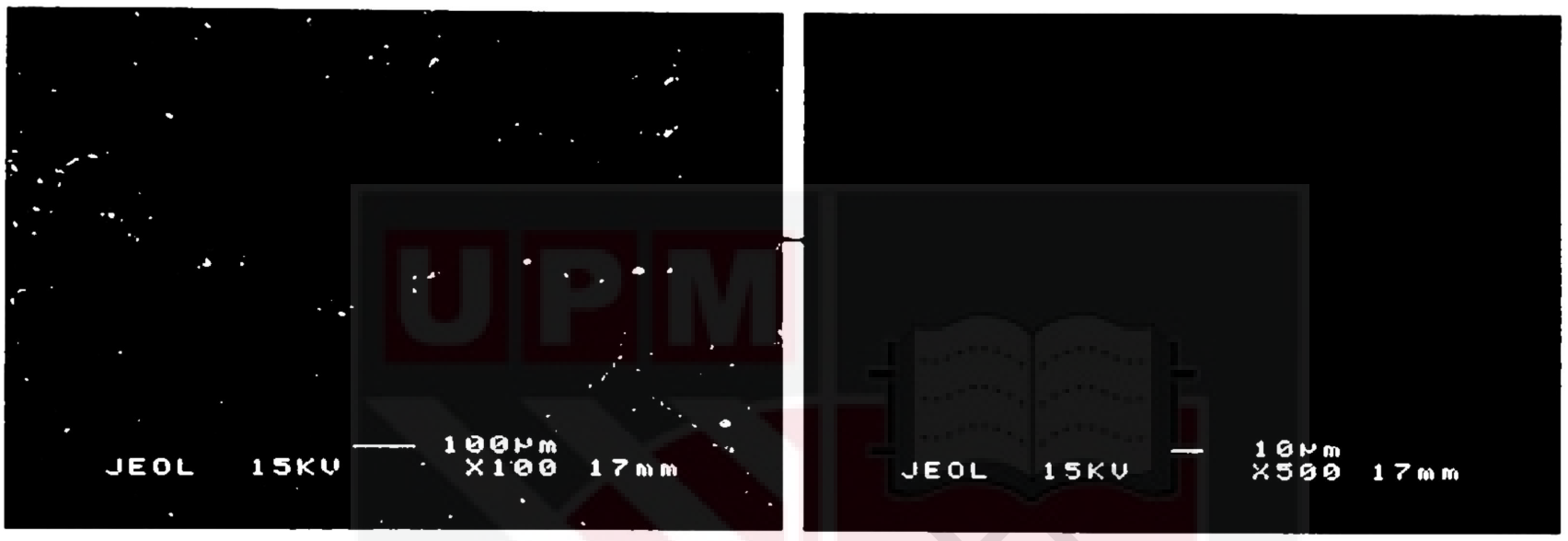
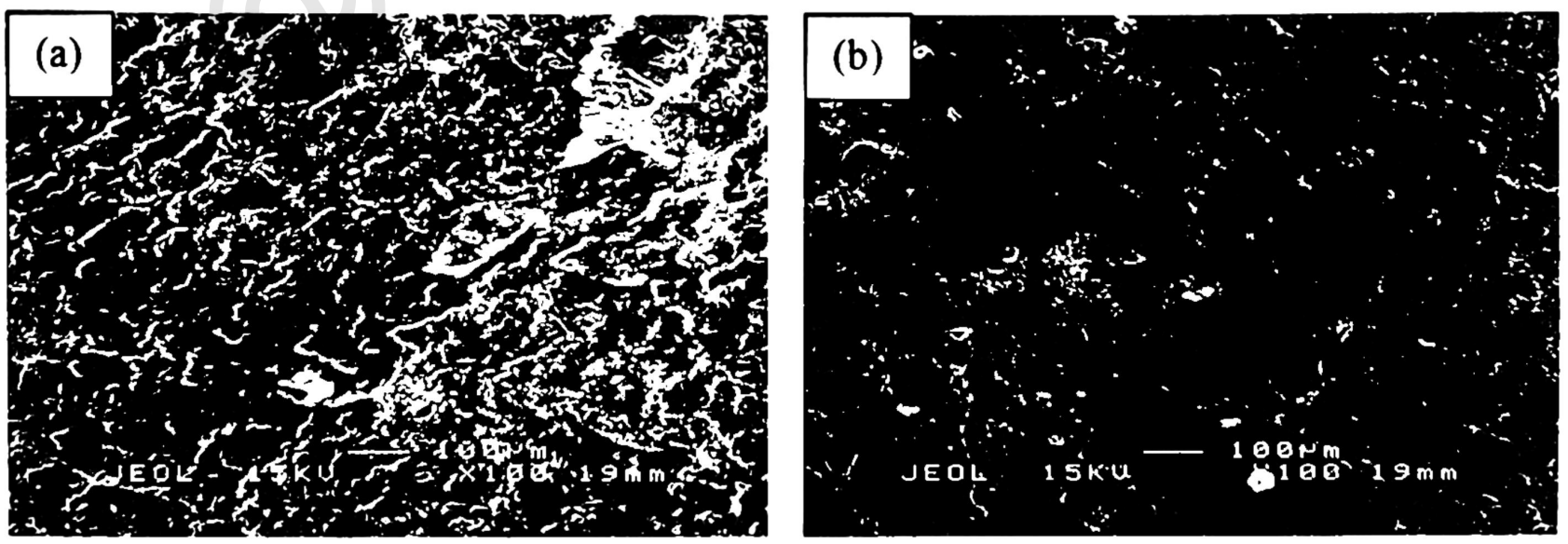


Figure 4.9: SEM micrographs of dry Mangosteen leaves at 100x and 500x magnification

Glycerol was found to be the most significant effect on colour intensity as compared to that of other additives. Therefore, the SEM was conducted to investigate the effect of different amount of Glycerol on the microstructure of the Mangosteen leaves, which subsequently affect the amount of colour extracted from the leaves.



