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**QUANTITATIVE ANALYSIS OF INDIGO BY ULTRAVIOLET-VISIBLE SPECTROPHOTOMETER AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY**

**Development of Analysis Methods**

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## ABSTRACT

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<b>Name of thesis</b> QUANTITATIVE ANALYSIS OF INDIGO BY ULTRAVIOLET-VISIBLE SPECTROPHOTOMETER AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY. Development of Analysis Methods		
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<p>Indigo is one of the oldest dyes which was utilized by mankind since ancient times. After travelling for thousands of years around the world, the indigo derived from Woad or Morsinko (in Finnish) plant has found its way into Finnish fields. Natural Indigo Oy located in Nivala has run a pilot project for cultivating woad in Pyhäsalmi mine by utilizing innovative technology. This research work aims at developing reliable quantification methods of indigo with the consideration of accessibility, availability, economic aspect, and limitations.</p> <p>Indigo is insoluble in water and most common solvents but soluble in ethyl acetate, chloroform and dimethyl sulfoxide. Two methods used to separate indigo from aqueous solution for analysis are liquid-liquid extraction (with ethyl acetate and chloroform) and centrifugal separation to dissolve in dimethyl sulfoxide. The result differences and efficiency between different solvents, extraction methods, and detection instruments (Ultraviolet-Visible spectrophotometer and high-performance liquid chromatography) are discussed. The commercially synthetic indigo is used as the standards to support the reliability of methods.</p>		

### Key words

Chloroform, Ethyl Acetate, DMSO, HPLC, Indigo, Isatis tinctoria, UV-Vis.

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Nguyen Ngoc Phuong Thao

## CONCEPT DEFINITIONS

Abs	Absorbance
ACN	Acetonitrile
AVG	Average
CAS	Chemical Abstracts Service
Conc	Concentration
DMSO	Dimethyl Sulfoxide
EU	European Union
HPLC	High-Performance Liquid Chromatography
l	Liter
LC	Liquid Chromatography
LLE	Liquid-liquid extraction
$\lambda_{\max}$	Maximum absorbance wavelength
$\mu\text{m}$	Micrometer
mAU	Milli-Absorbance units
mg	Milligram
min	Minute
ml	Milliliter
mm	Millimeter
MPa	Megapascal unit (1 MPa = 10 bar)
PTFE	Polytetrafluoroethylene
rpm	Round per minute
SD	Standard Deviation
TFA	Trifluoroacetic acid
UV-Vis	Ultraviolet-Visible Spectrophotometer

**ABSTRACT**

**ACKNOWLEDGEMENTS**

**CONCEPT DEFINITIONS**

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## 1 INTRODUCTION

Indigo known as iconic color of blue hue denim has been used for coloration of textiles since antiquity. The precursors of indigo are found in approximate a hundred and fifty varieties of plant around the world. Before the popularity of synthetic indigo in 1870, plant-derived indigo was widely cultivated to meet the high demand of worldwide consumption. In the Middle Ages, woad (*Isatis tinctoria*) was an important crop in Europe because of bringing immense wealth to the woad traders. The central trades of indigo were in Southern Europe such as France and Germany. The discovery of sea-route to India in the 17<sup>th</sup> century brought Indian-cultivated indigo (*Indigofera*) to Europe in bigger scale, replacing almost woad consumption in European countries. Nowadays, indigo is mainly synthesized from by-products of fossil fuels which are non-sustainable resources. The growing awareness and interest of renewability and sustainability have brought many attentions to natural indigo production, especially woad production in Europe. Woad plant prefers chilled summer nights of Finland yet it cannot stand the Finnish wintertime. Fortunately, the ideas of growing indigo underground in the old mine of Pyhäsalmi is highly potential to not only cultivate woad in Finland but also utilize the excellent facilities of the old mine. Natural Indigo Oy has operated a pilot project for growing in the mines. The aim of this project is to find out the cultivating methods that can yield the highest amount of indigo production with the lowest amount of impurities (Kauranen 2018).



FIGURE 1. Pilot project of growing woad in Pyhäsalmi mine (Natural Indigo Oy)



Unlike other natural dyes, indigo does not exist in the plants but is formed during the extraction process from its precursors accumulated by the plants. The precursors are compounds containing the indoxyl group which is released and oxidized by atmospheric oxygen to indigo during the extraction process. Before the mass extraction process is dealt with, the efficiency of extraction and the quantitative determination of indigo are considered carefully. This study is to focus on one of these considerations, which is methods of determining indigo.

In order to quantify the yield of indigo gets from a crop, a time-saving and reliable method of determining indigo formation is needed. However, there is no available standards or simple methods to quantify indigo concentration. Indigo is a tricky compound to analyze. It is insoluble in water and most common solvents. The methods to determine indigo concentration in this study is based on the literature review of indigo properties and the solvent miscibility chart. Fortunately, it is discovered that indigo can be dissolved in ethyl acetate, chloroform, and Dimethyl Sulfoxide (DMSO) with different shades of blue, which means the Ultraviolet-Visible spectrophotometer (UV-Vis) instrument are possibly used to detect indigo concentration in solvents by the loss of absorbance. Additionally, indigo in DMSO can be measured by High-performance liquid chromatography (HPLC) with a certain settings of retention time, elution solutions and temperature. The disadvantage of these methods is that they require a careful control and repeatable experiments to obtain a reliable results and standard deviations.

In this study, the comparison of indigo separation techniques and instrument accuracy is mainly presented by experiment results and discussed. The commercially synthetic indigo (95% purity) is used as standards in testing and calibrations to confirm the reliability of quantitative methods. The considerations of accessibility, availability, economic aspect, and limitation for each method are also mentioned in this study.

## 2 AIM OF STUDY

This study starts at investigating the structure and properties of indigo, the extraction and formation of plant-derived indigo from woad, along with the artificial indigo synthesis method and its pros and cons. Besides, the knowledge related analytic chemistry and organic chemistry paves a strong foundation to research on the solubility of indigo in different solvents and the applicability of UV-Vis and HPLC instruments in quantification of indigo. The results of literature review may be used as basic for development quantitative analysis methods for natural indigo.

The aim of this study is to develop the reliable method to find out the approximate unknown concentration of indigo after extracting from woad. Liquid-liquid extraction (LLE) is carried out with two solvents (ethyl acetate and chloroform) with different number of extractions and amount of solvent for each extraction. Concurrently, samples from specimen goes through the centrifuge to extract indigo paste and dissolve in DMSO for further analysis. The comparison of different separating techniques and instruments might give the credible results of developing analysis methods.

### 3 REVIEW OF LITERATURE

Dyestuffs such as Sulphur and vat dyes, especially indigo, plays an important role in textile industry and dyeing industry. Indigo accounts for approximately 3% of all dyes used globally and about 15% of the dyes used for cotton in particular (Ghaly, Ananthashankar and Ramakrishnan 2014). This literature review consists of sections with the history of indigo, properties of indigo, precursors of indigo, indigo formation, traditional and modern extraction methods of indigo in dyeing and textile industry, along with analysis methods of determining indigo compound focused on separation methods and UV-Vis.

#### 3.1 Brief history of Indigo

Indigo blue (also known as Indigotin) is one of the oldest and most popular dyestuffs which have been used by mankind since ancient times (FIGURE 2). Evidence from the excavations in the Indus valley show that indigo was firstly discovered by Indian (Roshan 2015, 39). Phoenician traders and migrating people spread through Mesopotamia, Egypt, Greece, Rome, Britain, Peru, and African (Kriger and Collen 2006). Balfour-Paul (2000) and Gilbert & Cooke (2001) also mentioned that mummies from Egyptian tombs have covered by indigo dyed cloths since about 2500 BC. Thus, historians estimated the history of indigo started at 5000 BC (Roshan 2015, 39).

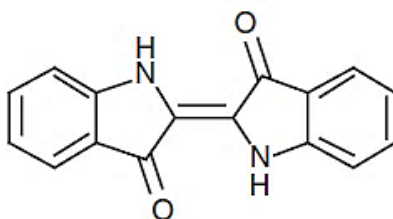


FIGURE 2. Indigo (Indigotin)

Not only was indigo used in dyeing fabrics, it was a popular colorant in medieval illuminations by some of the great master in antiquity and the pre-modern eras due to a characteristic of light stability (Hommes 2004). Different species of indigo plant sources were cultivated around the world, such as *Indigofera* species (*I. tinctoria*) in the tropics like India, dyer's woad (*Isatis tinctoria*) in Europe (Clarke 2004), dyer's knotweed (*Polygonum tinctorium*) in China and Japan, and the native *Indigofera caroliniana*, the introduced *I. tinctoria* and *Indigofera suffruticosa* species in colonial North America (Roshan 2015, 39). During the industrial revolution, the demand of indigo dramatically increased due to the popularity of

Levi Strauss's blue denim jeans (Roshan 2015, 40). The natural extraction process was expensive and could not meet the demand of growing and garment industry, thereby requiring the new methods for synthetic indigo production. Since cost effectiveness and availability of synthetic indigo, the use of natural indigo had been neglected from about 100 years but have recently come back into the limelight due to the consideration of health and environment (Vuorema 2008, 8-9).

In Chinese medicine, indigo (Qing Dai in Chinese) is commonly used in medicine as a heat remover to treat various ailments. It is believed that cooling properties of indigo affects the functions of the human liver, resulting in the elimination of heat from the body and a drop of blood's temperature (Stasiak, Kukula-Koch and Glowniak 2014, 216-219). Indigo can be used either alone or together with other herbs based on Chinese medicine recipe to treat sore throat, eczema, psoriasis, saliva gland, ulcers in the mouth a gingivitis (Tang and Eisenbrand 1992). Therefore, natural indigo still plays an important part in Chinese medicinal consumption which cannot be replaced by synthetic indigo due to health effects. Nowadays, the trend of using plant-based dyes for clothing which is not only sustainable and biodegradable solutions, also preserves the ecological balance has caught global attentions. The use of natural dyes is believed to avoid the allergic symptoms for consumers due to chemical reactions, thereby making a steps in cosmetic industry.

### **3.2 Natural indigo**

For textile dyeing industry with natural dyes, indigo plays a unique position as the most important blue natural dye. Before the advent of synthetic indigo, in 19<sup>th</sup> century, especially between the first cotton gin and the Civil War, the value of natural indigo produced in South Carolina (UAS) was much higher than that of rice or cotton (Roessler 2003, 8). Recently, the consumer's concern for environmental impacts and sustainability of manufactured products has revived the interest in plant-derived indigo products. Unlike other natural dyes, indigo does not exist in the plants. The indoxyl groups from the precursors in plant species are released during the extraction process. They are spontaneously oxidized by the atmospheric oxygen to indigo (Bechtold and Mussak 2009, 112).

### 3.2.1 Properties of indigo

Indigo blue or indigotin (c.i. Vat blue, IUPAC name 3H-indol-3-ylidene)-1,2-dihydro-, chemical formula  $C_{16}H_{10}N_2O_2$ , CAS number 482-89-3) exists as dark blue-violet needles or leaves with a reddish bronze metallic appearance under normal pressure and ambient temperature (Roessler 2003, 11; Božič and Kokol 2008, 299-309). The color of indigo is changed due to its environment. In solid form, in polar solvents as well as being applied to textiles as a vat dye, indigo owns its blue color; however, in gas phase, it exists in its monomeric form which causes red color; and it is violet in non-polar solvents (Christie 2009, 51-56). The blue color of indigo and its derivatives was a fascinating subject during latter half of 20<sup>th</sup> century. It was discovered that the substitution in different ring position of indigo caused significant shifts in both visible long-wavelength and UV band (Sadler 1956, 316-319). Furthermore, if there is the presence of indirubin as an impurity in plant, its results in the purple-blue color of indigo (Meijer, et al. 2006). Melo, et al. (2004, 6978-6979) mentioned that indirubin in dimethyl formamide has the maximum absorbance wavelength at 546 nm; while Ahn et al. (2013, 107) reported that indirubin in DMSO has the maximum absorbance at 542 nm.

Indigo is insoluble in water and soluble in some organic solvents (Vuorema 2008, 13). Green (1989, 15-16) and Steingruber (2004) indicated that indigo is more soluble in polar organic solvents than non-polar solvents. The extremely low solubility is explained by the strong intermolecular and intramolecular hydrogen bonds which are formed in indigo crystals (Holt ja Sadler 1958, 495-505). Despite the possibility of cis-trans isomerism of the central C=C, hydrogen bonding stabilizing trans-isomer was observed, causing poor solubility, high melting point, and the vibrational and electronic absorption spectra of indigo (Kiessinger and Lüttke 1966). As a result of the relatively high boiling point (~300°C), indigo is very resistant to elevated temperatures and light in either presence of air or not. At 390°C, indigo decomposes. Thus, in the purification process of indigo, impure indigo extracted from the crop is heated to or just above 300°C; then, the vapor indigo can be easily separated from impurities remaining in solid phase (Bechtold and Mussak 2009, 126). Pure solid indigo can be collected when cooling down below 300°C. Figure 3 shows a block diagram of purification process constituted by two phases: sublimation and desublimation.

