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Institute of Organic Chemistry

# Development of Versatile Hair Dyes Based on *Indigofera tinctoria* L.: A Contribution to Sustainable Cosmetic Chemistry

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| | |
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**Theses**

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| 2024ID00047 | Deaktivierung der Enzymaktivität im Indigo Pflanzenpulver für die Stabilisierung des enthaltenen Indikans, Reaktivierung mit beta-Glucosidase und Modifizierung des Farbergebnisses | 2024 | Inventor | A1 |
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| 2023ID00347 | Deaktivierung der Enzymaktivität in gemahlenen Indigoblättern für die Stabilisierung des enthaltenen Indikans und Aufbereitung dieser in Pulverform | 2023 | Inventor | A1 |
| 2022ID00307 | Deaktivierung der Enzymaktivität im Indigo Pflanzenpulver fr̈ die Stabilisierung des enthaltenen Indikans und Modifizierung des Farbergebnisses | 2023 | Inventor | A1 |
| 2022ID00305 | Modifizierung des Indigo-Färbeergebnisses durch Zugabe verschiedener Zusätze | 2022 | Inventor | A1 |
| 2022ID00296 | Deaktivierung der Enzymaktivität im Indigo Pflanzenpulver für die Stabilisierung des enthaltenen Indikans und Verhinderung der charakteristischen Farbveränderung | 2022 | Inventor | A1 |

To be continued on the next page

| Number | Titel | Year | Role | Status |
|---|---|---|---|---|
| 2022ID00280 | Beeinflussung des Haarfärbeergebnisses mit Indigo Pflanzenpulver (Indigo crushed leaves) durch verschiedene Nachbehandlungen | 2022 | Inventor | A1 |
| 2022ID00270 | Intensivierung des Farbergebnisses von Reseda (Weld extract) durch die Zugabe eines Cellulase-Blends zur Haarfärbung | 2022 | Inventor | A1 |
| 2022ID00174 | Hennafärbung von Proteinfasern im Zwei-Komponenten-System | 2022 | Contributor | A1 |
| 2022ID00173 | Stabilisierung von glykosyliertem Lawson und glykosylierten Lawson-Derivaten mittels thermischer Enzyminaktivierung von Hydrolasen im Henna | 2022 | Contributor | A1 |
| 2022ID00172 | Methode zur Trennung von glykosyliertem Lawson/ glykosylierten Lawson-Derivaten und hennaeigenen Hydrolasen mittels alkoholischer Extraktion | 2022 | Contributor | A1 |
| 2022ID00159 | Veränderung des Farbeindrucks natürlicher Haarfärbung mit anthocyanhaltigen Pflanzen durch die Addition von Hydrolasen | 2022 | Contributor | A1 |
| 2022ID00149 | Kombination von Matcha (Camelia sinensis) und einem Silbersalz zur Haarfärbung | 2022 | Inventor | A1 |

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# Abbreviations

| | |
|---|---|
| **AI** | Artificial Intelligence |
| **Å** | Ångström |
| **a*** | Red-Green Dimension in CIELAB Color Space |
| **ACN** | Acetonitrile |
| **b*** | Blue-Yellow Dimension in CIELAB Color Space |
| **°C** | Degree Celsius |
| **CC** | Column Chromatography |
| **cf** | Confer |
| **CIELAB** | Commission Internationale de l'Éclairage – L*a*b* |
| **cm** | Centimeter |
| **CMC** | Cell-Membrane-Complex |
| $\Delta E$ | Change in Color |
| $\delta$ | Chemical Shift in ppm |
| **DMSO** | Dimethyl Sulfoxide |
| **EU CPR** | European Parliament and of the Council on Cosmetic Products (Regulation 1223/2009) |
| **EtOH** | Ethanol |
| **EtOAc** | Ethyl Acetate |
| **FA** | Formic Acid |
| **g** | Gram |
| **HCl** | Hydrogen Chloride |
| **HPLC** | High Performance Liquid Chromatography |
| **Hz** | Hertz |
| **InCL** | *Indigofera tinctoria* L. Crushed Leaves |
| **InHD** | Heat Denatured *Indigofera tinctoria* L. |
| **InHD liq.** | Heat Denatured *Indigofera tinctoria* L. Liquid |
| **InHD p.** | Heat Denatured *Indigofera tinctoria* L. Powder |
| **InEE** | *Indigofera tinctoria* L. Ethanolic Extract |
| **InEER** | *Indigofera tinctoria* L. Ethanolic Extraction Residue |
| **J** | Coupling Constant in Hz |