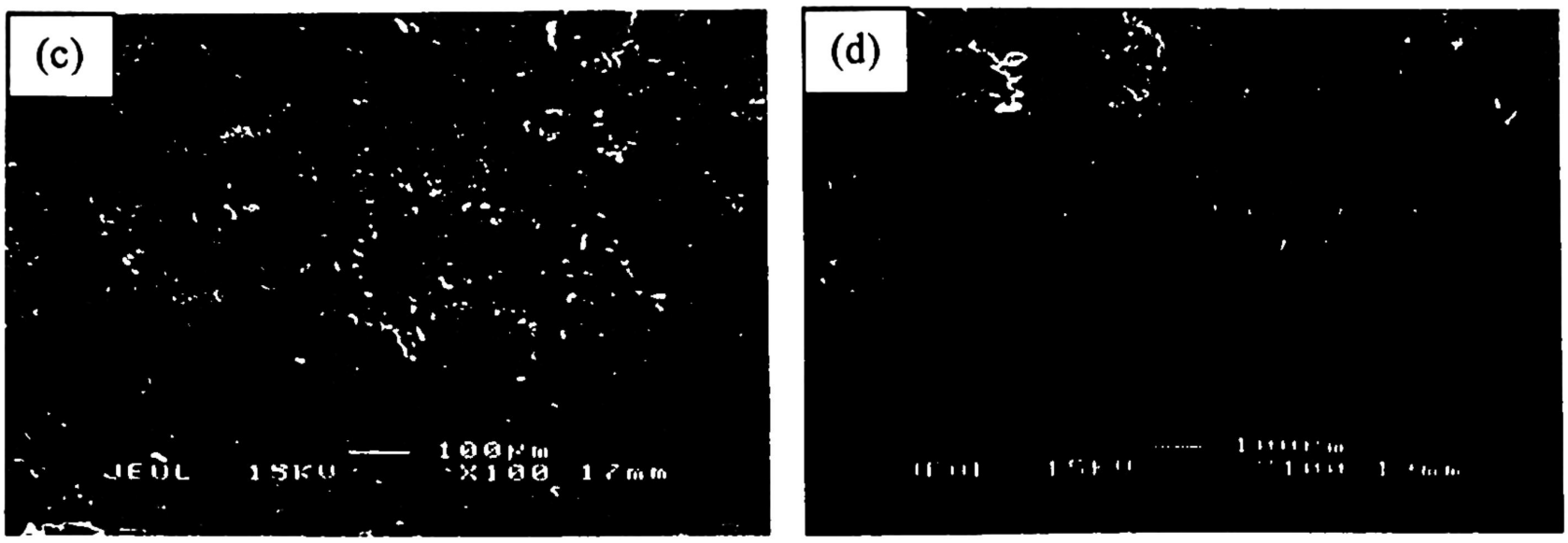


Figure 4.10: SEM micrographs of (a) boiled Mangosteen leaves without Glycerol, (b) sample S35 (good colour intensity properties), (c) sample S19 (moderate properties), and (d) sample S26 (poor properties) at 100x magnification.

Based on Figure 4.10, the SEM micrographs of all samples showed a corrugated surface due to cell shrinkage. In Figure 4.10(a), the cells shrunk with most of the cell wall structure still intact to each other. Under high-temperature conditions, the leaves developed a folded cuticle and cell wall on the adaxial epidermis layer (Salem, 2011).

The microstructures of Figure 4.10(a) and (d) was found to be quite similar since the cell wall structures were still intact to each other. However, it can be seen that the structures of the cell wall structure of sample S35 in Figure 4.10(b) is started to collapse in certain areas and small pores were developed. It shows the highest amount of collapsed cell wall structures. It is because the leaves are boiled with the highest amount of Glycerol which is capable of altering the cell structure. Hence, Glycerol is generally used as a solvent in water to extract natural pigment. Glycerol also acts as a pre-treatment agent and affects the final colour yield by improving colour performance (Hou, 2019). Figure 4.10(c) shows a large area of the sample S19 covered by the collapsed cell walls structure. Sample S26 as shown in Figure 4.10(d) experienced the least amount of collapsed cell wall structures because it was boiled with least amount of Glycerol. Therefore, this sample has the least area of cell structure that was altered by Glycerol.

It can be said that an increase in the amount of Glycerol in boiled water may alter the cell wall structure of the leaves and subsequently increased the colour yield extracted from the leaves. This is due to the ability of Glycerol as an extraction solvent, thus has a significant effect during the anthocyanin extraction process. The role of Glycerol as anthocyanin extraction solvent has two main effects: first, the weakening of the membrane structures of the cells in which the anthocyanin are store in the vacuole and second, the transformation of the extracted anthocyanin to their flavylium form, which is their most stable form (Amor, 2009). This condition has been extensively used in technological processes to extract anthocyanin from natural sources such as fresh berries and grape pomace (Nabli, 2012).