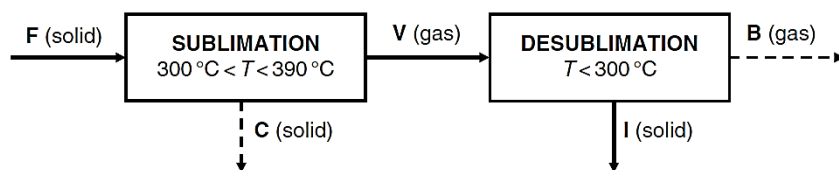
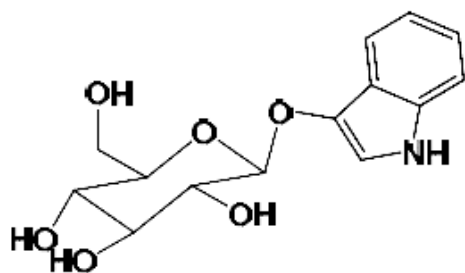


FIGURE 3. Purification process of indigo based on sequential sublimation–desublimation. F = feed, C = carbonaceous residue, V = gas phase containing sublimated indigo; I = purified indigo; B = by-products, adapted from (Bechtold and Mussak 2009, 126)

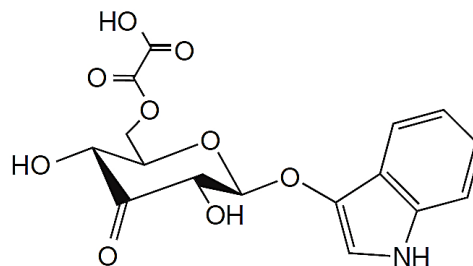
### 3.2.2 Indigo formation from plant-derived precursors and indigo reduction for dyeing

There are three types of plants with more or less than 300 species for producing indigo in many parts of the world: Leguminosae (pea family), known as *Indigofera tinctoria*, is grown in India, South East Asia, and the Middle East; Cruciferae (cabbage family), known as *Isatis tinctoria* or woad, used to be cultivated in the Mediterranean and Western Asia, currently being grown in North America and Europe; Polygonaceae (dock family) is commonly named after the places it is grown, Japanese or Chinese indigo plant. *Indigofera sumatrana* is the most important species (Roshan 2015, 39-41). Other species include *Strobilanthes cusia* in Japan's Ryukyu Islands and Taiwan, *I. suffruticosa* (Anil) and *Indigofera arrecta* (natal indigo) in Central and South America, *Polygonum tinctorum* (dyer's knotweed) in some temperate climate regions (Cardon 2007).

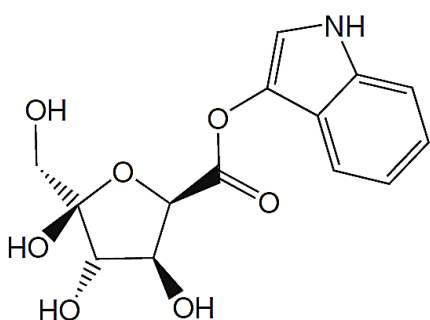
Indigo does not exist in plant species, but it can be produced by its precursors. Precursors of indigo are mainly found in the leaves (vacuoles), meanwhile, the roots, stems, flower buds, flowers, and cotyledons contain about 3% concentration of precursors less than leaves (Minami, et al. 2000, 218-225). Indican (FIGURE 4-a) (indoxyl- $\beta$ -D-glucoside, 1-O-(1 H-indol-3-yl)- $\beta$ -D-glucoside) has been identified as indigo precursor in *Indigofera* species and *Polygonum tinctorium* since 1900 (Oberthür, et al. 2004, 178). Isatan B (FIGURE 4-c, d) (Indoxyl-5-ketogluconate) is another main precursor of indigo in *Isatis tinctoria* (Balfour-Paul 2000). Lately, two other precursors (isatan C and isatan A) has been found in plant components. Isatan C was proposed to be an ester of dioxindole, yielding mainly isoindirubin (Bechtold and Mussak 2009, 9). It has not been fully identified and characterized but it is low potential to conversion to indigo due to its low concentration in compared to indican and isatan B in plant species (Maugard, et al. 2001, 897-904). Isatan A (FIGURE 4-b) ( $\beta$ -D-ribohex-3'-ulopyranoside) was extracted in *Isatis* leaves about 5-20% of leaf dry weight (Oberthür, et al. 2004, 178-182).



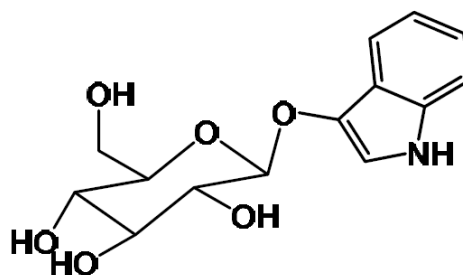
a) Indican = indoxyl-β-D-glucoside



b) Isatan A = β-D-ribohex-3'-ulopyranoside



c) Isatan B = Indoxyl-5-ketogluconate



d) Isatan B = Indoxyl-5-ketogluconate

FIGURE 4. Indigo precursors: a) Indican (Vuorema 2008, 12), b) Isatan A (Oberthür, et al. 2004, 180), c) isatan B (Epstein, Nabors and Stowe 1967, 548), d) isatan B (Vuorema 2008, 12)

The reaction leading to the formation of indigo is shown in Figure 5a. The precursors, both indican and isatans, contain glucoside which can be hydrolyzed to release the free indoxyl with or without alkali as a catalyst (Lestari 1998, 20-29). Indoxyl radical firstly forms leuco-indigo which is then oxidized to precipitation of indigo. Coston & Holt (1958, 506-519) found that conversion of indoxyl to indigo is always less than 100% because of the generation of isatin from indoxyl in an oxygen-rich environment as a side reaction. The condensation of isatin and free indoxyl radical gives rise of indirubin which is known as the red shade of indigo (FIGURE 6) (Kokubun, Edmonds and John 1998, 80). Besides, natural indigo also contains other impurities such as indigo-brown, indigo gluten and mineral matter.

Since indigo is insoluble in water, it must be converted to soluble form to dye the fabrics. In ancient time, conversion of indigo insoluble form to leuco indigo soluble form was called reduction process, carrying out un wooden vat; thus, indigo is categorized in vat dyes class (Roshan 2015, 49). In dyeing process, cotton fabric is dipped multiple times in reduced indigo solution, known as leuco indigo, then

it is hung to dry out where leuco indigo is oxidized by atmospheric oxygen to indigo. Indigo color particle is large enough to get stuck in the cotton fabric and stay last long. The reduction and oxidation process of indigo and leuco indigo is presented in Figure 5b.

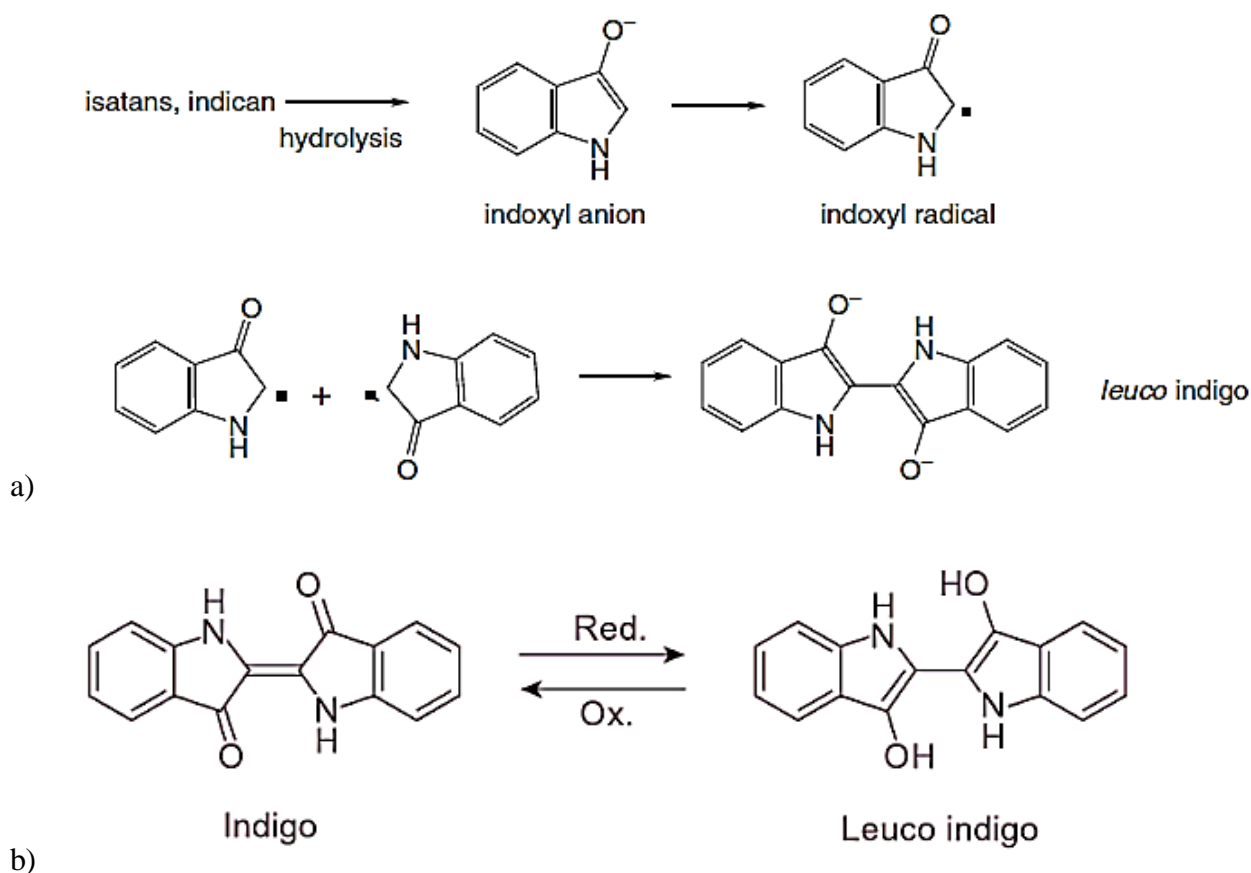


FIGURE 5. a) Indoxyl radicals generated by the hydrolysis of the indigo precursors are formed into leuco indigo (Bechtold and Mussak 2009, 112); b) Indigo oxidation and reduction (Roshan 2015, 49)

Several reduction methods have been invented for the application of indigo in dyeing, starting from the fermentation vat or bacterial reduction which was used for centuries before modern methods came (Vuorema 2008, 14-15). Chemical reduction is a highly efficient method which universally uses sodium hydrosulphite as a reducing agent. However, the use of sodium hydrosulphite causes the formation of non-environment friendly products such sulphite, sulphate, and thiosulphate (Božič and Kokol 2008). Chemical reduction of indigo is non-recycle process because the reducing power of reducing agents cannot be regained; electrochemical reduction, catalytic hydrogenation and electrocatalytic hydrogenation are potential alternatives (Vuorema 2008, 24-29).



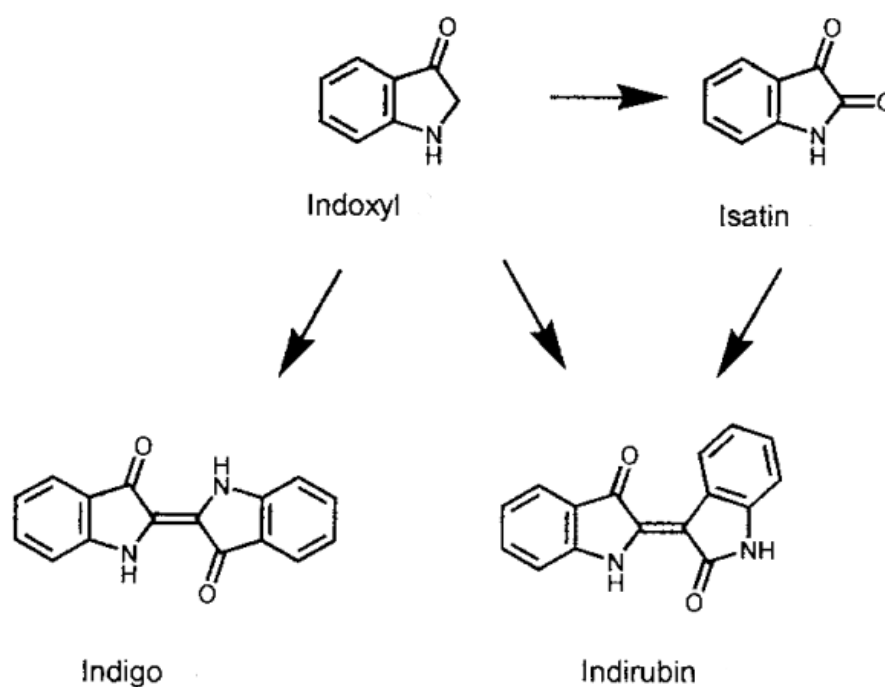


FIGURE 6. Side reaction leading to the formation of indirubin (red shade of indigo), adapted from (Kokubun, Edmonds and John 1998, 80)

### 3.2.3 Extraction process of indigo from plant

There are two available methods of indigo extraction procedures: Dry method using crushed leaf material and Wet method by steeping leaf in water. In traditional methods, there are slight differences in indigo extraction process between different plants (Roshan 2015, 41); meanwhile, modern methods can be applied widely for indigo extraction from plant species.

The basic principle of traditional process is to allow indigo formation within the leaf material by crushing the leaves. The crushed leaf then goes through the “couching” process to reduce its mass and fibrous nature, resulting in air contact for indigo formation and concentrated indigo in readiness for dyeing. This is an intensely time and labor consuming process, yet the final product is impure and undefined (Bechtold and Mussak 2009, 114). After harvesting, fresh woad leaves are usually crushed to a pulpy paste and kneaded to a ball for drying in the frames or racks. These balls can be stored for later uses. In contrast, Polygonum leaves are chopped and dried for a day in the sun, then overnight in drier and store in straw bags. Before vat dyeing, dried balls or dried leaves are watered and fermented with careful

temperature and moisture in several weeks. It was discovered that the woad ball dried in the long time which causes delaying the formation of stable indigo end-product, extending the life of unstable indoxyl and leuco indigo intermediates; consequently, indirubin and other impurities are relatively high (Kokubun, Edmunds and John 1998, 79-87).