| | |
|---|---|
| **L*a*b*** | CIELAB Color Space Dimensions |
| **L*** | Brightness Dimension in CIELAB Color Space |
| **LOD** | Loss on Drying |
| **mbar** | Millibar |
| **µg** | Microgram |
| **mg** | Milligram |
| **MHz** | Megahertz ($10^6$ Hz) |
| **µL** | Microliter |
| **mL** | Milliliter |
| **min** | Minute |
| **MV** | Mean Value |
| **NaCl** | Sodium Chloride |
| **NaOH** | Sodium Hydroxide |
| **nat** | Native |
| **nm** | Nanometer |
| **NMR** | Nuclear Magnetic Resonance |
| **NMT** | Not More Than |
| **PE** | Petroleum Ether |
| **ppb** | Parts Per Billion, Concentration Indication |
| **rcf** | Relative Centrifugal Force |
| **RH** | Relative Environmental Humidity |
| **RM** | Raw Material |
| **RT** | Room Temperature |
| **SCCS** | Scientific Commitee on Consumer Safety |
| **SD** | Standard Deviation |
| **SLES** | Sodium Laureth Sulfate |
| **TDS** | Total Dissolved Solids |
| **TLC** | Thin Layer Chromatography |
| **UPW** | Ultra Pure Water |
| **UV** | Ultra Violet |
| **w/v** | Weight per Volume |
| **v/v** | Volume per Volume |

# 1 Zusammenfassung

Pflanzliche Haarfärbemittel auf Basis von *Indigofera tinctoria* L. zeigen ein hohes Potenzial für die Entwicklung nachhaltiger und leistungsstarker Haarfärbeformulierungen. Die enzymatische Hydrolyse des Farbstoffvorläufers Indican durch $\beta$-D-Glucosidase in wässriger Umgebung führt zur Bildung von Indoxyl, das in die Haarfaser eindringt und dort zu Indigo und Indirubin oxidiert. Beide Farbstoffmoleküle sind wasserunlöslich und dauerhaft im Haarkortex verankert.

Die Haarstruktur beeinflusst die Farbausprägung, insbesondere die nachträgliche Bildung von Indirubin, maßgeblich. Dies führt vom initialen Blauton zu einem violetten Farbeindruck. Mit 5% (w/v) getrockneten und gemahlenen Indigoblättern in wässriger Lösung zeigt sich nach 14 Tagen eine deutliche Farbverschiebung von $\Delta E = 33.0$, bei gleichzeitig permanenter Farbstabilität über 30 Haarwäschen. Zusätzlich lässt sich durch Zusatz von Isatin und Cystein oder Ascorbinsäure erstmalig eine sofortige und stabile Indigo-Rotfärbung auf dem Haar erzielen ($\Delta E$ Tag 0 und Tag 14 = 7.6), konform mit den EU-Kosmetikrichtlinien.

Die Kombination aus Indican, $\beta$-D-Glucosidase und Wasser, essenziell für die Farbbildung aus *Indigofera tinctoria* L., muss bei der Extrakterstellung für die Produktentwicklung berücksichtigt werden. Ein Indican-haltiger, hitzedenaturierter, wässriger Indigo-Extrakt bietet Vorteile für die Produktentwicklung und ermöglicht die kontrollierte Farbstofffreisetzung durch gesteuerte Zugabe von $\beta$-D-Glucosidase unmittelbar vor der Anwendung. Außerdem zeigt er besonders geringe Farbveränderungen innerhalb von 14 Tagen auf dem Haar ($\Delta E = 1.6$). Zudem lassen sich wichtige Faktoren für Anbau und Verarbeitung der Pflanze in Indien identifizieren, um die Farbstoffqualität zu sichern.