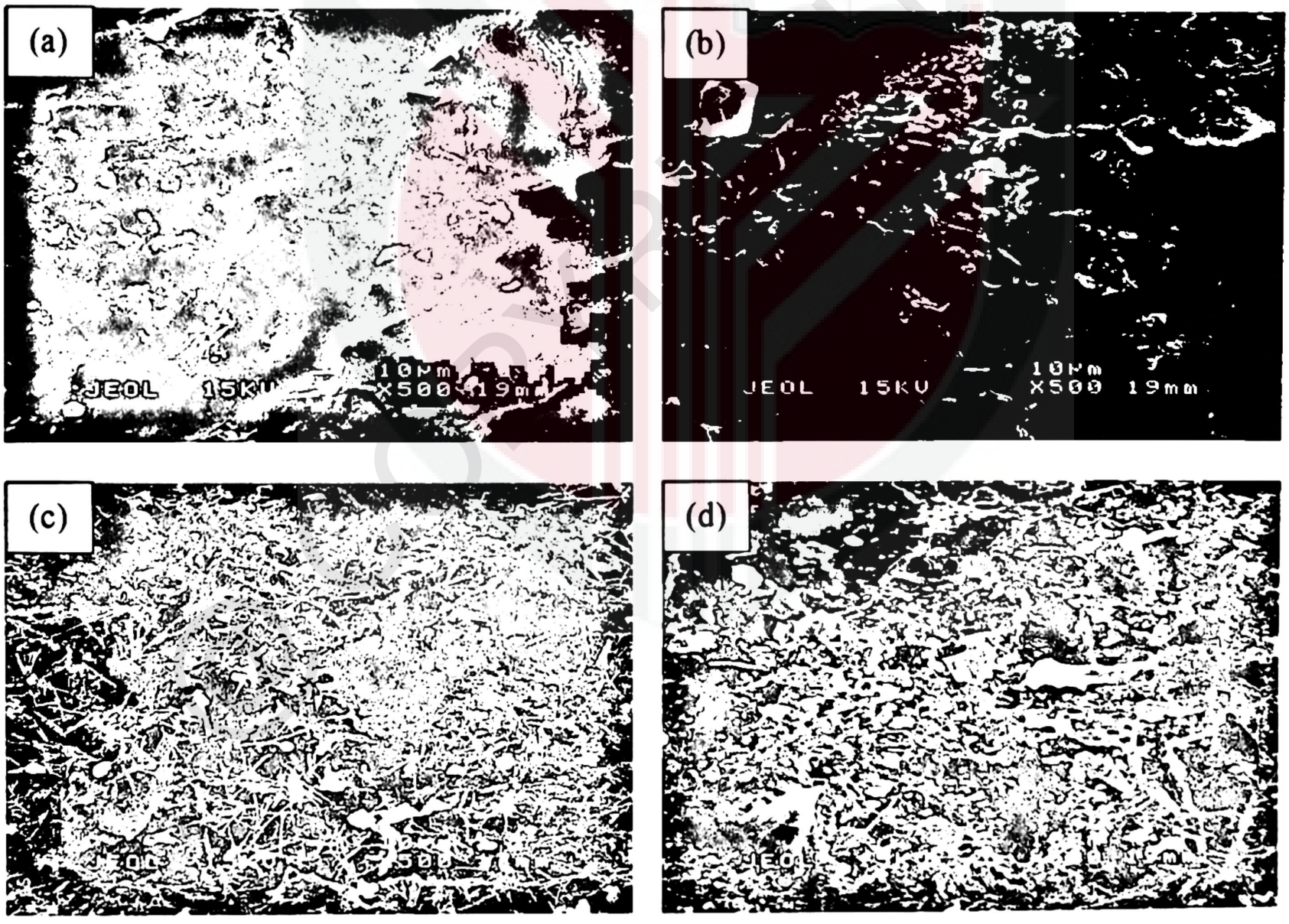


Figure 4.11: SEM micrographs of (a) boiled Mangosteen leaves without Glycerol, (b) sample S35 (good colour intensity properties), (c) sample S19 (moderate properties), and (d) sample S26 (poor properties) at 500x magnification.

Further magnification at 500x revealed a more definite structure of the leave as compared to that of the 100x magnification. Based on Figure 4.11(a), boiled Mangosteen leaves without Glycerol shows a good bonding between cell walls structure and considerable smooth surface. The colour was able to be extracted from the leaves by travelling through the small pores between the cells. These pores are observed at the intercellular space, between neighbouring epidermal cell. The moderate and large size of irregular oblong pores and disrupted orientation of membranes were formed due to heat stress during the boiling process. A few reports have described the effect of high temperature on the anatomical and ultrastructural changes occurred in the leaves including the organization of leaves, the protective outer layer of epidermis and cuticle as well as stomata (Baniwal, 2004). The result is in agreement with Salem et al. (2011) that proved a high-temperature condition can result in the folded cuticle and cell wall on the adaxial epidermis layer of leaves. Sample S35 (good colour intensity properties) in Figure 4.11(b) shows the largest area of collapsed cell walls structure while sample S19 (moderate properties) in Figure 4.11(c) was observed with a thin needle-like and small-sized cell wall structures collapsed which majority of the structures covered most of the surface. Sample S26 (poor properties) as shown in Figure 4.11(d) covered the largest surface with collapsed large-sized and thick cell walls structure.

The thicker cell wall structures of sample S26 (poor properties) as compared to that of sample S19 (moderate properties) can be seen based on the colour intensity of the aggregated structures. Formation of the large-sized and thick cell walls structure was due probably to the aggregation of the high amount of small-sized cell wall structures. Due to high surface area and strong attractive interaction between particles, the aggregation process was likely occurring and in agreement with other researchers (Jouault, 2009). Aggregation cause the fracture mechanism to be changed, and the fracture starts and develops from the aggregation center (Esbati, 2018). The thick and aggregated cell wall structures may result in blockage or

interference of the intercellular spaces which are located between neighbouring epidermal cells. Therefore, anthocyanin, which is the source of colour and located in the vacuoles of leaf mesophyll tissue or epidermal cells, is not able to be fully extracted out (Zhang, 2016). In contrary, sample S19 (moderate properties) shows thinner cell wall structure because of the gradual removal of colour resulted by a dramatic decrease in the anthocyanin content. The thickness and density of the cell structure were progressively reduced as anthocyanin was being extracted out from the leaves.

4.4.2 Thermogravimetric Analysis (TGA)

Thermogravimetric analysis (TGA) was found to be an alternative method in determining the drying rate of a solution by measuring the sample weight loss during the drying process (Kim, 2001). Drying rate was found to be affected by the viscosity of a fluid and the rate of evaporation of a solvent (Munekata, 2013). In this research, four inks which exhibited poor, moderate, good and optimized colour intensity and viscosity properties were tested, and the results are shown in Figure 4.13. To better visualize the weight loss of the ink during the TGA test, a graph of weight loss (in percentage) as a function of time was plotted as shown in Figure 4.12.