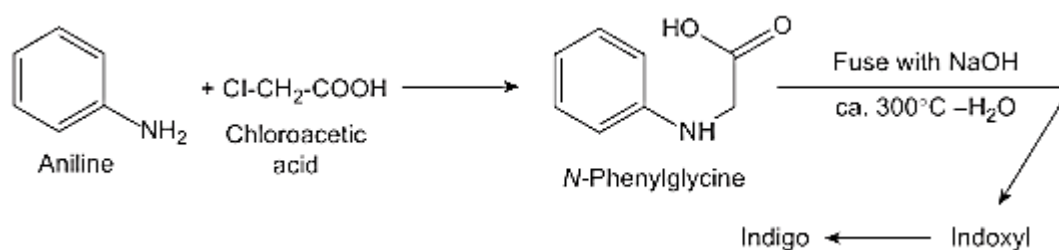
Wet method or steeping-in-water method is the production of indigo by steeping leaves in water where the precursors are water-soluble and easy to release free indoxyl. Due to the aeration, free indoxyl forms blue precipitation indigo, settling down as the bottom products. The basic steps of water extraction include extraction, alkalization, oxidation, and filtration. Prior to water extraction process, leaves are rinsed to remove soil particles which may react with indoxyl to form non-indigotin oxidation products and contaminate the final pigment products (Wenner 2017, 17). In the first stage of water extraction, harvested woad plants are soaked tightly in the stepping vat for certain period of time in which anaerobic fermentation happens and results in yellow-green solution. The precursors of indigo are hydrolyzed within the decaying leaf tissue and the indoxyl is leached from the leaves. Prolonging the fermentation stage causes the decay of indoxyl to undesired products because of side reactions (Perkin and Everest 1918). Right after the fermentation stage, the addition of a small amount of alkali such as ammonia, sodium carbonate or calcium hydroxide, can accelerate the rate the indoxyl oxidation to indigo, resulting in the increase of indigo yield; however, an excess of alkali decreases the yield of indigo (Darrac and van Schendel 2006). Vuorema (2008) noted that a pH of 11 is the most suitable for indigo production when using calcium hydroxide as an alkalizer. In the oxidation stage, the yellow-green solution run into beating vat where the mechanical paddles introduce air by beating the solution, by creating a spray water, or by pumping air in. In this second stage (in beating vat), oxidative conditions lead to the formation of a blue precipitation indigo, settling down in the bottom of tank. The last stage is purification of indigo by heating, filtering, washing, and finally drying in form of cake (Bechtold and Mussak 2009, 117). The wet process of *Isatis tinctoria* leaf extraction is almost the same as that of *Indigofera* leaf extraction, excepts that *Isatis tinctoria* leaves are steeped in heated water for a certain of time. This is because isatan is predominantly precursors (indicant is a minor precursor); isatan must be treated with acidified hot water, then made alkaline and oxygenated (Stoker, Cooke and Hill 1998, 316-317). Historically, the Japanese have used a slightly different method called Sukumo to extract Indigo from *Polygonum* leaves, which is hydrolyzed leaves are mixed with wheat husk powder, limestone powder and lye ash and be fermented for about a week to form the dye pigment (Roshan 2015, 41-42).

### 3.3 Synthetic indigo

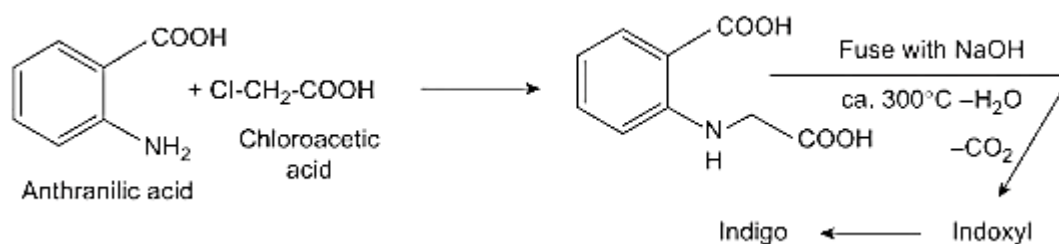
Adolf von Baeyer's research of indigo structure in 1869 paved the way for the first commercially successful synthesis of indigo published by Heumann in 1890 (Vandenabeele and Moens 2003, 187-193). Hermann's first synthesis with N-phenylglycine as a starting chemical could yield very low product of indigo (Roshan 2015, 43-46). In the second synthesis, anthranilic acid was converted by fusion with sodium hydroxide into indoxyl which was quickly oxidized by atmospheric oxygen, dimerizing into indigo (Christie 2001). This synthesis route obtained a high yield of indigo but anthranilic acid was expensive. The 2<sup>nd</sup> version of Heumann's process was scaled up to an industrial level (several thousands of tons per year) by BASF in 1897 (Seefelder 1994). In the first decade of 20<sup>th</sup> century, BASF was accounted for 80% of the world's synthetic indigo, leading to that the natural indigo was entirely replaced by 1913 (Freeman 1997). Besides, modified and improved versions of Heumann's indigo synthesis, e.g. Heumann-Pfleger indigo synthesis (1901), improved synthesis of N-Phenylglycine BASF (1925), have been commercially available to meet the highly global demand of indigo during the 1960s and 1970s. These synthesis routes are shown in Figure 7. The introduction and invasion of synthetic indigo ended the colonial production of indigo, particularly in British, French and Iberian colonies (Bechtold and Mussak 2009, 10-11). The purity of plant-derived indigo is relatively low compared to synthetic indigo even though modern extraction methods have been applied. It is reported that the purity of *Indigofera* indigo and woad are from 50% to 77% and from 20% to 40% respectively from Stoker et.al. (1998) and Bechtold et.al. (2002); synthetic indigo, meanwhile, always produces over 90% (Garcia-Macias and John 2004).

Indigo production with hydrocarbon degrading bacteria expressing mono-oxygenase or dioxygenases has been known and investigated of possible alternative for synthetic indigo production since 1920s (Gray 1928, 263-280). *Escherichia coli*. have been developed for fermentation process for biotech indigo from glucose (Roessler 2003, 11). However, this method produced indirubin causing undesirable red color to the dyeing.

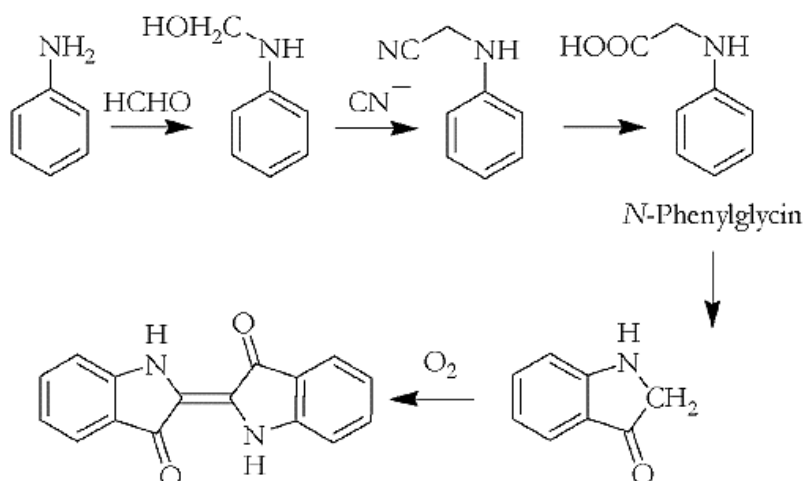
## Heumann version 1 (1890)



## Heumann version 2 (1890)



## Pflger – Heumann (1901)



## Improved synthesis of N-Phenylglycine BASF (1925)

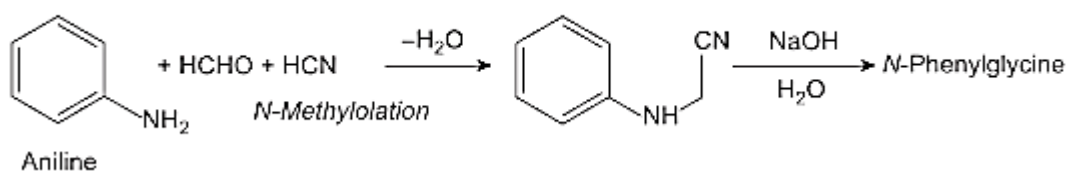


FIGURE 7: Commercially available indigo synthesis (adapted from Roessler (2003, 46) and Roshan (2015, 10))

### 3.4 Market demand and future trend of indigo

Certain information and statistics from official government sources prove that dyestuff industry and demin demand has ceaselessly grown for couples of years. Figure 8 shows that volume growth is estimated to be slower than value growth, which means increasing prices due to stringent environmental requirements and strong end-use demand. According to JCR-VIS (Choangalia 2018), global demin market is estimated to grow approximately 6.4% annually from \$57 billion in 2016 to \$75 billion in 2021. Until 2020, Europe is expected to place in second position of dye demand (FIGURE 9) (Consulting SRI and A.T. Kearney 2017). However, most of EU countries import dyestuff (including indigo) from mainly Bangladesh, Mexico, China, Pakistan, Turkey, Hong Kong, Italy, India, Spain and Brazil.

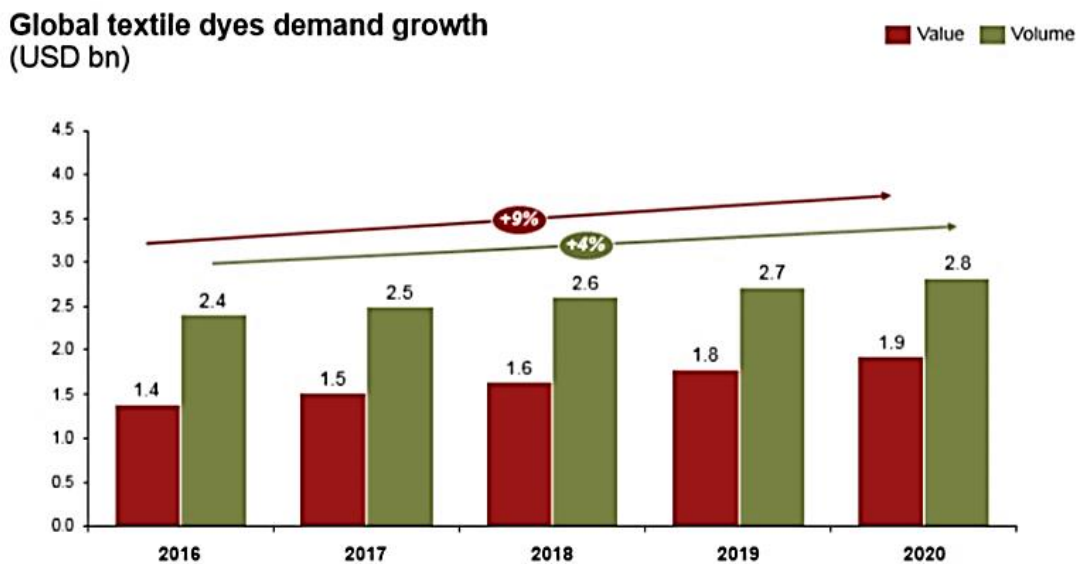


FIGURE 8. Global textile dyes market by volume and value (Consulting SRI and A.T. Kearney 2017, 88)

Besides, a growing awareness for health and environmental protection has led to reduce the ecological impact of production processes. Worldwide consumers are demanding eco-friendly and biodegradable products. Traditional synthesis of indigo from chemicals such as aniline has been researched and alternated to bio synthesis processes. BASF AG is one of the main indigo producers/importers in EU, according to EU risk assessment report, Aniline (2004). It is reported that long time exposure to aniline may cause reduction of Methemoglobin content in blood and skin irritation or allergies. Recent years, the demand for natural dyes has increased in many countries due to health hazards and environment pollution issues related to synthetic dyes (Chavan 2004). Future trends of indigo production are likely

to focus on the revival of plant-derived indigo and microbial-derived indigo which is cellular-cloned bacteria for indigo production, and development of environmentally-friendly reduction techniques (Roshan 2015, 59).

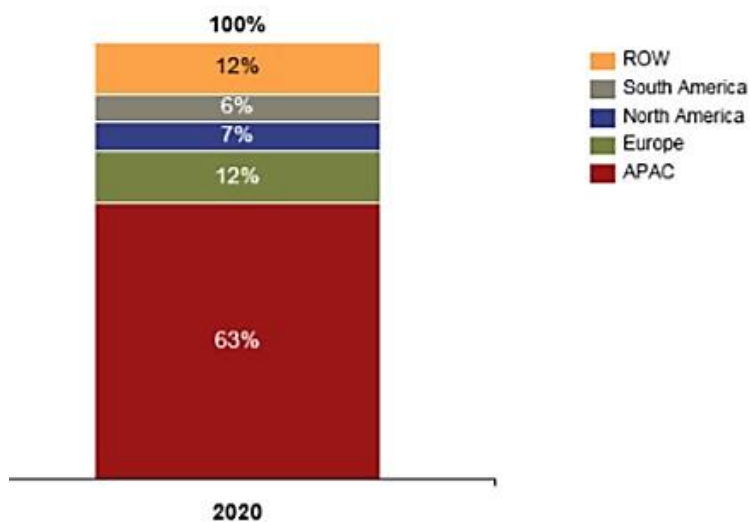


FIGURE 9. Estimated dye market by major regions in 2020 (Consulting SRI and A.T. Kearney 2017, 90)

## 4 EXPERIMENTALLY QUANTITATIVE METHODS OF INDIGO DETERMINATION

As mentioned in literature review part, a rapid and reliable analysis method of measuring indigo is not available due to the insolubility of indigo in water and other commonly used solvents. After plant extraction process (water steeping process), indigo is precipitated particles in aqueous solution. In this experiment part, indigo is separated from original aqueous solution and some impurities by liquid-liquid extraction and centrifuge separation. The chosen solvents for the experiments are based on the Solvent Miscibility Chart (Appendix 1) adapted from Paul Sadek (2002). This practical experiment work aims to make the comparison of different analysis methods of indigo quantity determination and develop the reliable quantitative methods for indigo in the aspect of accuracy, time, safety, and economy.