Abschließend wird auf Grundlage der Untersuchungen ein neuer Farbbildungsweg auf dem Haar, ausgehend von der Speicherform in Pflanzen, dem Indican, vorgestellt. Die Ergebnisse belegen, dass Funktionalität und Nachhaltigkeit in der Haarfärbung mit *Indigofera tinctoria* L. vereinbar sind. Die gezielte Steuerung der Farbbildung ermöglicht die Entwicklung bio-basierter Haarfärbemittel mit hoher Performance und ökologischer Verträglichkeit - eine vielversprechende Alternative zu konventionellen synthetischen Produkten.

# 2 Abstract

Plant hair dyes based on *Indigofera tinctoria* L. show great potential for the development of sustainable and effective hair dye formulations. The enzymatic hydrolysis of the dye precursor indican by $\beta$-D-glucosidase in an aqueous environment leads to the formation of indoxyl, which penetrates the hair fiber and oxidizes there to indigo and indirubin. Both dye molecules are insoluble in water and permanently anchored in the hair cortex.

The hair structure has a significant influence on the color expression, in particular the subsequent formation of indirubin. This leads from the initial blue tone to a violet color impression. With 5% (w/v) dried and ground indigo leaves in an aqueous solution, a clear color shift of $\Delta E = 33.0$ is evident after 14 days, with permanent color stability over 30 hair washes. Furthermore, the addition of isatin and cysteine or ascorbic acid achieves an immediate and stable indigo-red coloration on the hair for the first time ($\Delta E$ day 0 and day 14: = 7.6), in accordance with EU cosmetics guidelines.

The combination of indican, $\beta$-D-glucosidase and water, essential for color formation from *Indigofera tinctoria* L., must be taken into account when preparing extracts for product development. A heat-denatured, aqueous indigo extract containing indican offers advantages for product development and enables controlled dye release through the targeted addition of $\beta$-D-glucosidase immediately before application. In addition, it shows particularly low color changes on the hair within 14 days ($\Delta E = 1.6$). Furthermore, important factors for the cultivation and processing of the plant in India can be identified in order to ensure dye quality.

Finally, based on the totality of investigations, a new color formation pathway of *Indigofera tinctoria* L. on the hair is presented, starting from the storage form in plants, indican. The results prove that functionality and sustainability are compatible in hair coloring with *Indigofera tinctoria* L.. The targeted control of color formation enables the development of bio-based hair dyes with high performance and ecological compatibility - a promising alternative to conventional synthetic products.

# 3 Introduction

For thousands of years, people have been using plant-based dyes for aestheticization of textiles, skin and hair – serving both aesthetic and cultural purposes. In ancient Egyptian times, dyed hair was considered an expression of status and individuality. [1] Natural colorants derived from plants, roots, insects, animals or minerals were widely used [2]. Among those natural dyes, henna and indigo are especially notable for their historical and cultural significance in dyeing. [3, 4]

The industrial revolution and the advent of synthetic dyes shifted the focus away from natural solutions. Today, synthetic hair dyes dominate the market due to their broad color range, reproducibility, and suitability for mass production. However, their petrochemical origin and potentially harmful ingredients have raised increasingly health and environmental concerns. [2, 4, 5] Allergies, respiratory issues, and even carcinogenic effects have been linked to their use [6]. In addition, the trend towards sustainable cosmetic products ensures that the development of plant-based hair coloring systems is becoming increasingly important. [7, 8] Besides Henna (*Lawsonia inermis* L.), Indigo (*Indigofera tinctoria* L.) is regaining attention as a direct natural dye. [3, 4]

The indigo molecule from the *Indigofera tinctoria* L. plant (cf. figure 3.1) is one of the oldest and best-known dyes in human history and is still used in the textile industry today for dyeing silk, cotton, wool and jeans as well as food industry in its synthetic form. [9] While its chemical structure and dyeing behavior are well understood in textiles and microbial fermentation, its application and mechanism in hair dyeing is still limited. Until now, it has mainly been used as a traditional hair dye where the dried and shredded leaves are mixed with water and applied on hair. Its coloration occurs blue freshly after application. [10, 11]