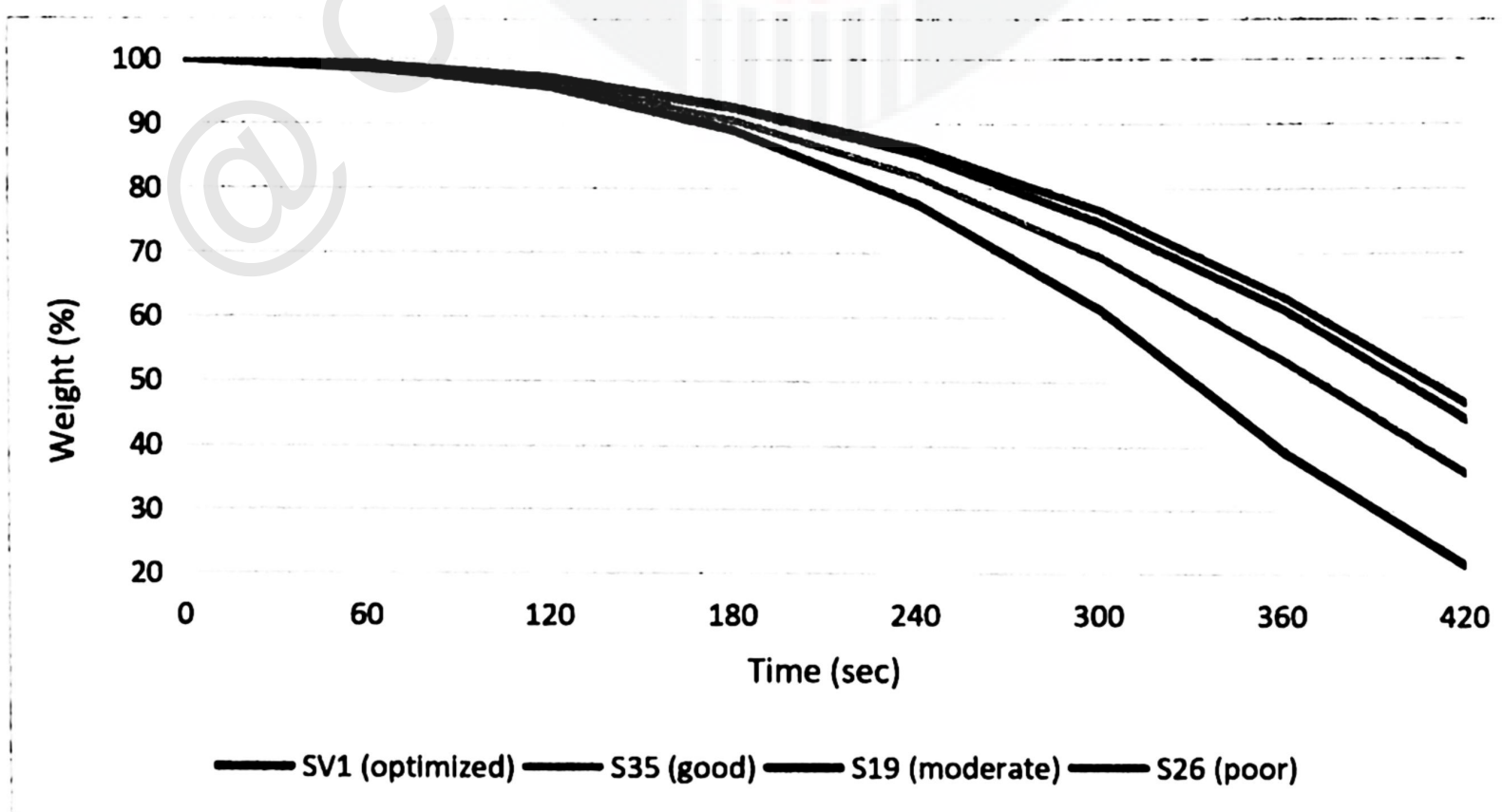


Figure 4.12: Weight loss as a function of time of sample (a) S35 (good properties), (b) S19 (moderate properties), (c) S26 (poor properties) and (d) SV1 (optimized properties).

The weight loss for all samples for the first 120 seconds was very minimal, with a maximum of 5 percent loss recorded from sample SV1. As the temperature for the test increased by 25°C for every 60 seconds, an increase of temperature of 50 degree celcius increased the initial drying rate. External conditions of a volatile matter, including higher air velocity, lower air humidity, and higher air temperature contribute to the increase of drying rate (Berk, 2009). The drying curve represents the loss of volatiles first by evaporation from a saturated surface on the ink film followed in turn by a period of evaporation from a saturated surface of gradually decreasing area and finally when the volatiles evaporate from the interior of the ink's solid (Varadarajan, 1990). Sample SV1 recorded the highest percentage of weight lost of 78.5 percent followed by sample S35 (64.1 percent), S19 (55.8 percent), and S26 (53.4 percent). It can be concluded that the optimized sample SV1 requires the fastest time to dry.

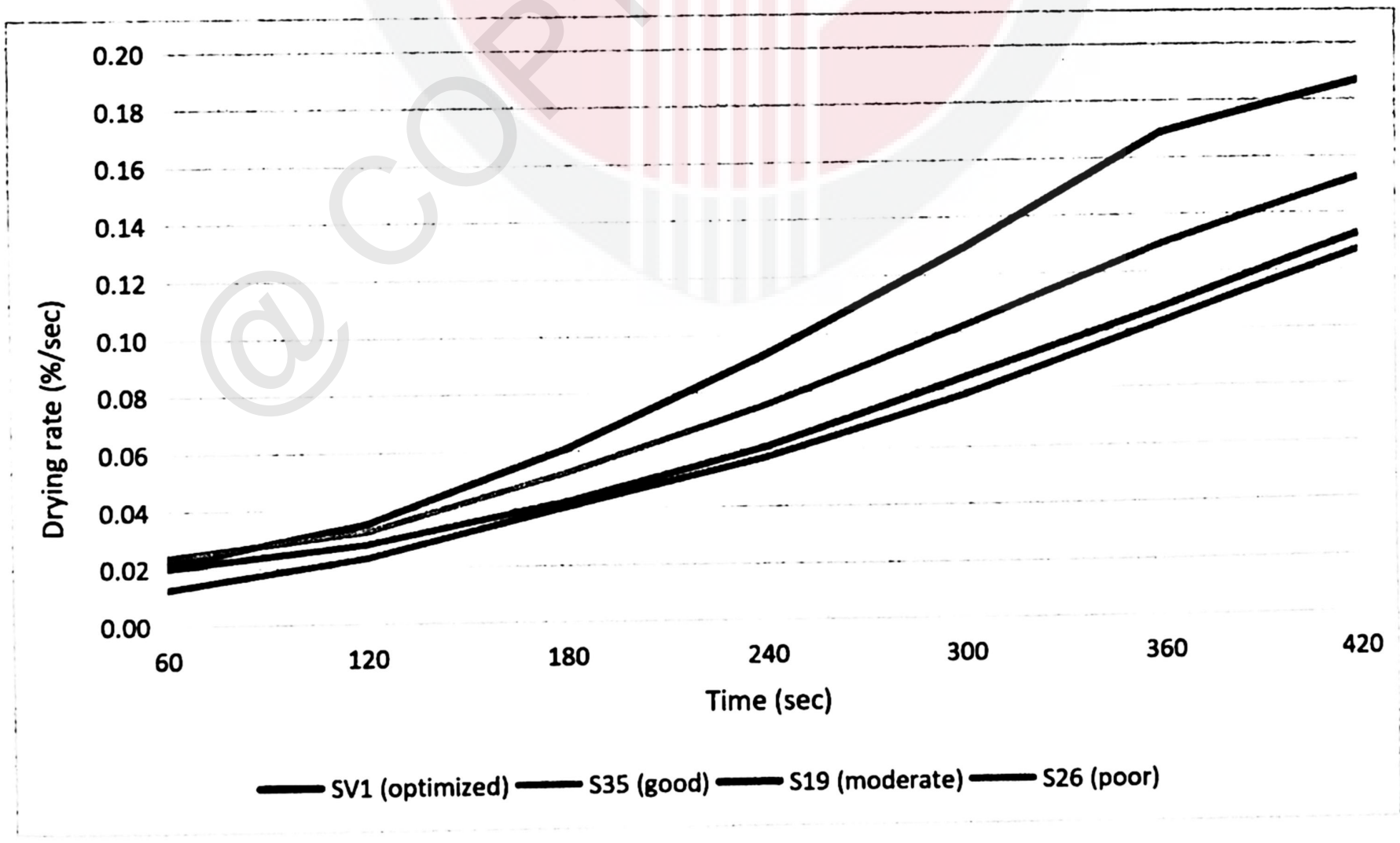


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Figure 4.13: Drying rate of sample (a) S35 (good properties), (b) S19 (moderate properties), (c) S26 (poor properties) and (d) SV1 (optimized properties).