### 4.1 Materials and instruments

Natural Indigo – company located in Nivala has run a pilot project about cultivating woad plant (Morsinko in Finnish) in Pyhäsalmi mine. LUKE is their partner company who has been working with specimens from woad extraction containing indigo. Plant-extracted indigo specimens in aqueous solution utilized to analyse in this thesis were provided by LUKE in December 2018 after the harvesting period of Natural Indigo. Specimens were stored in refrigerator to maintain the quality and quantity of indigo content (FIGURE 10).

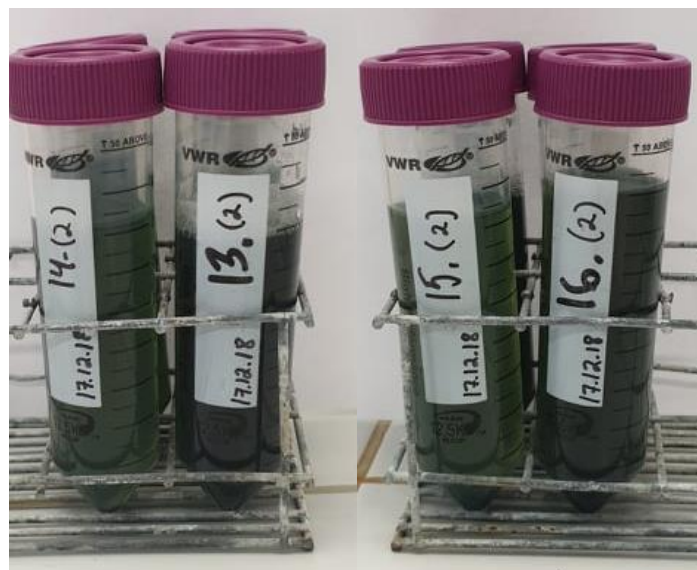


FIGURE 10. Indigo specimens 13(2), 14(2), 15(2), and 16(2) from LUKE

TABLE 1. List of utilized chemicals and manufactures

Chemicals	CAS number	Manufacturers
Acetonitrile (ACN)	75-05-8	Merck
Chloroform, GR for analysis	67-66-3	Merck
Dimethyl sulfoxide (DMSO), GR for analysis	67-68-5	Merck
Ethyl acetate	141-78-6	J.T Baker
Indigo, synthetic (95% purity)	482-89-3	Acros Organics
Trifluoroacetic acid (TFA)	76-05-1	Merck

Chemicals used in experiments are listed in TABLE 1, along with CAS number and manufactures. Those chemicals were provided by the Chemistry Laboratory of Centria University of Applied Sciences and Centria Research and Development Department. Chemical handling and storage were handled under manufacturer's instructions.



PICTURE 1. Shimadzu UV-1800

The main analysis instruments used for determining the quantity of indigo were Shimadzu UV-1800 Spectrophotometer (PICTURE 1) and Shimadzu HPLC series equipped with superior solvent delivery performance LC-20AD, autosampler SIL-20AC, photodiode array detector SPD-M20A, refractive index detector RID-20A and accommodate multiple column oven CTO-20A (PICTURE 2). Other supporting equipment includes vortex, ultrasonic bath and centrifuge.





PICTURE 2. Shimadzu HPLC series instrument

#### 4.2 Liquid-liquid extraction with Ethyl acetate and UV-Vis analysis instrument

Synthetic indigo was dissolved completely in ethyl acetate (9.98 mg/l) by sonicating about 30 minutes. This stock solution of indigo in ethyl acetate was used for measuring the wavelength at which the absorbance was maximum by UV-Vis spectrum range 400-800 nm. As a result,  $\lambda_{\text{max}}$  was 600 (FIGURE 11). Stock solution was diluted to several concentrations for calibration curve (TABLE 2).

TABLE 2. Dilutions of indigo in Ethyl acetate for calibration curve, UV-Vis analysis method

	Amount of stock solution (ml)	Total volume (ml)	Concentration (mg/l)
STD0 (blank)	0.00	5.00	0.00
STD1	0.10	5.00	0.20
STD2	0.25	5.00	0.50
STD3	0.50	5.00	1.00
STD4	1.00	5.00	2.00
STD5	1.50	5.00	2.99

In sample preparation, indigo content in aqueous specimens was determined by different multiple LLE process and different volume of ethyl acetate solvent (2ml and 3ml ethyl acetate in particular). Each experiment was made with three replicas simultaneously to get more precise mean results and standard deviation. The results were used to compare the efficiency and indigo content by the different methods of extraction. Specimens 13(2), 14(2) (FIGURE 10) were mixed until homogeneous state before LLE.

In each LLE experiment, 0.75ml of specimen was added by ethyl acetate solvent with a certain amount and vortexed for 30 seconds. Sample was let to stand for 30 minutes to reach equilibrium state. Blue layer or upper layer (organic phase) was extracted carefully for UV-Vis analysis (PICTURE 3 -left).

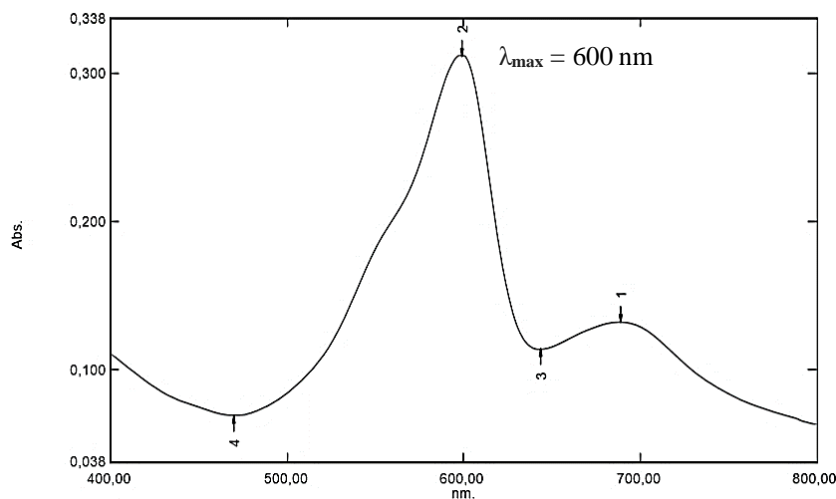


FIGURE 11. Absorbance spectrum of indigo in Ethyl acetate

#### 4.3 Liquid-liquid extraction with Chloroform and UV-Vis analysis instrument

Synthetic indigo in chloroform with the concentration 9.88 mg/l was made as a stock solution for making calibration standards and measuring the maximum absorbance wavelength of indigo in chloroform. The  $\lambda_{\max}$  was recorded at 604 nm (FIGURE 12). Calibration curve of indigo in Chloroform was obtained by dilutions of stock solution (TABLE 3).

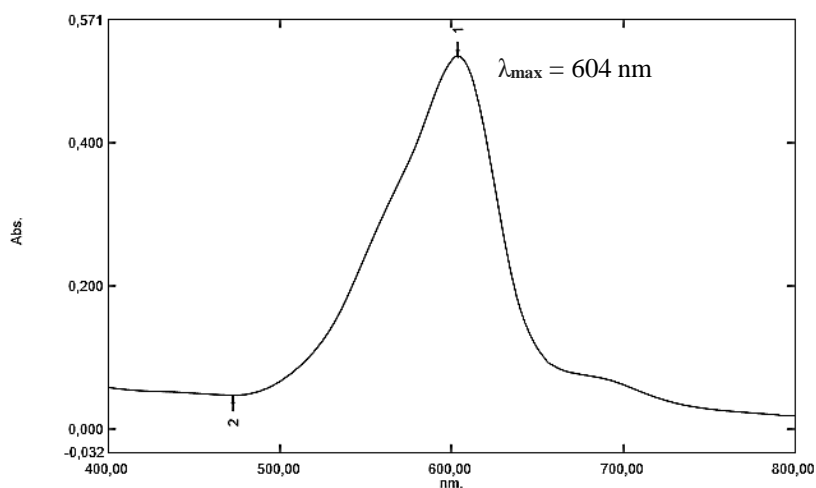
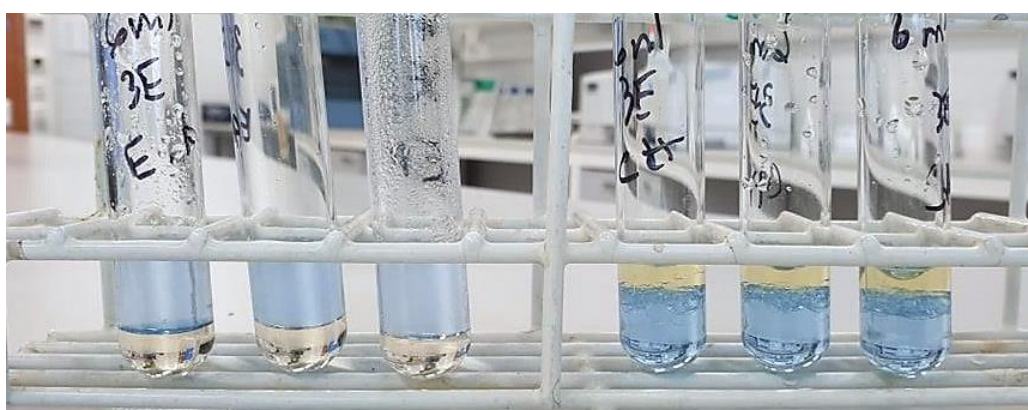


FIGURE 12. Absorbance spectrum of indigo in Chloroform

TABLE 3. Dilutions of indigo in Chloroform for calibration curve, UV-Vis analysis method

	Amount of stock solution (ml)	Total volume (ml)	concentration (mg/l)
STD0 (blank)	0.00	5.00	0.00
STD1	0.10	5.00	0.20
STD2	0.25	5.00	0.49
STD3	0.50	5.00	0.99
STD4	1.00	5.00	1.98
STD5	1.50	5.00	2.96
STD6	2.50	5.00	4.94

Specimens 13(2), 14(2), and 16(2) (FIGURE 10) were used for analyzing in this method. The same methods of LLE with ethyl acetate, LLE with chloroform was performed in different amount of solvent and different number of extractions to figure out which one was able to yield the highest amount of indigo by UV-Vis analysis. Furthermore, original specimen was diluted with two different dilute factors to know whether the concentration of aqueous samples effect on LLE performance. In each LLE method, the experiment was made in triplicate. The mean value of replicas and standard deviation were calculated. Regularly, 0.75 ml of indigo-aqueous solution was mixed with a certain amount of solvent by vortex for 30 seconds and allowed to stand for 30 minutes. Blue layer or lower layer (organic phase) was carefully separated for analyzing with UV-Vis (PICTURE 3- right).



PICTURE 3. Liquid-liquid extraction of indigo with ethyl acetate (three replicas on the left) and chloroform (three replicas on the right)

#### 4.4 Centrifuge separation and UV-Vis analysis instrument

Stock solution of indigo standards was prepared by dissolving 1.04 mg synthetic indigo (95%) in DMSO solvent to 10 ml volumetric flask. Solution was sonicated for about 30 minutes in boiling water until all indigo was dissolved. Indigo concentration in stock solution obtained 98.80 mg/l. Maximum absorbance wavelength was determined with spectrum detecting range 400-800 nm by measuring stock solution. The  $\lambda_{\text{max}}$  was 620 nm (FIGURE 14). Several dilutions of stock solution in DMSO were made by following TABLE 4. The dilutions were measured as standards for calibration curve by Shimadzu UV-1800.