**Figure 3.1: *Indigofera tinctoria* L. Plant.** Own recording.

However, natural hair dyes must meet many criteria to present an equal alternative and compete with synthetic ones. A better understanding of the coloring mechanism of *Indigofera tinctoria* L. enables influence on the reaction and taking advantage of it to make natural hair colors more convenient. This represents a significant research gap, but at the same time holds great potential for sustainable innovation in the cosmetics industry. [12]

## 3.1 Human Hair

Human hair, along with fingernails and toenails, sebaceous glands and sweat glands, is a skin appendage. There are about two to five million hair follicles on the entire human body, around one ninth of which are on the scalp. The number of follicles is fixed at birth and does not naturally change over the course of a lifetime. As a result of evolution, hair no longer fulfills one of its original functions: thermal insulation. In addition to their function as a sensory organ through sensitivity to touch, they primarily have a psycho-social function. Head hair in particular plays a major role in a person's self-presentation and aesthetics [10].

### 3.1.1 Structure of Human Hair

Human scalp hair consists of the keratinized hair shaft, which is visible from the outside, and the living hair root. The hair root is the part of the hair that is connected to the skin via the dermis invagination of the scalp, the cone-like hair papilla. The hair root has a sebaceous gland, the erector pili muscle, blood capillaries and nerve tracts (cf. figure 3.2). Cell division and biological changes take place here, and metabolic products can also be transported to the skin via the connection. The continuous cell division of matrix cells in the hair root leads to the newly formed cells being pushed towards the skin surface. During this process, further cell differentiation into individual hair components takes place. The initial soft protein cells in the hair root first transform into pre-keratin and then into keratin. The finally keratinized hair grows out of the hair root [10,13,14]. Depending on the environmental conditions to which it is exposed, the virgin hair has a moisture content of 7 to 12% [15–17].

A terminal hair is between 50 and 150 µm thick [10]. Just above the papilla, up to three essential components of the hair shaft can be recognized: the medulla, the cortex and the cuticle (cf. figure 3.2). The medulla is the hair's marrow, which is located inside the hair, consists mainly of keratin and is very soft. It has little influence on the effect of hair treatments and is not found in all hair and only in thicker terminal hair. [10, 18, 19]

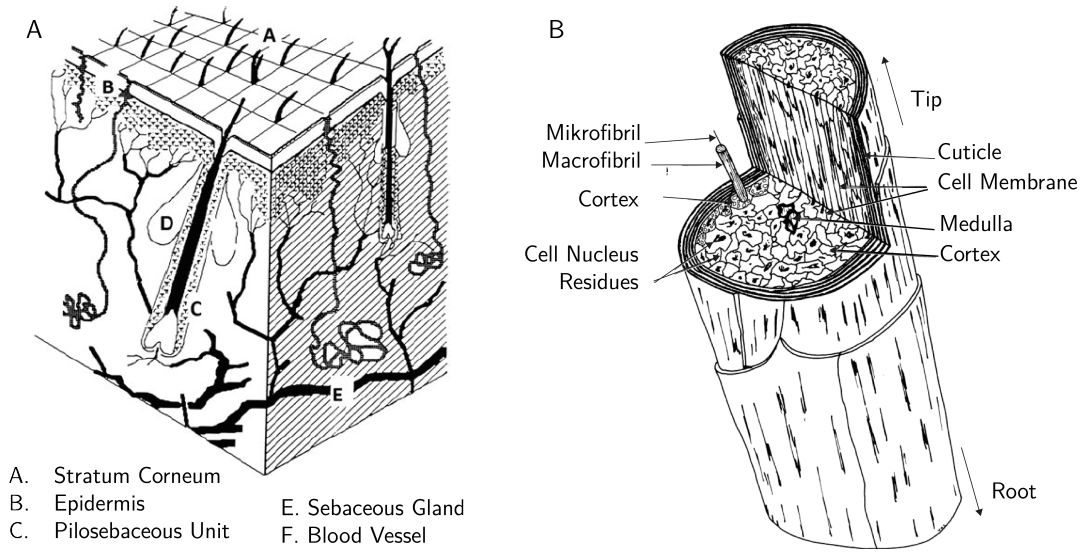



**Figure 3.2: Schematic Representation of Hair Follicle and Hair Fiber.** A: Cross-section of epidermis, dermis and subcutis with the localization of the skin appendage in the skin. Adapted from Robbins (2012) [19]; B: Structure of the hair fiber. Adapted from Swift (1977) [18].