Figure 4.13 shows the drying rate curves as a function of the time in second. The drying rate for sample S19 and S26 recorded a comparable drying rate between 0.01 and 0.13 percent per seconds. The optimized sample SV1 shows the highest drying rate of approximately 0.19 percent per seconds at 420 seconds. Due to low viscosity of sample SV1, the diffusion of solvent molecules (water) proceeds faster than in a higher viscosity particle which subsequently affects their ability to evaporate (Vaden, 2011).

The slower drying rate of highly viscous samples is due to diffusional mass transport limitations in the particle phase arising from the viscous phase of the particles or due to a combination of oligomer degradation and mass transfer limitations (Vaden, 2011). If a viscous phase is formed, the mixing within the particle bulk will be kinetically limited, and an equilibrium process cannot well represent the gas-particle partitioning. The drying rate is mainly governed by the reversible decomposition of oligomers back to monomers (Roldin, 2014).

CHAPTER 5: Conclusion and Recommendation

5.1 Conclusion

The research results permit the following conclusions:

1. Experimental designs centred on development and optimization of plant-based marker ink using Mangosteen leaves were shown to be useful in conducting experimental work. Screening process using Fractional Factorial Design (FrFD) and optimization process using Response Surface Methodology (RSM) was appropriate in identifying the significant factors (additives) and optimizing the responses, respectively.

Experiments were conducted based on the design matrix generated by the MINITAB software. Each experiment was conducted on a laboratory scale following appropriate standards.

a) Based on the Pareto chart, CMC was found to be the most significant factor on viscosity since it extended the most beyond the reference line. All factors were statistically significant at 95% confidence level. Compared to other interactions, glycerol collectively had the strongest effect on responses which is the colour intensity.

b) Based on the main effect plot, glycerol has the greatest positive effect on the colour intensity, while CMC has the greatest positive effect on the viscosity.

c) The interaction plot proved that glycerol and CMC reacted well with other additives to produce ideal ink properties.

d) CMC and glycerol are selected as the main factors to determine the optimum formulation of plant-based marker ink.

2. Statistical analysis was successfully conducted to determine the effect of individual factor and their interactions on the viscosity and colour intensity of the plant-based marker ink using FrFD. Optimization and validation process is successfully performed using RSM. The resulting conclusions are as follows:

   a) For colour intensity test, a linear regression model indicated that all factors and interaction effects were highly significant ($P < 0.000$) except for CMC (V_4) with $P < 0.113$ and V_4*V_4 with $P < 0.529$. Values for $R^2 = 0.9873$ and R^2 (adjusted) = 0.9858 were considered high, which indicated that 98.73% of the sample variation in the response was attributed to the factors.

b) For viscosity test, a linear regression model showed the P of all factors and their interactions were highly significant ($P < 0.000$) except for V1 with $P < 0.382$. Value for R2 = 0.9292 and R2 (adjusted) = 0.9209 were considered very high, which indicated that 92.92% of the sample variation in the response was attributed to the independent variables.

c) ANOVA and the regression models generated contour plots that illustrated the effect of glycerol (V_1) and CMC (V_4) on the responses. From the contour plot, it shows that high amount of glycerol resulted in high colour intensity. Low amount of CMC resulted in low viscosity.

d) The optimum formulation of marker ink can be achieved when the ratio of glycerol, benzalkonium chloride, ferrous sulphate, and CMC is set as 5g, 5g, 1g, and 2.85g, respectively. The desirability of optimization was calculated as 0.82815, indicating that all factors were within the target, which was to obtain the desirable ink properties. The value of composite desirability was calculated as 0.82815, which is high and proved that the prediction is accurate.

3. Microstructure test

SEM micrograph observed the highest amount of cell walls structure collapsed on leaves boiled with the highest amount of glycerol as compared to that of other leaves boiled with the lower amount of glycerol. The result permitted that a high concentration of glycerol may alter the leaves structure to increase the amount of colour extracted from plant cells.

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4. Thermogravimetric test (TGA)

   From the test, the optimized sample recorded the highest percentage of weight lost of 78.5 percent followed by sample with good (64.1 percent), moderate (55.8 percent), and poor (53.4 percent) properties. It can be concluded that the optimized sample requires the fastest time to dry. The slower drying rate of the sample with poor properties is due to diffusional mass transport limitations in the particle phase arising from the viscous phase of the particles or due to a combination of oligomer degradation and mass transfer limitations.

5.2 Recommendation

Several potential improvements were identified in this study that may improve the development and optimization of plant-based marker ink. The followings are recommended for future investigation:

1. To use different raw material from biological sources such as herbal plant sources that can produce edible ink. For example, Beta valgaris (Beet Root) Chenopodiaceae, Citrus limonene (Citrus peel) family Rutaceae, Pentas lanceolata (Butterfly Flow petals) family Rubiaceae, Bauhinia purpurea (Butterflytree) family Caesalpiniaceae, etc.
2. To evaluate the ink by using different factors such as water proof ability, odour, taste, brightness, drying time, flowability, non-clogging nature, viscosity, permanency of colour and stability.
3. To compare the ink sample with the commercial ink (control) to determine the best concentration level for optimal ink penetration on selected paper surfaces such as cardboard, wax paper, book paper and printing paper.

4. To analyze the molecular structure and chemical composition on the ink sample by using Fourier Transform Infrared Spectrophotometer (FTIR). This method can measure the hardening processes of inks and drying processes of solvents contained in inks and output the results on infrared spectra so that the drying speed can be evaluated simply but with high accuracy.
5. To add other additives such as stabliliser for enhancing the ink property.