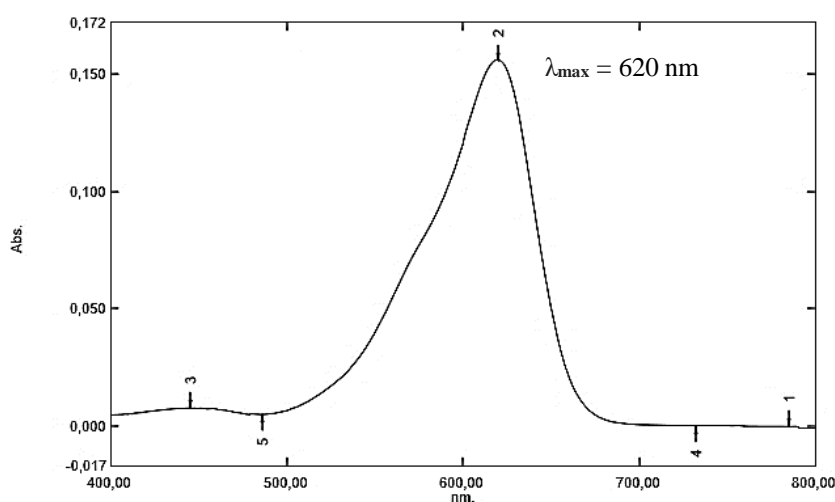


FIGURE 13. Absorbance spectrum of indigo in DMSO

TABLE 4. Dilutions of indigo in DMSO for calibration curve, UV-Vis analysis method

	Amount of stock solution (ml)	Total volume (ml)	concentration (mg/l)
STD0 (blank)	0.00	5.00	0.00
STD1	0.10	5.00	1.98
STD2	0.20	5.00	3.95
STD3	0.50	5.00	9.88
STD4	0.75	5.00	14.82
STD5	1.00	5.00	19.76

Indigo aqueous specimens 14(2) and 16(2) (FIGURE 10) were shaken to homogeneous state prior to sampling for analysis. For every specimen, the experiment process was performed in triplicate, measured

with wavelength 620nm by UV-Vis. The mean value of replicas and their standard deviations were calculated. For the sample preparation process, 0.75 ml of homogeneous solution from a certain specimen was centrifuged at 13000 rpm for 5 minutes in which denser particles (precipitated form of indigo) settle to the bottom of the tube (PICTURE 4). After aqueous solution was carefully removed (PICTURE 5), indigo paste was transferred to test tube and 3 ml of DMSO was added. The test tube was vortexed for 10 seconds and placed in hot water for 10-15 min until residue was dissolved completely (PICTURE 6). The indigo in DMSO samples were transferred to cuvettes for UV-Vis measuring.



PICTURE 4. Sample after centrifuge



PICTURE 5. Indigo paste



PICTURE 6. Indigo in DMSO

#### 4.5 Centrifugal separation and HPLC analysis instrument

Shimadzu HPLC series was used for sample analysis. LC separation was achieved by Agilent Poroshell 120 SB-C18 column (ID: 687975-902), 75mm length, 4.6mm internal diameter, 2.7  $\mu\text{m}$  pore size. The gradient elution applied in the analysis using solvent A (ACN + 0.4% TFA) and solvent B Ultra-pure  $\text{H}_2\text{O}$  + 0.4% TFA) was mentioned in TABLE 5 with total flow 1.00ml/min, and the sample injection volume was 10 $\mu\text{L}$ . Detection wavelength for indigo was set for 611 nm with the spectrum detection range 450-800nm. The column temperature was 30 $^\circ\text{C}$  and pressure limits is 30 MPa.

TABLE 5. Gradient HPLC program setting

	Time (min)	Module	%A	%B	Comment
1	0.01	Pumps	20	80	
2	5.00	Pumps	50	50	
3	15.00	Pumps	100	0	
4	17.00	Pumps	100	0	
5	20.00	Pumps	20	80	Stabilization of gradient run

Stock solution of indigo in DMSO, concentration 101.65 mg/l, was used to make several dilutions for calibration curve, which can be seen in TABLE 6. All the diluted solutions were filtered over a PTFE Membrane, 0.45  $\mu\text{m}$  syringe filter prior to injection to HPLC vials. Due to the quick degradation of

standard solutions, they were measured immediately after preparing. The calibration curve was used as standards for measuring and calculating the concentration of indigo by HPLC method.

TABLE 6. Dilutions of indigo in DMSO for calibration curve, HPLC analysis method

	<b>Amount of stock solution (ml)</b>	<b>Total volume (ml)</b>	<b>Concentration (mg/l)</b>
STD1	0.10	5.00	2.03
STD2	0.25	5.00	5.08
STD3	0.50	5.00	10.17
STD4	1.00	5.00	20.33

Samples prepared for HPLC analysis were made from specimens 14(2) and 16(2) (FIGURE 10). The 0.75 ml of every specimen was made in triplicate and centrifuged at 13000 rpm for 5 minutes. The indigo paste collected after centrifuge was dissolved in 3 ml DMSO by being vortexed for 10 seconds and placed in hot water bath for 10-15 minutes. The samples were filtered over a PTFE Membrane, 0.45  $\mu$ m syringe filter prior to injection to HPLC vials. Each process was made in triplicate to calculate mean and standard deviation.

## 5 RESULTS AND DISCUSSION

In the chapter, the experiment results are shown in the mean value and its standard deviations. The comparisons of different methods are also presented and explained. Phenomena and problems observed in the experiments are mentioned as well.

### 5.1 Results of total indigo content determination by extraction of ethyl acetate and detection of UV-Vis

Calibration curve for indigo quantification was measured by UV/Vis according to technique mentioned in chapter 4.2. The goal was to find the amounts of absorbances at a certain wavelength which matches with the known indigo concentrations. It is established that the regression equation is  $y = 0.06608x - 0.00046$  and the correlation coefficient is  $r^2 = 0.99988$  (FIGURE 18).

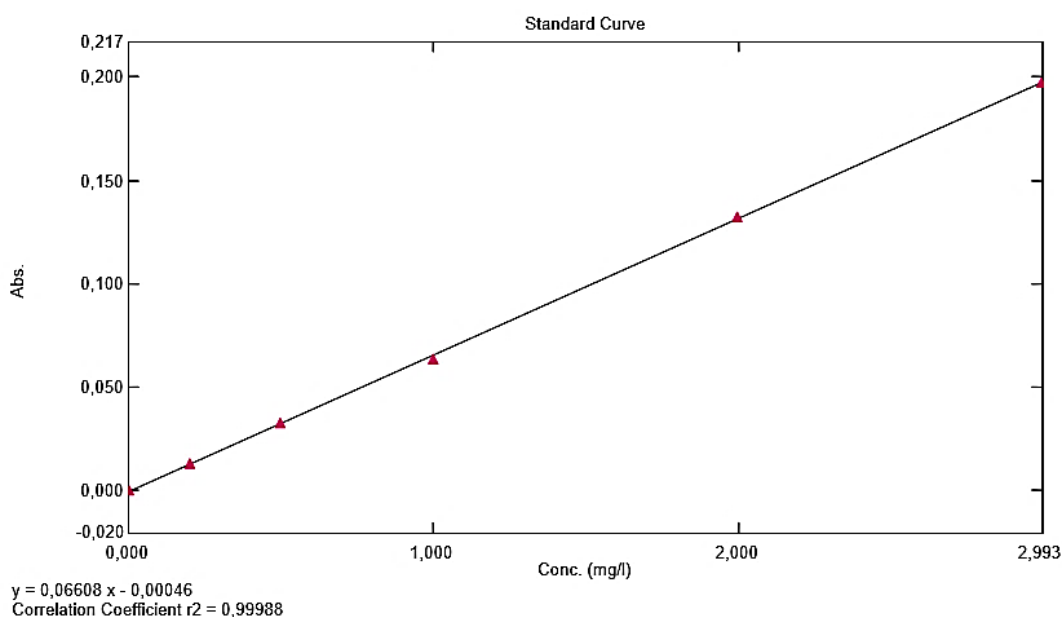


FIGURE 14. Calibration curve of synthetic indigo in Ethyl acetate by UV-Vis

With the same extraction technique and four extractions, the different amounts of ethyl acetate yield the difference in dispersion concentration of indigo. The extracted amount of indigo in the first extraction is significantly higher than the later extractions; then, the indigo concentration was measured by UV/Vis decreases along the extraction times. Figure 19 shows the trend of average indigo concentration from



three replicas decreasing by the extraction times. For specimen 13(2), four extractions are the maximum number of extractions for both 2ml and 3ml ethyl acetate each time because of the limitation of UV-Vis detection. Further extraction times caused the instability of absorbance detection and noises.

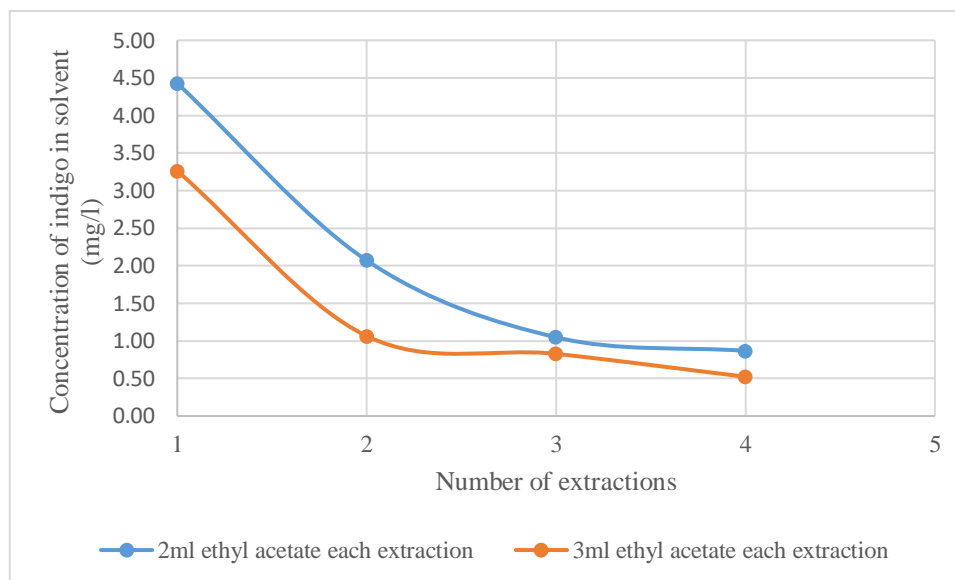


FIGURE 15. Decline of indigo concentration on the related amount of ethyl acetate and number of extraction times (specimen 13(2))

TABLE 7. Quantification of indigo in specimen 13(2) by different LLE methods with ethyl acetate and their standard deviations

LLE Methods		Indigo concentration in specimen 13(2) (mg/l)	
<b>One extraction</b>	4ml solvent	12.73	$\pm 0.76$
	6ml solvent	11.05	$\pm 2.25$
<b>Two extractions</b>	2ml solvent (total 4ml)	17.62	$\pm 0.56$
	3ml solvent (total 6ml)	21.10	$\pm 0.74$
<b>Three extractions</b>	2ml solvent (total 6ml)	20.89	$\pm 1.32$
<b>Four extractions</b>	2ml solvent (total 8ml)	22.44	$\pm 1.35$
	3ml solvent (total 12ml)	22.71	$\pm 1.22$

As can be seen in Table 7 and Figure 22, the more extraction times yields the higher indigo quantification analysis for the same specimen 13(2). With the same total of 6 ml ethyl acetate, one extraction resulting in the indigo concentration in specimen is  $11.05 \pm 2.25$  mg/l; two extractions (3 ml each time) and three

extractions (2 ml each time), meanwhile, show higher results  $21.10 \pm 0.74$  mg/l and  $20.89 \pm 1.32$  mg/l respectively. The same situation with total 4 ml ethyl acetate, extracting the same amount 0.75 ml specimen twice gets the result of  $17.62 \pm 0.56$  mg/l indigo concentration, which is higher than extracting once with 4 ml solvent,  $12.73 \pm 0.76$  mg/l. Overall, four-time extraction with 2 ml and 3 ml solvent are relatively yielding the same best results for indigo quantification; however, on the aspect of economy, 2 ml ethyl acetate each extraction and total 8 ml ethyl acetate is more efficient.

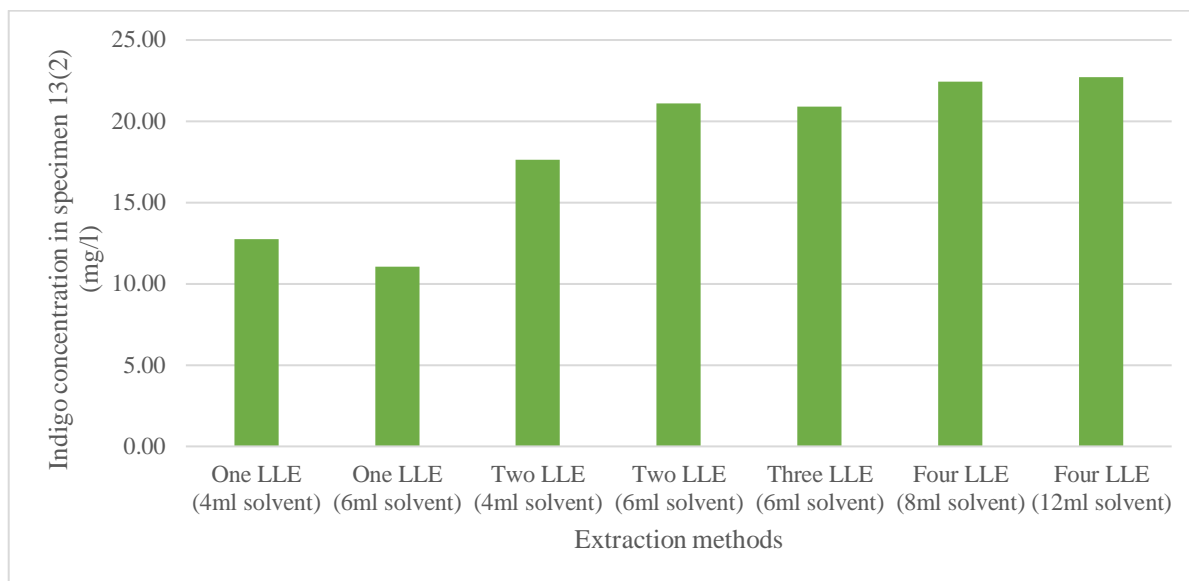


FIGURE 16. Quantification of indigo in specimen 13(2) by different LLE methods with ethyl acetate

## 5.2 Results of total indigo content determination by extraction of chloroform and detection of UV/Vis

The calibration curve of indigo in chloroform was obtained from standard preparation technique in chapter 4.3 by UV-Vis at wavelength 604 nm. The equation  $y = 0.06468x + 0.00193$  and correlation coefficient  $r^2 = 0.99926$  are shown in figure 23. This calibration curve was used as a standard to quantify the extracted indigo from specimens with chloroform as a solvent.