The cortex cells make up the largest part of the hair mass with up to 90%. They are found between the medulla and the cuticle. Each of the spindle-shaped cortex cells has a length of approx. 50-100 µm and a diameter of approx. 3 µm and is aligned in the direction of the long hair axis towards the tip of the hair. The cells consist of macrofibrils, which are composed of microfibrils and an amorphous matrix of sulfur proteins. These microfibrils in turn consist of intermediate filaments made up of tetramers. Keratin strands are the main component of the tetramers. They are composed of $\alpha$-helices (cf. figure 3.3) and rich in cysteine compounds. [19–21]

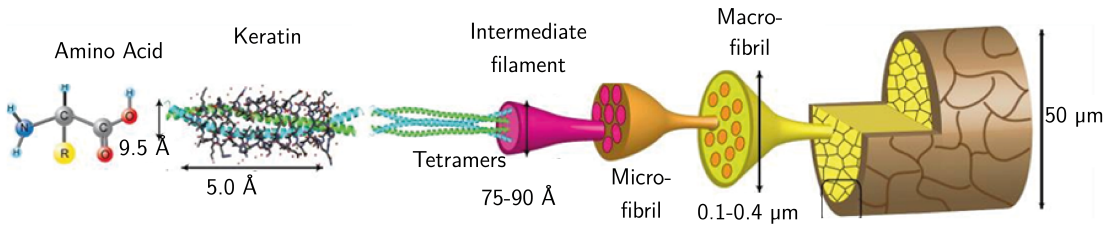



**Figure 3.3: Structure of the Human Hair to Amino Acid Level.** Adapted from Yang *et al.* (2014). [20, 21]

Even though the exact amount of cysteine in cortical cells is unknown, the proportion is significantly higher than in the cuticle [22]. The cortex is largely responsible for

the mechanical properties of the hair due to its numerous disulfide bonds and its cell membrane complex and for the hair color due to the storage of the hair's natural color molecule called melanin [19, 23–25].

The cuticle is the outermost hair layer and surrounds the cortex cells. Depending on the condition of the hair, it consists of six to eleven layers of flat, approx. 35-40 µm thick cells (scales), which overlap towards the tip of the hair and extend from the scalp to the tip of the hair. The cuticle is separated from the underlying cortex by intercellular material, the cell membrane complex (CMC), which consists of non-keratinous proteins and lipids [19,26]. Each cuticle layer in turn is also separated from other neighboring cuticle layers by CMC [27]. Further, a cuticle cell is divided into three different components: The epicuticle consists of 12% cysteine and is coated with lipids. It is responsible for the hydrophobic properties of the hair and determines its frictional properties [19]. The middle layer, the exocuticle, can in turn be divided into the A and B layers. At 35%, the A layer is rich in cysteine, while the B layer is somewhat poorer at 15%. The inner endocuticle has a cysteine content of 3% and consists largely of various other amino acids (cf. figure 3.4) [23]. The high stability of the hair and its insolubility can be explained by the sulphur content of the hair associated with the cysteine content (cf. figure 3.4, **2**). Plowman *et al.* (2003), Parry *et al.* (2006) and Deb-Choudhury (2018) state that accessibility of cysteine contained in merino wool and in human hair is heterogeneous: A subgroup of the cysteine residues of the proteins is easily accessible, while others are shielded by the protein structures. Cysteine itself is colorless. [28–30]