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APPENDICES

Appendix 1: Fractional Factorial Design (FrFD)

Run Order	Sample	Glycerol	Benzalkonium	Ferrous Sulphate	CMC	Viscosity	Colour intensity
1	S1	5	5	1	5	0.3266	31.85
2	S2	5	5	5	1	0.0948	29.08
3	S3	5	25	1	1	0.0971	27.10
4	S4	1	25	5	1	0.0513	27.41
5	S5	1	5	1	1	0.0656	26.75
6	S6	5	25	1	1	0.1096	27.10
7	S7	1	5	5	5	1.0409	29.33
8	S8	1	5	5	5	1.0451	29.34
9	S9	1	5	1	1	0.0555	26.67
10	S10	5	5	1	5	0.3504	31.39
11	S11	5	25	1	1	0.1196	27.08
12	S12	1	25	5	1	0.0505	27.44

13	S13	5	5	5	1	0.0811	29.10
14	S14	5	25	5	5	0.3153	27.77
15	S15	5	25	5	5	0.3499	27.72
16	S16	5	25	5	5	0.3522	27.87
17	S17	1	25	5	1	0.0524	27.37
18	S18	1	25	1	5	0.0402	26.02
19	S19	5	5	1	5	0.3345	31.80
20	S20	1	25	1	5	0.0347	25.87
21	S21	1	25	1	5	0.0475	25.94
22	S22	1	5	5	5	1.0463	29.35
23	S23	5	5	5	1	0.0989	29.07
24	S24	1	5	1	1	0.0611	26.50

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Appendix 2: Response Surface Methodology (RSM)

Run Order	Sample	Coded Factor		Uncoded Factor		Viscosity	Colour intensity
		Glycerol	CMC	Glycerol	CMC		
1	S1	-2	0	5	3	0.0221	24.70
2	S2	0	2	15	5	0.7278	25.83
3	S3	1	-1	20	2	0.0132	27.20
4	S4	-1	1	10	4	0.2320	25.11
5	S5	-2	0	5	3	0.0163	24.73
6	S6	0	0	15	3	0.1137	25.79
7	S7	-1	-1	10	2	0.0076	24.93
8	S8	0	0	15	3	0.1090	25.78
9	S9	2	0	25	3	0.0091	28.80
10	S10	0	0	15	3	0.1117	25.62
11	S11	0	0	15	3	0.1098	25.58
12	S12	0	-2	15	1	0.0045	25.83
13	S13	1	-1	20	2	0.0111	27.16
14	S14	0	0	15	3	0.1083	25.80
15	S15	-2	0	5	3	0.0203	24.78
16	S16	-1	-1	10	2	0.0109	25.14
17	S17	-1	-1	10	2	0.0084	24.99
18	S18	0	0	15	3	0.1042	25.61
19	S19	1	1	20	4	0.1595	27.40 (moderate)
20	S20	0	0	15	3	0.0948	25.78
21	S21	0	0	15	3	0.1077	25.84
22	S22	0	0	15	3	0.1064	25.85
23	S23	1	-1	20	2	0.0211	27.13
24	S24	1	1	20	4	0.1571	27.48
25	S25	0	0	15	3	0.1063	25.74
26	S26	-1	1	10	4	0.2245	25.09 (poor)
27	S27	0	-2	15	1	0.0045	25.70
28	S28	0	2	15	5	0.7082	25.85
29	S29	0	0	15	3	0.1037	25.63
30	S30	0	0	15	3	0.1083	25.72
31	S31	2	0	25	3	0.0096	28.78
32	S32	0	-2	15	1	0.0045	25.62
33	S33	-1	1	10	4	0.2173	25.22
34	S34	0	0	15	3	0.0973	25.72
35	S35	2	0	25	3	0.0100	28.78 (good)
36	S36	1	1	20	4	0.1344	27.00
37	S37	0	0	15	3	0.0960	25.73
38	S38	0	2	15	5	0.6262	25.72
39	S39	0	0	15	3	0.1083	25.76

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Appendix 3: Viscosity Test- FrFD

Factorial Fit: Viscosity versus Glycerol, Benzalkonium, ...

Estimated Effects and Coefficients for Viscosity (coded units)

Term	Effect	Coef	SE Coef	T	P
Constant		0.2592	0.002122	122.17	0.000
Glycerol	-0.0801	-0.0400	0.002122	-18.87	0.000
Benzalkonium	-0.2484	-0.1242	0.002122	-58.53	0.000
Ferrous Sulphate	0.2447	0.1223	0.002122	57.66	0.000
CMC	0.3622	0.1811	0.002122	85.35	0.000
Glycerol*Benzalkonium	0.2579	0.1290	0.002122	60.79	0.000
Glycerol*Ferrous Sulphate	-0.2523	-0.1261	0.002122	-59.45	0.000
Glycerol*CMC	-0.1242	-0.0621	0.002122	-29.27	0.000

S = 0.0103945 PRESS = 0.00388965
R-Sq = 99.93% R-Sq(pred) = 99.84% R-Sq(adj) = 99.90%

Analysis of Variance for Viscosity (coded units)

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Main Effects	4	1.55488	1.55488	0.388720	3597.74	0.000
Glycerol	1	0.03849	0.03849	0.038488	356.22	0.000
Benzalkonium	1	0.37012	0.37012	0.370116	3425.55	0.000
Ferrous Sulphate	1	0.35921	0.35921	0.359210	3324.61	0.000
CMC	1	0.78707	0.78707	0.787068	7284.58	0.000
2-Way Interactions	3	0.87370	0.87370	0.291233	2695.46	0.000
Glycerol*Benzalkonium	1	0.39921	0.39921	0.399214	3694.86	0.000
Glycerol*Ferrous Sulphate	1	0.38191	0.38191	0.381907	3534.67	0.000
Glycerol*CMC	1	0.09258	0.09258	0.092579	856.85	0.000
Residual Error	16	0.00173	0.00173	0.000108		
Pure Error	16	0.00173	0.00173	0.000108		
Total	23	2.43031				

Obs	StdOrder	Viscosity	Fit	SE Fit	Residual	St Resid
1	2	0.32660	0.33717	0.00600	-0.01057	-1.25
2	6	0.09481	0.09159	0.00600	0.00322	0.38
3	20	0.09713	0.10878	0.00600	-0.01165	-1.37
4	7	0.05131	0.05139	0.00600	-0.00008	-0.01
5	17	0.06559	0.06073	0.00600	0.00486	0.57
6	12	0.10960	0.10878	0.00600	0.00082	0.10
7	21	1.04090	1.04410	0.00600	-0.00320	-0.38
8	13	1.04510	1.04410	0.00600	0.00100	0.12
9	9	0.05547	0.06073	0.00600	-0.00526	-0.62
10	18	0.35040	0.33717	0.00600	0.01323	1.56
11	4	0.11960	0.10878	0.00600	0.01082	1.28
12	15	0.05048	0.05139	0.00600	-0.00091	-0.11
13	14	0.08106	0.09159	0.00600	-0.01053	-1.24
14	8	0.31530	0.33913	0.00600	-0.02383	-2.81R
15	24	0.34990	0.33913	0.00600	0.01077	1.27
16	16	0.35220	0.33913	0.00600	0.01307	1.54
17	23	0.05237	0.05139	0.00600	0.00098	0.12
18	19	0.04022	0.04082	0.00600	-0.00060	-0.07
19	10	0.33450	0.33717	0.00600	-0.00267	-0.31
20	3	0.03470	0.04082	0.00600	-0.00612	-0.72
21	11	0.04753	0.04082	0.00600	0.00671	0.79
22	5	1.04630	1.04410	0.00600	0.00220	0.26
23	22	0.09889	0.09159	0.00600	0.00730	0.86
24	1	0.06112	0.06073	0.00600	0.00039	0.05

R denotes an observation with a large standardized residual.