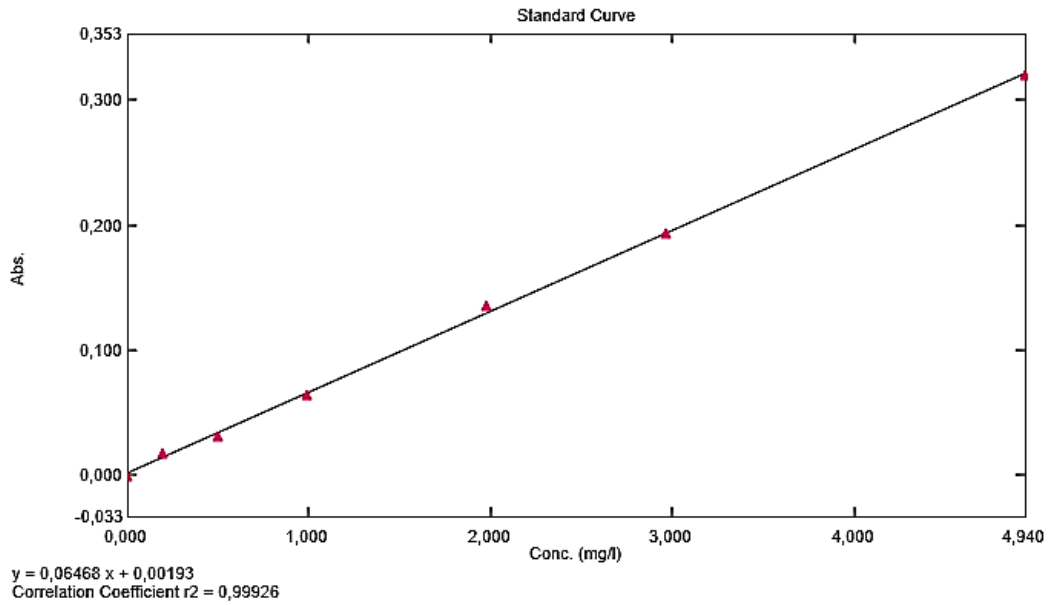


FIGURE 17. Calibration curve of synthetic indigo in Chloroform by UV-Vis

Figure 22 expresses the decreasing trend of indigo concentration in solvent after number of extraction times. Regularly, the concentration of indigo in a certain stage (extraction) is approximately decreased by half of the previous stage (extraction). With the same specimen 13(2), indigo LLE with chloroform seems to be better than with ethyl acetate (FIGURE 19).

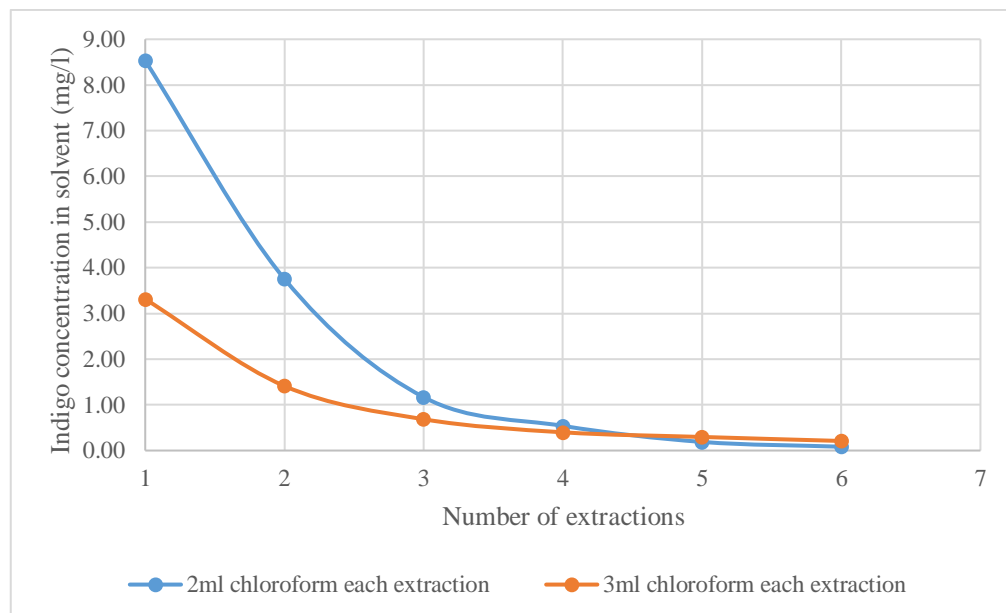


FIGURE 18. Decline of indigo concentration on the related amount of chloroform and number of extraction times (specimen 13(2))

In term of number of extraction times, specimen 13(2) could be extracted up to 6 times with chloroform and 4 times with ethyl acetate. Chloroform shows the ability of extracting more indigo than ethyl acetate in the method of 2 ml solvent for each extracting time. In the first extraction with 2 ml solvent, indigo concentration in chloroform is averagely 8.53 mg/l, better than that in ethyl acetate, 4.43 mg/l. In case of using 3 ml solvent for each extraction, ethyl acetate and chloroform are relatively same in performance. The more indigo can be extracted from aqueous specimen, the more accuracy the method can get. However, it was observed that using chloroform as a solvent to extract indigo may form the emulsion, leading to non-separation of contact area between two phases (PICTURE 7). It causes the difficulty in extracting the organic phase for analysis.



PICTURE 7. Emulsion formation when using chloroform to extract indigo

The same as ethyl acetate, chloroform shows the different quantification of indigo with different extraction times and amount of solvent. As can be seen from figure 24 and table 8, the more amount of chloroform used for each extraction time, the less indigo transfers from aqueous phase to organic phase, resulting in underestimating the concentration of indigo in specimen, such as one extraction with 6 ml chloroform gives the lowest results of indigo quantification,  $8.65 \pm 0.91$  mg/l. On aspect of 2 ml chloroform for each extraction, the results of two extractions and three extraction seems to be relatively the same,  $25.24 \pm 1.32$  mg/l and  $26.10 \pm 2.85$  mg/l respectively; however, three extractions method yields lower average indigo concentration than two extractions, which is  $22.74 \pm 1.03$ . It is probably because the formation of emulsion causes the stabilization and prevents indigo from transferring to organic phase, leading to the big standard deviations for methods.

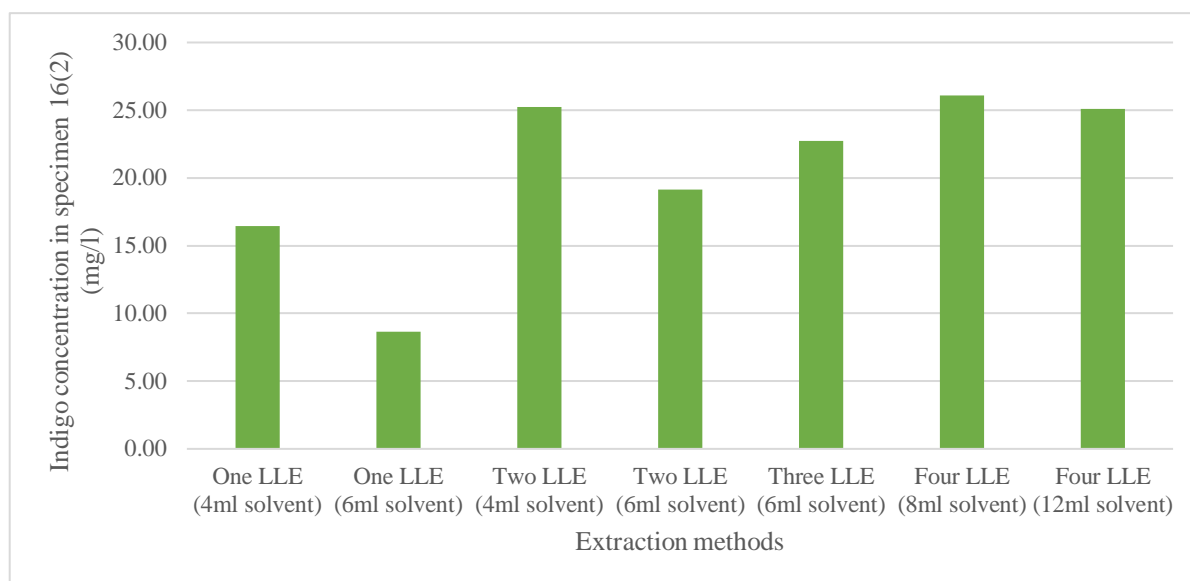


FIGURE 19. Quantification of indigo in specimen 16(2) by different LLE methods with chloroform

TABLE 8. Quantification of indigo in specimen 16(2) by different LLE methods with chloroform and their standard deviations

LLE methods		Indigo concentration in specimen 16(2) (mg/l)	
<b>One extraction</b>	4ml solvent	16.44	$\pm 1.25$
	6ml solvent	8.65	$\pm 0.91$
<b>Two extractions</b>	2ml solvent (total 4ml)	25.24	$\pm 1.32$
	3ml solvent (total 6ml)	19.12	$\pm 1.14$
<b>Three extractions</b>	2ml solvent (total 6ml)	22.74	$\pm 1.03$
<b>Four extractions</b>	2ml solvent (total 8ml)	26.10	$\pm 2.85$
	3ml solvent (total 12ml)	25.10	$\pm 1.94$

The comparison when using different indigo concentration in aqueous phase was performed in the same extraction method (0.75 ml aqueous solutions extracting with 2 ml solvent each extraction was repeated 3 times, using total 6 ml solvent). It was observed during the experiments that the water-diluted aqueous phase could made less emulsion forming. Once chloroform is used as solvent, 1:2 diluted specimen 16(2) expresses the highest amount of indigo in specimen,  $30.58 \pm 1.16$  mg/l, following by the 1:3 diluted specimen with  $26.25 \pm 0.14$ . The original specimen extracted with chloroform shows the underestimation of indigo concentration result, which is  $22.74 \pm 1.03$  mg/l. On the other hand, using ethyl acetate to

extract indigo may have the same results for both 1:2 and 1:3 diluted specimens,  $24.97 \pm 2.68$  mg/l and  $25.58 \pm 1.90$  mg/l respectively. The results of those experiments are shown in Table 9 and Figure 25.

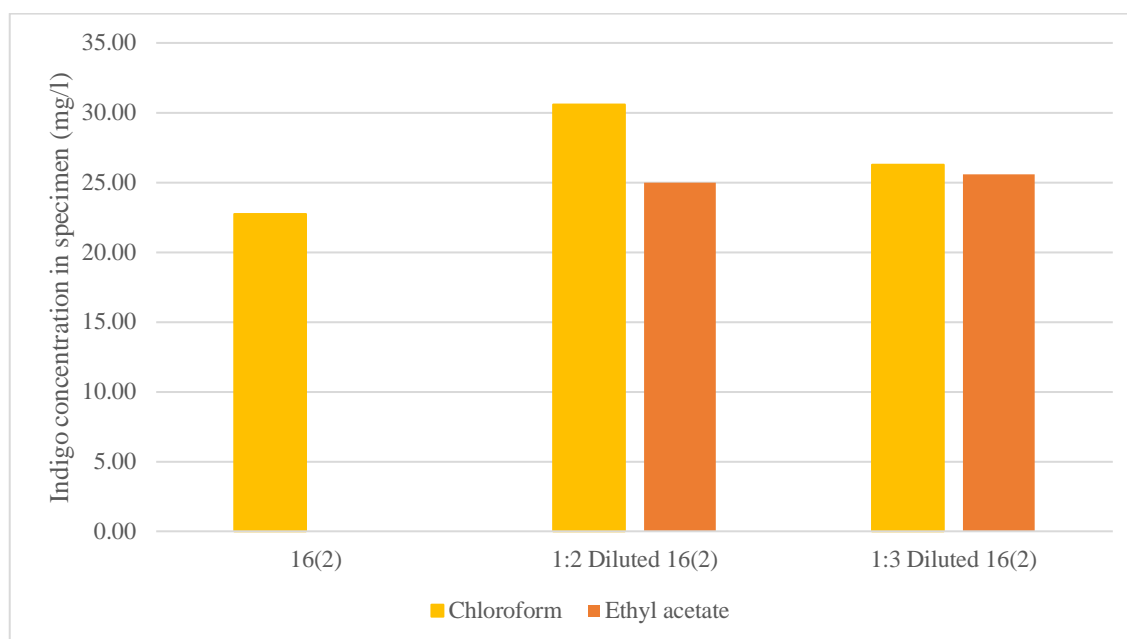


FIGURE 20. Comparison of indigo quantification results with the different indigo concentration in aqueous phase

TABLE 9. Quantification of indigo with the different concentration of indigo in aqueous phase

	Indigo concentration in specimen 16(2) (mg/l)		
	16(2)	1:2 Diluted 16(2)	1:3 Diluted 16(2)
Chloroform	$22.74 \pm 1.03$	$30.58 \pm 1.16$	$26.25 \pm 0.14$
Ethyl acetate	-	$24.97 \pm 2.68$	$25.58 \pm 1.90$

### 5.3 Comparative results of total indigo content determination by centrifugal separation and detection of UV-Vis and HPLC

Synthetic indigo dissolving in DMSO was used as the standards for calibrating in both UV-Vis and HPLC, as mentioned in chapters 4.4 and 4.5. The calibration curve operated by UV-Vis detector at wavelength 620 nm displays the equation  $y = 0,07790 x + 0,00001$ , along with the correlation coefficient  $r^2 = 0,99999$  (FIGURE 26). Meanwhile, the calibration curve was obtained by HPLC (FIGURE 27) with  $y = (3.3674e-5)x + (0)$  and  $r^2 = 0.9991875$ . In the testing process with synthetic indigo, high-concentrated

synthetic indigo in DMSO by HPLC caused the large differences between the real concentration of standards and the HPLC results as well as the noisily-unknown peaks. Therefore, the concentration of indigo under 20 mg/l may yield more precise and accurate result.