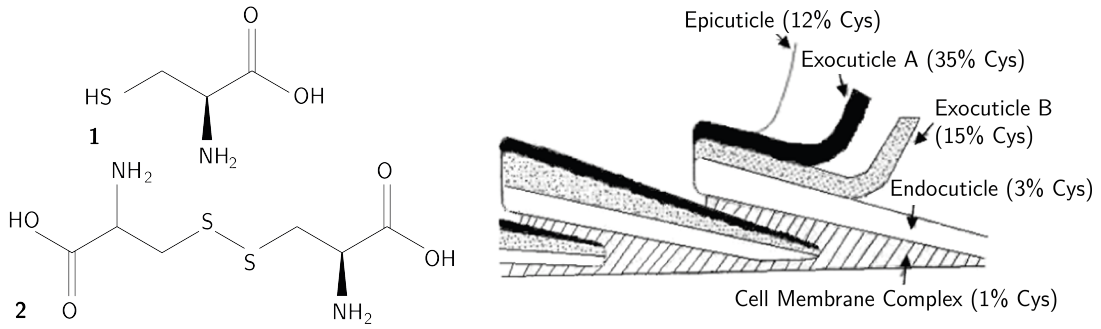



**Figure 3.4: (*L*)-Cysteine (1), Cystine (2) and Lamellar Arrangement of Cuticle Cells.** Left: *L*-Cysteine (**1**); center: Cystine (**2**) from two (*L*)-Cysteine-Molecules, which are linked via a disulfide bridge. Lamellar arrangement of the cuticle cells with the assignment of the cysteine content. Adapted from Swift (1991). [23]

A human hair contains around 10% water, which increases to 20% through washing [31]. Cosmetic treatments of the hair have a direct effect on the cuticle of the hair or spread through the cuticle into the interior of the hair [19, 32]. The size of dye molecules that can potentially penetrate the hair is assessed by their charge (anionic, cationic and non-ionic) and through the molecular lateral diameter ($L_D$) introduced by Morel *et al.* (2008) [33]. $L_D$ is defined using a parallelepiped enclosing the corresponding molecule and measuring all three dimensions of the same with the indices $S_7$, $S_8$ and $S_9$. The two smallest indices are then used for calculation of $L_D$ (cf. eq. 3.1). Assuming $S_7$ and $S_8$ having the lowest values, results in the following equation.

$$L_D = \sqrt{(S_7^2 + S_8^2)} \tag{3.1}$$

Virgin hair can be penetrated by non-ionic molecules up to a size of $L_D = 9.5$ Å [33].

### 3.1.2 Bonds in Human Hair

The protein structure of keratin, the main component in hair, is characterized by various covalent and non-covalent interactions between its amino acids. On the one hand, these can take place intramolecularly and thus ensure the stability and characteristic $\alpha$-helical structure of the protein. On the other hand, intermolecular interactions can take place between protein and e.g. color molecules.

Intramolecularly, covalent disulfide bridges play a major role, which are formed between the thiol groups of cysteine (cf. figure 3.4, 1 & 2). They ensure the stability and mechanical properties of hair. Furthermore, non-covalent interactions in the form of hydrogen bonds, van der Waals and hydrophobic interactions between the amino acids of the protein are possible. These forces are essential for the stability of the protein and ensure the characteristic $\alpha$-helical structure. Depending on the pH value, ionic interactions are also conceivable, e.g. when the amino group is protonated. When considering intermolecular interactions, non-covalent interactions are particularly important, as they represent the main interaction between the color molecule and the hair protein. [17, 34]

### 3.1.3 Structure of Yak Belly Hair Compared to Human Hair

Like human hair, yak belly hair consists mainly out of keratine. In general, both hair types have a similar chemical composition. The exact content of cysteine in yak hair, however, has not been clarified [35]. As yak belly hair does naturally not contain melanin, it appears white. [36] Even though the hair types are alike on a molecular level, there are structural differences (cf. figure 3.5).