Appendix 4: Colour Intensity Test- FrFD

Factorial Fit: Colour versus Glycerol, Benzalkonium, Ferrous Sulp, CMC

Estimated Effects and Coefficients for Colour (coded units)

Term	Effect	Coef	SE Coef	T	P
Constant		28.122	0.02203	1276.62	0.000
Glycerol	1.578	0.789	0.02203	35.83	0.000
Benzalkonium	-2.128	-1.064	0.02203	-48.31	0.000
Ferrous Sulphate	0.565	0.282	0.02203	12.82	0.000
CMC	1.132	0.566	0.02203	25.69	0.000
Glycerol*Benzalkonium	-0.813	-0.407	0.02203	-18.46	0.000
Glycerol*Ferrous Sulphate	-1.517	-0.758	0.02203	-34.43	0.000
Glycerol*CMC	0.513	0.257	0.02203	11.65	0.000

S = 0.107916 PRESS = 0.41925
R-Sq = 99.74% R-Sq(pred) = 99.41% R-Sq(adj) = 99.62%

Analysis of Variance for Colour (coded units)

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Main Effects	4	51.7250	51.7250	12.9313	1110.38	0.000
Glycerol	1	14.9468	14.9468	14.9468	1283.45	0.000
Benzalkonium	1	27.1788	27.1788	27.1788	2333.78	0.000
Ferrous Sulphate	1	1.9153	1.9153	1.9153	164.47	0.000
CMC	1	7.6840	7.6840	7.6840	659.81	0.000
2-Way Interactions	3	19.3518	19.3518	6.4506	553.90	0.000
Glycerol*Benzalkonium	1	3.9691	3.9691	3.9691	340.81	0.000
Glycerol*Ferrous Sulphate	1	13.8017	13.8017	13.8017	1185.12	0.000
Glycerol*CMC	1	1.5811	1.5811	1.5811	135.76	0.000
Residual Error	16	0.1863	0.1863	0.0116		
Pure Error	16	0.1863	0.1863	0.0116		
Total	23	71.2631				

Obs	StdOrder	Colour	Fit	SE Fit	Residual	St Resid
1	2	31.8500	31.6800	0.0623	0.1700	1.93
2	6	29.0800	29.0833	0.0623	-0.0033	-0.04
3	20	27.1000	27.0933	0.0623	0.0067	0.08
4	7	27.4100	27.4067	0.0623	0.0033	0.04
5	17	26.7500	26.6400	0.0623	0.1100	1.25
6	12	27.1000	27.0933	0.0623	0.0067	0.08
7	21	29.3300	29.3400	0.0623	-0.0100	-0.11
8	13	29.3400	29.3400	0.0623	0.0000	0.00
9	9	26.6700	26.6400	0.0623	0.0300	0.34
10	18	31.3900	31.6800	0.0623	-0.2900	-3.29R
11	4	27.0800	27.0933	0.0623	-0.0133	-0.15
12	15	27.4400	27.4067	0.0623	0.0333	0.38
13	14	29.1000	29.0833	0.0623	0.0167	0.19
14	8	27.7700	27.7867	0.0623	-0.0167	-0.19
15	24	27.7200	27.7867	0.0623	-0.0667	-0.76
16	16	27.8700	27.7867	0.0623	0.0833	0.95
17	23	27.3700	27.4067	0.0623	-0.0367	-0.42
18	19	26.0200	25.9433	0.0623	0.0767	0.87
19	10	31.8000	31.6800	0.0623	0.1200	1.36
20	3	25.8700	25.9433	0.0623	-0.0733	-0.83
21	11	25.9400	25.9433	0.0623	-0.0033	-0.04
22	5	29.3500	29.3400	0.0623	0.0100	0.11
23	22	29.0700	29.0833	0.0623	-0.0133	-0.15
24	1	26.5000	26.6400	0.0623	-0.1400	-1.59

R denotes an observation with a large standardized residual.

Appendix 5: Viscosity Test- RSM

Response Surface Regression: Viscosity versus Glycerol, CMC

The analysis was done using coded units.

Estimated Regression Coefficients for Viscosity

Term	Coef	SE Coef	T	P
Constant	0.093769	0.011913	7.871	0.000
Glycerol	-0.007338	0.008282	-0.886	0.382
CMC	0.143055	0.008282	17.273	0.000
Glycerol*Glycerol	-0.023524	0.005993	-3.925	0.000
CMC*CMC	0.059319	0.005993	9.897	0.000

S = 0.0496920 PRESS = 0.117395
R-Sq = 92.92% R-Sq(pred) = 90.10% R-Sq(adj) = 92.09%

Analysis of Variance for Viscosity

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	4	1.10239	1.10239	0.275598	111.61	0.000
Linear	2	0.73867	0.73867	0.369333	149.57	0.000
Glycerol	1	0.00194	0.00194	0.001939	0.79	0.382
CMC	1	0.73673	0.73673	0.736728	298.36	0.000
Square	2	0.36372	0.36372	0.181862	73.65	0.000
Glycerol*Glycerol	1	0.12184	0.03804	0.038041	15.41	0.000
CMC*CMC	1	0.24188	0.24188	0.241882	97.96	0.000
Residual Error	34	0.08396	0.08396	0.002469		
Lack-of-Fit	4	0.07713	0.07713	0.019282	84.74	0.000
Pure Error	30	0.00683	0.00683	0.000228		
Total	38	1.18635				