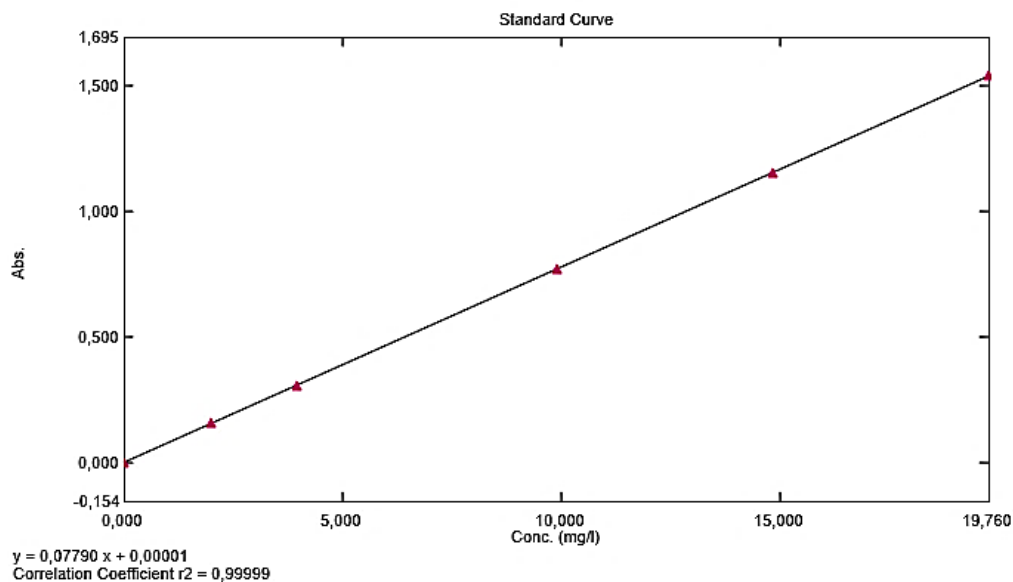


FIGURE 21. Calibration curve of synthetic indigo in DMSO by UV-Vis

Natural indigo in aqueous solution was prepared by centrifugal separation and dissolving in DMSO. Since natural indigo in specimens were extracted from plant, there were some small part of plant tissue found after indigo was completely dissolved in DMSO. For that reason, the suggestion is that samples should be filtered with membrane pore size at least  $0.45 \mu\text{m}$  to prevent the impurities from causing mislead in quantification.

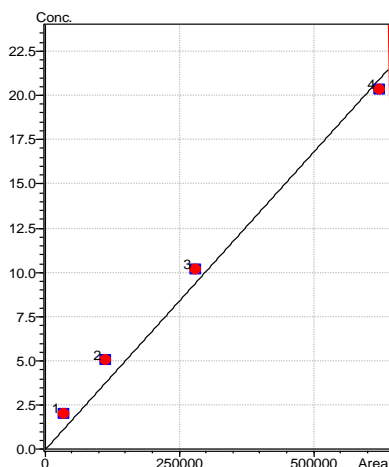


FIGURE 22. Calibration curve of synthetic indigo in DMSO by HPLC

Indigo paste samples dissolved in DMSO show different quantification results between UV-Vis and DMSO as a detector (FIGURE 28, TABLE 10). UV-Vis detector gives better results for both specimen 16(2) and 14(2) than HPLC detector. By using UV-Vis, the quantified concentration of indigo in specimen 16(2) and 14(2) are  $22.30 \pm 0.61$  mg/l and  $10.74 \pm 0.30$  mg/l respectively; however, HPLC shows much lower results,  $13.25 \pm 1.25$  mg/l and  $5.35 \pm 0.87$  mg/l respectively. Although UV-Vis is better choice in term of measuring indigo in DMSO solution, three extraction times for total 6 ml of chloroform displays the higher quantitative results of unknown concentration indigo in sample, which is  $22.74 \pm 1.03$  mg/l for specimen 16(2) and  $12.82 \pm 0.17$  mg/l for specimen 14(2). On the aspect of time-consuming, solvent extraction takes more time to conduct than centrifugal separation; therefore, in case of dealing with many samples, centrifuge separation is considered as a good option.

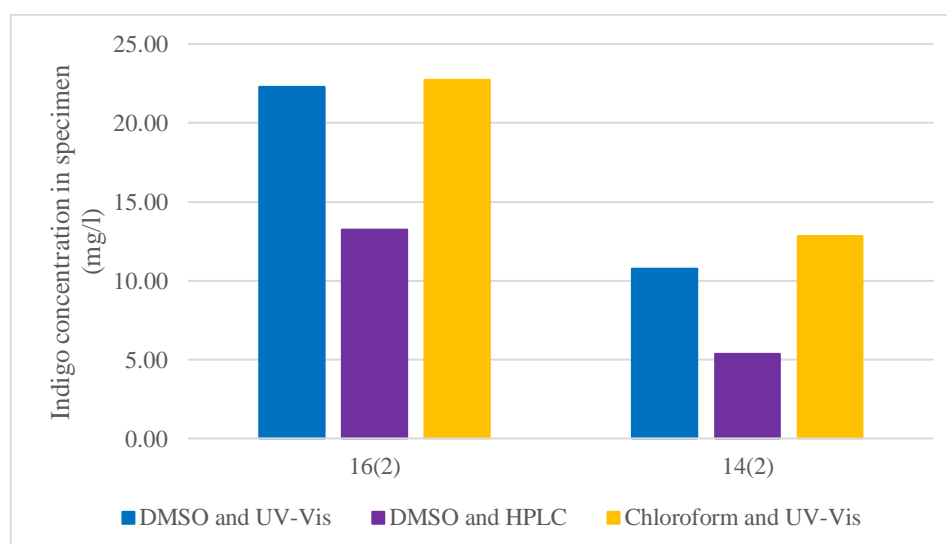


FIGURE 23. Comparison of indigo quantitative results between detection methods of UV-Vis and HPLC

TABLE 10. Comparison of indigo quantitative results between detection methods of UV-Vis and HPLC

Methods	Indigo concentration in specimen (mg/l)	
	16(2)	14(2)
DMSO and UV-Vis	$22.30 \pm 0.61$	$10.74 \pm 0.30$
DMSO and HPLC	$13.25 \pm 1.25$	$5.35 \pm 0.87$
Three LLE with total 6ml chloroform and UV-Vis	$22.74 \pm 1.03$	$12.82 \pm 0.17$



Apart from the quantification of indigo, HPLC method can simultaneously detect others compounds or impurities which are soluble in DMSO from the specimens. From Figure 29 and 30, the peak starting at about 6.6 (min) and stopping before 7.0 (min) is indigo. Another peak coming after that is an unknown compound. In the literature part, it was already mentioned that indirubin (known as red shade of indigo or impurity) usually forms simultaneously with indigo because of the overoxidation. Some researchers have found that the maximum wavelength absorbance of indirubin is around 542 nm and indirubin also dissolve in DMSO. There is a potential that the impurity compound showing up in HPLC results of both specimen 16(2) and 14(2) is small amount of indirubin. However, the exact identity and amount of this impurity is impossible to determine without its synthetic standard to compare and measure.

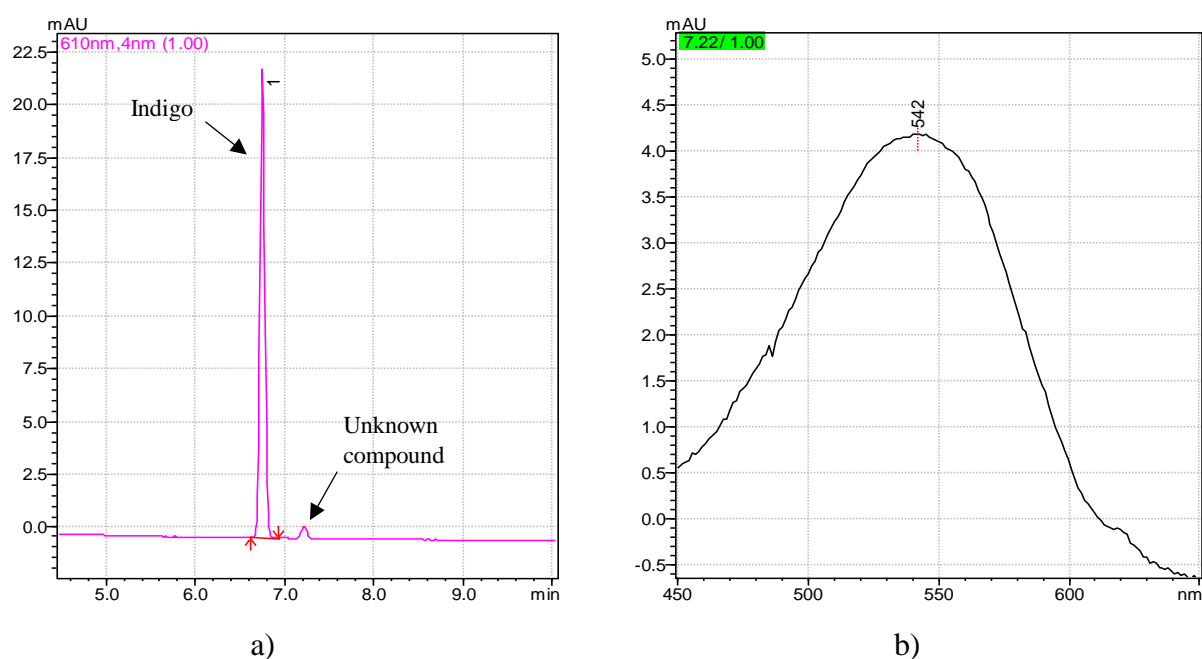


FIGURE 24. a) HPLC retention time of indigo; b) Unknown impurity peak (Specimen 16(2))

In case of analyzing many samples, the joint work of centrifugal separation and HPLC detection is a better choice. Even though HPLC takes longer time to analyze a single sample, it can operate automatically. Furthermore, it is the only method that can detect others compound existing in sample. However, HPLC may cause the remarkable underestimation of indigo quantification.

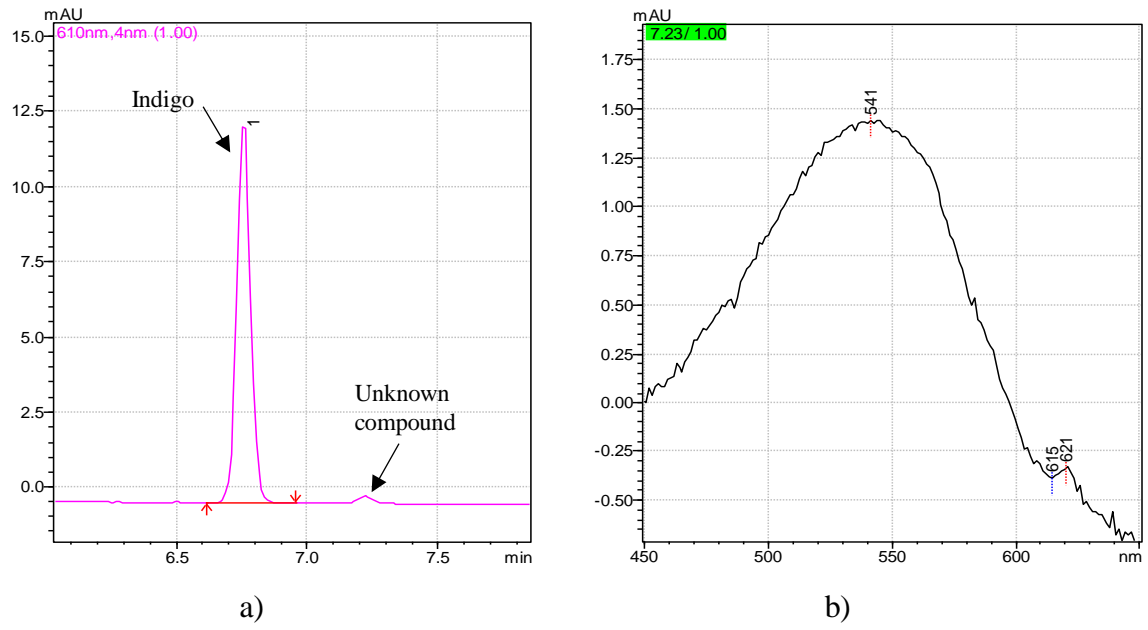


FIGURE 25. a) HPLC retention time of indigo; b) Unknown impurity peak (Specimen 14(2))

## 6 CONCLUSION

The aim of this study was to determine the level of indigo content in different specimens of plant extraction so that it can be used to estimate the overall indigo production from a certain crop. Therefore, the accuracy of quantitative determination methods is crucially important in either researching to improve the yields of indigo or estimating the efficiency of the plant extraction process. In the effort to achieve the objectives of this study, positive and encouraging results were used for comparison.