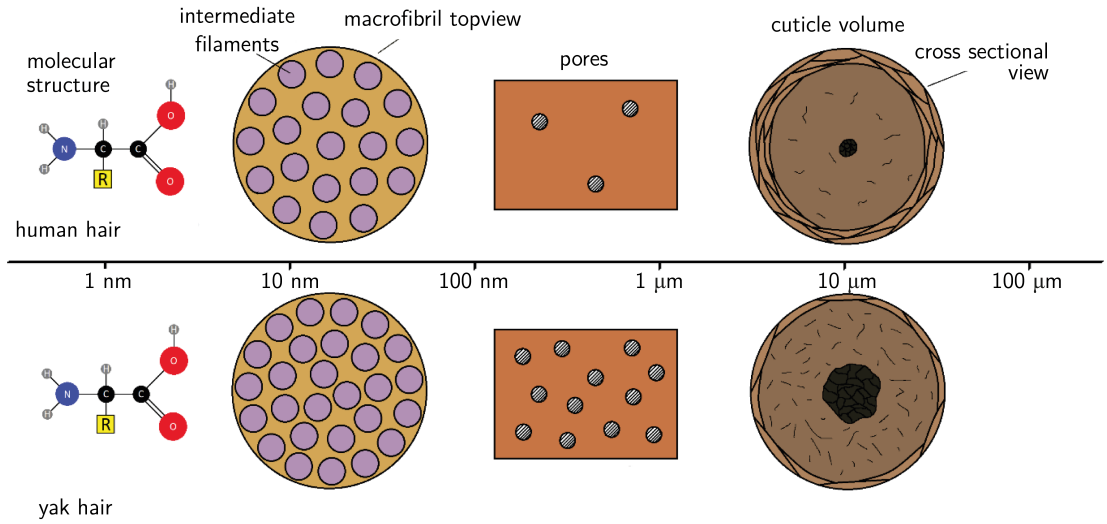



**Figure 3.5: Scheme to Visualize the Comparison Between Structures of Human and Yak Belly Hair.** Human and yak hair show the same molecular structure, an intermediate filaments, whereby those of yak hair is less ordered and has less matrix in between. Human hair shows lower amount of pores and more cuticle volume. Yak hair has more cracks, tiny structures and larger medulla. Adapted from Müllner *et al.* (2020). [36].

The yak hair shows smaller distances between intermediate filaments and a lower matrix thickness (1.3 nm in yak vs. 2.9 nm in human). Further, the porosity of yak hair is higher at all hierarchical levels. It often has a pronounced medulla, which leads to a lower hair density. On the surface of hair fibers, yak belly hair has has fewer overlapping keratin scales, which makes it easier for applied substances to penetrate. [36, 37]

## 3.2 Hair Coloration

Since human head hair today is almost exclusively of socio-psychological significance, changing hair color is a means of expressing personality and social status. [38] The hair colors used for this purpose can be classified according to various criteria. One criterion includes the basic distinction between synthetic and natural hair colors.

### 3.2.1 Synthetic Hair Dyes

Synthetic hair dyes can be divided into temporary, semi-permanent, demi-permanent and permanent dyes due to intensity of interaction with the hair. Further, they can be divided by their mechanism of action into oxidative and non-oxidative colors. [10,39]

Non-oxidative hair dyes achieve their result through colored molecules with chromophoric groups, e.g. azo dyes with a high molar mass, which adhere to the hair due to an anionic interaction with the hair fiber. They are usually applied to wet, freshly washed hair and are not washed out afterwards. Due to their size, their interaction is limited to the cuticle and, in rare cases, the cortex, which means that they only achieve low resistance and are classified as temporary (removed after one hair wash) or, at most, semi-permanent colors which last for six to eight hair washes. [10, 19, 39, 40]

Demi-permanent dyes are washable after about 10-15 shampoos as they reach the cortex partly. To reach this, the cuticle scales are opened by their basic pH. However, they do not contain ammonia or ethanolamine but low concentrations of $H_2O_2$. [41]

Petroleum-based, oxidative, permanent hair dyes are the commercially most important ones: They offer long-term color change for more than 24 washes, the widest range of colors, grey hair coverage and the ability to either darken or lighten hair [39, 42]. They are comprised of a two-component system – a developer-coupler duo and an oxidation base, e.g. $H_2O_2$. Both components are added together immediately before use and applied to the hair. The color impression is then created in the hair from the so-called precursors, a *o*- or *p*-substituted aromatic amine, the *developer*, and an electron-rich, aromatic *coupler*. [43] Figure 3.6 shows an exemplary reaction proposal for the reaction of developer and coupler to the color molecule.