Obs	StdOrder	Viscosity	Fit	SE Fit	Residual	St Resid	
1	31	0.022	0.014	0.026	0.008	0.18	
2	34	0.728	0.617	0.026	0.111	2.59	R
3	15	0.013	-0.021	0.014	0.034	0.71	
4	16	0.232	0.280	0.014	-0.048	-1.01	
5	5	0.016	0.014	0.026	0.002	0.05	
6	35	0.114	0.094	0.012	0.020	0.41	
7	14	0.008	-0.006	0.014	0.014	0.29	
8	38	0.109	0.094	0.012	0.015	0.32	
9	6	0.009	-0.015	0.026	0.024	0.57	
10	26	0.112	0.094	0.012	0.018	0.37	
11	12	0.110	0.094	0.012	0.016	0.33	
12	7	0.004	0.045	0.026	-0.040	-0.95	
13	2	0.011	-0.021	0.014	0.032	0.67	
14	25	0.108	0.094	0.012	0.015	0.30	
15	18	0.020	0.014	0.026	0.006	0.14	
16	1	0.011	-0.006	0.014	0.017	0.36	
17	27	0.008	-0.006	0.014	0.015	0.30	
18	23	0.104	0.094	0.012	0.010	0.22	
19	17	0.160	0.265	0.014	-0.106	-2.22	R
20	24	0.095	0.094	0.012	0.001	0.02	
21	9	0.108	0.094	0.012	0.014	0.29	
22	11	0.106	0.094	0.012	0.013	0.26	
23	28	0.021	-0.021	0.014	0.042	0.88	
24	30	0.157	0.265	0.014	-0.108	-2.27	R
25	39	0.106	0.094	0.012	0.013	0.26	
26	3	0.225	0.280	0.014	-0.055	-1.16	
27	33	0.004	0.045	0.026	-0.040	-0.95	
28	8	0.708	0.617	0.026	0.091	2.13	R
29	13	0.104	0.094	0.012	0.010	0.21	

30	22	0.108	0.094	0.012	0.015	0.30	
31	32	0.010	-0.015	0.026	0.025	0.58	
32	20	0.005	0.045	0.026	-0.040	-0.95	
33	29	0.217	0.280	0.014	-0.063	-1.32	
34	10	0.097	0.094	0.012	0.003	0.07	
35	19	0.010	-0.015	0.026	0.025	0.59	
36	4	0.134	0.265	0.014	-0.131	-2.75	R
37	37	0.096	0.094	0.012	0.002	0.05	
38	21	0.626	0.617	0.026	0.009	0.21	
39	36	0.108	0.094	0.012	0.015	0.30	

R denotes an observation with a large standardized residual.

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Appendix 6: Colour Intensity Test- RSM

Response Surface Regression: Colour versus Glycerol, CMC

The analysis was done using coded units.

Estimated Regression Coefficients for Colour

Term	Coef	SE Coef	T	P
Constant	25.7739	0.03071	839.238	0.000
Glycerol	1.0331	0.02135	48.384	0.000
CMC	0.0347	0.02135	1.626	0.113
Glycerol*Glycerol	0.2607	0.01545	16.870	0.000
CMC*CMC	0.0098	0.01545	0.636	0.529

S = 0.128106 PRESS = 0.710658
R-Sq = 98.73% R-Sq(pred) = 98.38% R-Sq(adj) = 98.58%

Analysis of Variance for Colour

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	4	43.4413	43.4413	10.8603	661.76	0.000
Linear	2	38.4627	38.4627	19.2314	1171.85	0.000
Glycerol	1	38.4193	38.4193	38.4193	2341.05	0.000
CMC	1	0.0434	0.0434	0.0434	2.64	0.113
Square	2	4.9786	4.9786	2.4893	151.68	0.000
Glycerol*Glycerol	1	4.9719	4.6705	4.6705	284.59	0.000
CMC*CMC	1	0.0066	0.0066	0.0066	0.40	0.529
Residual Error	34	0.5580	0.5580	0.0164		
Lack-of-Fit	4	0.2540	0.2540	0.0635	6.27	0.001
Pure Error	30	0.3039	0.3039	0.0101		
Total	38	43.9993				

Obs	StdOrder	Colour	Fit	SE Fit	Residual	St Resid	
1	31	24.700	24.750	0.066	-0.050	-0.46	
2	34	25.830	25.883	0.066	-0.053	-0.48	
3	15	27.200	27.043	0.037	0.157	1.28	
4	16	25.110	25.046	0.037	0.064	0.52	
5	5	24.730	24.750	0.066	-0.020	-0.19	
6	35	25.790	25.774	0.031	0.016	0.13	
7	14	24.930	24.977	0.037	-0.047	-0.38	
8	38	25.780	25.774	0.031	0.006	0.05	
9	6	28.800	28.883	0.066	-0.083	-0.75	
10	26	25.620	25.774	0.031	-0.154	-1.24	
11	12	25.580	25.774	0.031	-0.194	-1.56	
12	7	25.830	25.744	0.066	0.086	0.78	
13	2	27.160	27.043	0.037	0.117	0.96	
14	25	25.800	25.774	0.031	0.026	0.21	
15	18	24.780	24.750	0.066	0.030	0.27	
16	1	25.140	24.977	0.037	0.163	1.33	
17	27	24.990	24.977	0.037	0.013	0.11	
18	23	25.610	25.774	0.031	-0.164	-1.32	
19	17	27.400	27.112	0.037	0.288	2.34	R
20	24	25.780	25.774	0.031	0.006	0.05	
21	9	25.840	25.774	0.031	0.066	0.53	
22	11	25.850	25.774	0.031	0.076	0.61	
23	28	27.130	27.043	0.037	0.087	0.71	
24	30	27.480	27.112	0.037	0.368	3.00	R
25	39	25.740	25.774	0.031	-0.034	-0.27	
26	3	25.090	25.046	0.037	0.044	0.36	
27	33	25.700	25.744	0.066	-0.044	-0.40	
28	8	25.850	25.883	0.066	-0.033	-0.30	
29	13	25.630	25.774	0.031	-0.144	-1.16	

30	22	25.720	25.774	0.031	-0.054	-0.43
31	32	28.780	28.883	0.066	-0.103	-0.93
32	20	25.620	25.744	0.066	-0.124	-1.13
33	29	25.220	25.046	0.037	0.174	1.42
34	10	25.720	25.774	0.031	-0.054	-0.43
35	19	28.780	28.883	0.066	-0.103	-0.93
36	4	27.000	27.112	0.037	-0.112	-0.91
37	37	25.730	25.774	0.031	-0.044	-0.35
38	21	25.720	25.883	0.066	-0.163	-1.48
39	36	25.760	25.774	0.031	-0.014	-0.11

R denotes an observation with a large standardized residual.

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