Even though indigo is soluble in ethyl acetate, chloroform and DMSO, it was found that indigo is the most soluble in chloroform. Synthetic indigo dissolved in ethyl acetate can slightly precipitate when storing overnight. Besides, it was observed that a small amount of indigo left in the inner surface of test tubes after LLE process, which means indigo cannot dissolve well in ethyl acetate. As a result, the analysis of indigo by ethyl acetate LLE and UV may cause the underestimation of indigo concentration. When DMSO is used as a solvent for dissolving indigo paste, indigo is dissolved well in DMSO as long as the hot water bath is applied. However, the degradation of indigo in DMSO is quick.

Technically, indigo samples in aqueous solution extracted by LLE with chloroform and detected by UV-Vis were found to be better to determine the unknown concentration of indigo in specimens. The different number of extracting times and the amount of chloroform used for each time gave the different quantification for indigo. For 0.75 ml sample from specimen, 2 ml chloroform for each extraction yields the higher indigo to transfer from aqueous phase to organic phase than 3 ml chloroform each extraction, thereby higher quantitative results of samples extracted with 2 ml each time. It is impossible to totally extract indigo from aqueous phase to chloroform due to equilibrium constant state. More number of extracting times, the more accuracy the quantification results can be achieved. However, LLE with chloroform tends to form emulsion when the concentration of indigo is high. The emulsion causes the improper separation between the aqueous phase and the organic phase and the difficulty to collect the organic solvent. Besides, the emulsion formation may trap indigo particles and prevent them to transfer to chloroform. In that case, the solution for this problem is to dilute the original aqueous sample before extraction. The experiment results show that specimen diluted with distilled water 1:2 yields the highest quantitative results of indigo. Considering the safety of solvents, chloroform is more harmful and unfriendly for using than ethyl acetate. Chloroform can cause skin and eye irritation, toxic if inhaled and make drowsiness or dizziness. The precautions and handling instruction must be followed strictly.

Furthermore, LLE is a time-consuming process and its results of the same process are slightly different between replicas. Replication is necessary to calculate the standard deviations.

In case of dealing with large amounts of specimens, indigo paste which is collected from centrifugal separation, dissolved in DMSO and measured by UV-Vis is a time-saving choice. The results can show the relative difference of concentration in different specimens. After that, the highest or lowest ones can be picked out for more accurate quantitative analysis by LLE with chloroform. Samples of indigo in DMSO can be analyzed by HPLC to find out any impurities such as indirubin in sample as well.

In conclusion, there is an increasing commercial demand for naturally sourced indigo that meets the purities standards set by the artificially synthetic indigo. This study concerns the development of reliable quantitative method for indigo made from leaves of woad (*Isatis tinctoria*) or Morsinko in Finnish. A rapid and reliable method of measuring indigo needs to be further investigated. In the comparison of methods used in this study, LLE with chloroform and detection by UV-Vis gives the best results yet it is time-consuming and problematic with emulsion formation. The more rapid but less accurate method is dissolving indigo paste from centrifugal separation in DMSO and measuring with UV-Vis.

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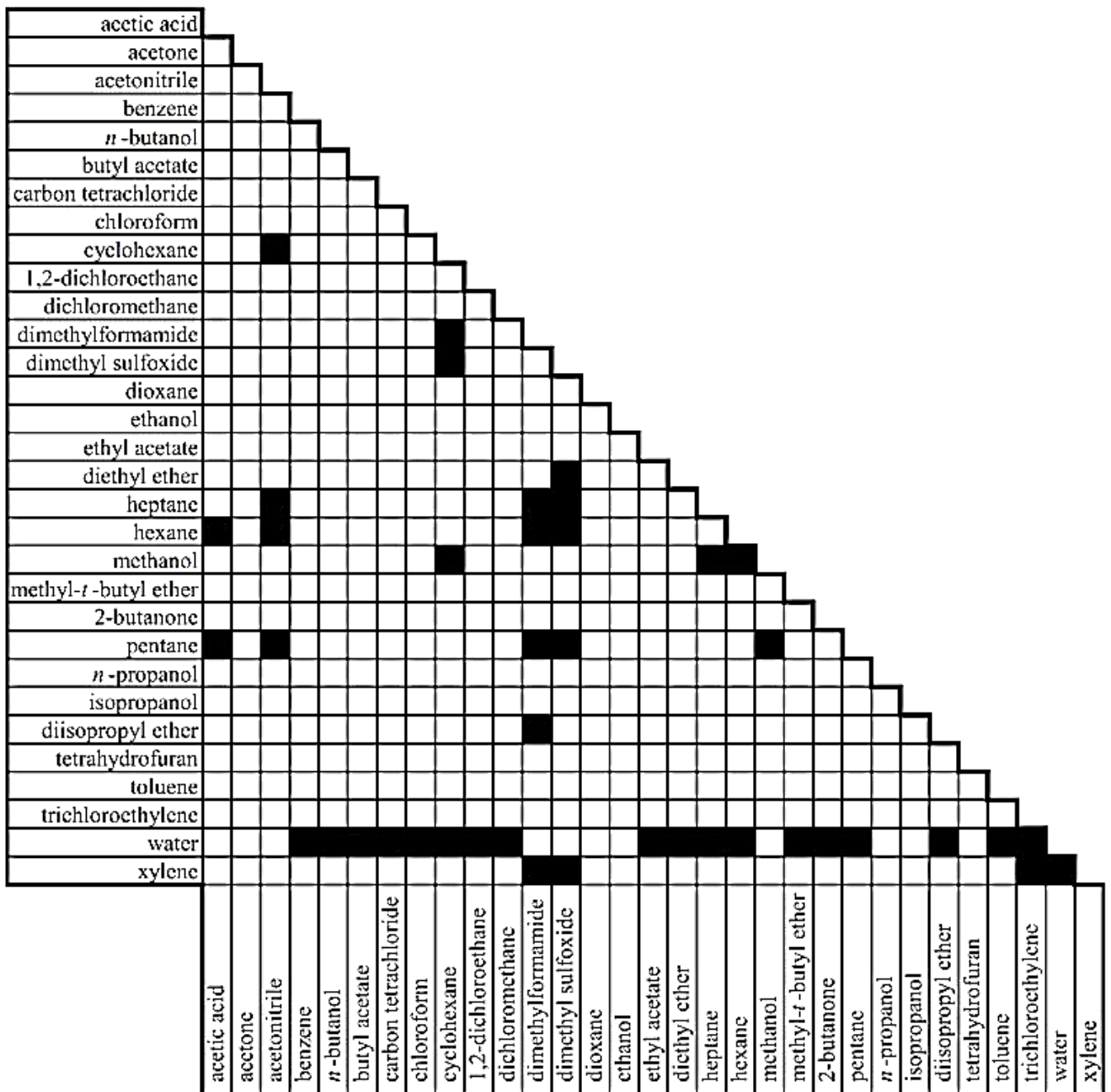
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SOLVENT MISCIBILITY CHART



■ = immiscible  
 □ = miscible

APPENDIX 2

**RESULT OF QUANTIFICATION ANALYSIS BY LIQUID-LIQUID EXTRACTION WITH ETHYL ACETATE AND UV-VIS DETECTION**

Specimen	Method		Replica 1 (mg/l)	Replica 2 (mg/l)	Replica 3 (mg/l)	Average concentration of indigo in solvent (mg/l)	Average extracted amount of indigo (mg)	Average concentration of indigo in specimen (mg/l)	Standard deviation (mg/l)
13(2)	One extraction	4ml solvent (total 4ml)	2.27	2.35	2.55	2.39	0.010	12.73	0.76
		6ml solvent (total 6ml)	1.70	1.25	1.19	1.38	0.008	11.05	2.25
	Two extractions	2ml solvent (total 4ml)	3.29	3.42	3.21	3.30	0.013	17.62	0.56
		3ml solvent (total 6ml)	2.64	2.54	2.73	2.64	0.016	21.10	0.74
	Three extractions	2ml solvent (total 6ml)	2.73	2.42	2.68	2.61	0.016	20.89	1.32
	Four extractions	2ml Solvent (total 8ml)	2.12	2.22	1.97	2.10	0.017	22.44	1.35
		3ml Solvent (total 12ml)	1.51	1.38	1.37	1.42	0.017	22.71	1.22
	Decline trend of indigo concentration in solvent (2 ml each LLE)	1 <sup>st</sup> extraction	5.00	4.14	4.14	4.43	0.009	11.81	1.32
		2 <sup>nd</sup> extraction	2.08	2.29	1.84	2.07	0.004	5.52	0.61
		3 <sup>rd</sup> extraction	0.83	1.33	0.99	1.05	0.002	2.79	0.68
		4 <sup>th</sup> extraction	0.56	1.13	0.91	0.87	0.002	2.31	0.77
	Decline trend of indigo concentration in solvent (3 ml each LLE)	1 <sup>st</sup> extraction	3.47	2.85	3.46	3.26	0.010	13.05	1.42
		2 <sup>nd</sup> extraction	1.17	1.27	0.75	1.06	0.003	4.26	1.09
		3 <sup>rd</sup> extraction	0.86	0.92	0.70	0.83	0.002	3.31	0.45
		4 <sup>th</sup> extraction	0.53	0.48	0.56	0.52	0.002	2.09	0.17
	16(2)	1:2 diluted 16(2)	Three extractions (total 6ml)	1.47	1.75	1.46	1.56	0.009	24.97
1:3 diluted 16(2)		Three extractions (total 6ml)	1.16	1.01	1.03	1.07	0.006	25.58	1.90

APPENDIX 3/1

**RESULT OF QUANTIFICATION ANALYSIS BY LIQUID-LIQUID EXTRACTION WITH CHLOROFORM AND UV-VIS DETECTION**

Specimen	Method	Replica 1 (mg/l)	Replica 2 (mg/l)	Replica 3 (mg/l)	Average concentration of indigo in solvent (mg/l)	Average extracted amount of indigo (mg)	Average concentration of indigo in specimen (mg/l)	Standard deviation (mg/l)	
13(2)	One extraction	4ml solvent (total 4ml)	2.27	2.35	2.55	2.39	0.010	12.73	0.76
		6ml solvent (total 6ml)	1.70	1.25	1.19	1.38	0.008	11.05	2.25
	Two extractions	2ml solvent (total 4ml)	3.29	3.42	3.21	3.30	0.013	17.62	0.56
		3ml solvent (total 6ml)	2.64	2.54	2.73	2.64	0.016	21.10	0.74
	Three extractions	2ml solvent (total 6ml)	2.73	2.42	2.68	2.61	0.016	20.89	1.32
	Four extractions	2ml solvent (total 8ml)	2.12	2.22	1.97	2.10	0.017	22.44	1.35
		3ml solvent (total 12ml)	1.51	1.38	1.37	1.42	0.017	22.71	1.22
	Decline trend of indigo concentration in solvent (2 ml each LLE)	1 <sup>st</sup> extraction	5.00	4.14	4.14	4.43	0.009	11.81	1.32
		2 <sup>nd</sup> extraction	2.08	2.29	1.84	2.07	0.004	5.52	0.61
		3 <sup>rd</sup> extraction	0.83	1.33	0.99	1.05	0.002	2.79	0.68
		4 <sup>th</sup> extraction	0.56	1.13	0.91	0.87	0.002	2.31	0.77
	Decline trend of indigo concentration in solvent (3 ml each LLE)	1 <sup>st</sup> extraction	3.47	2.85	3.46	3.26	0.010	13.05	1.42
		2 <sup>nd</sup> extraction	1.17	1.27	0.75	1.06	0.003	4.26	1.09
		3 <sup>rd</sup> extraction	0.86	0.92	0.70	0.83	0.002	3.31	0.45
		4 <sup>th</sup> extraction	0.53	0.48	0.56	0.52	0.002	2.09	0.17
	16(2)	1:2 diluted 16(2)	three extractions (total 6ml)	1.47	1.75	1.46	1.56	0.009	24.97
1:3 diluted 16(2)		three extractions (total 6ml)	1.16	1.01	1.03	1.07	0.006	25.58	1.90

APPENDIX 3/2

**RESULT OF QUANTIFICATION ANALYSIS BY LIQUID-LIQUID EXTRACTION WITH  
CHLOROFORM AND UV-VIS DETECTION**

Specimen	Method		Replica 1 (mg/l)	Replica 2 (mg/l)	Replica 3 (mg/l)	Average concentration of indigo in solvent (mg/l)	Average extracted amount of indigo (mg)	Average concentration of indigo in specimen (mg/l)	Standard deviation (mg/l)
14(2)	Three extractions	2ml solvent (total 6ml)	1.60	1.62	1.58	1.60	0.010	12.82	0.17

APPENDIX 4

**RESULTS OF INDIGO QUANTIFICATION BY CENTRIFUGAL SEPARATION WITH UV-VIS AND CENTRIFUGAL SEPARATION WITH HPLC**

Method	Specimen	Replica 1 (mg/l)	Replica 2 (mg/l)	Replica 3 (mg/l)	Average concentration of indigo in solvent (mg/l)	Average extracted amount of indigo (mg)	Average concentration of indigo in specimen (mg/l)	Standard deviation (mg/l)
UV-Vis	14(2)	2.71	2.74	2.60	2.69	0.008	10.74	0.30
	16(2)	5.72	5.59	5.41	5.57	0.017	22.30	0.61
HPLC	14(2)	1.27	1.16	1.58	1.34	0.004	5.35	0.87
	16(2)	3.02	3.28	3.64	3.31	0.010	13.25	1.25