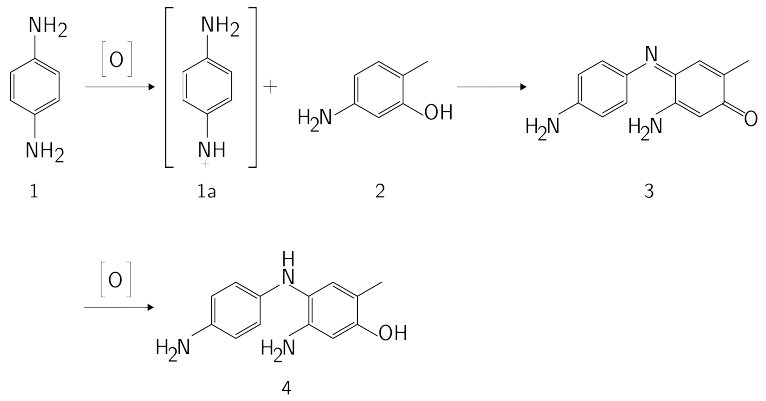



**Figure 3.6: Proposal of a Reaction Mechanism for the Formation of a Dinuclear Indo Color Molecule** (**2**). Starting from p-phenylenediamine (**1**) and 5-amino-o-cresol (**1a**) to a color less leuco product (**3**) forming the color molecule (**4**). Adapted from Morel *et al.* (2011). [43]

The developer-coupler duo contains an alkalizing agent which causes the cuticle to swell and open. This allows the small, colorless molecules of the dye precursors to penetrate the cortex. The added oxidizing agent (usually $H_2O_2$) triggers a reaction in the cortex where the reaction takes place, in which the coupler **1** is oxidized in a first step. The intermediate formed **1a**[1]. reacts with the developer **2** to form the initially colorless leuco product **3**, which reacts in a subsequent oxidation reaction to form the color molecule **4**. [43] The reaction product has a high molecular weight, which is anchored in the cortex by its size due to non-covalent interactions with the hair protein. This makes the color impression particularly resistant to external physical influences. [19]

The use of synthetic hair dyes is associated with risks to human health and nature. They have a high allergy potential and poor biodegradability. [43, 44] In addition, due to the amine-rich color precursors, there is a risk of the formation of carcinogenic nitrosamines through the reaction of secondary amines with atmospheric $NO_x$. [45]

### 3.2.2 Plant-Based Hair Dyeing

The use of natural dyes of plant or mineral origin for hair coloring dates back to ancient civilizations, with evidence from Egypt as early as 5,000 BCE [46–48]. Plant-derived pigments such as chlorophylls, carotenoids, flavonoids, and alkaloids are

[1]For clarity, only a mesomeric limiting structure is shown here

typically stored in various plant organs, including roots, stems, leaves, flowers, and fruits [49]. In addition to their sustainability and biodegradability, these natural colorants offer a significant advantage over synthetic dyes due to their low toxicity and safety for human health.

However, current limitations of plant-based hair dyes include laborious and time-consuming application procedures, a restricted color palette, and poor wash fastness. Moreover, they are rarely compatible with chemical treatments such as bleaching [10, 11]. Despite these drawbacks, plant-based dyes are valued for their use of renewable raw materials, low environmental impact, and complete biodegradability [50]. The Scientific Committee on Consumer Safety (SCCS) has classified the use of *Indigofera tinctoria* L. on human scalp hair at concentrations up to 25% as safe [51].

A distinction of plant-based hair dyes can be made between direct and mordant dyes. The direct dyes are particularly relevant for this work, which is why only these are referred to in the following. These primarily include *Lawson*, which precursor hennoside is stored in the leaves of the henna plant (*Lawsonia inermis* L.), and *Indigo*, which precursor indican is stored in the leaves of the indigo plant (*Indigofera tinctoria* L.). [50, 52] The following will focus on the latter in particular.

#### 3.2.2.1 Hair Dyeing with *Indigofera tinctoria* L.

In order to understand and specifically control the extraction and subsequent hair dyeing with *Indigofera tinctoria* L., it is crucial to consider the underlying biosynthetic pathways of dye formation. In the vacuoles of the plant leaves, the dye is stored in the form of the colorless, stable precursor indican (indoxyl-$\beta$-D-glucoside, **1**) [6, 8, 53–56]. The plant's own enzyme $\beta$-glucosidase is located in the chloroplasts. As soon as the leaf structure is destroyed and the leaves come into contact with water and oxygen, the enzyme initiates the hydrolytic cleavage of the sugar moiety (deglycosylation) of indican, resulting in the formation of indoxyl (**4a/4b**) [6, 9, 12, 57]. This is followed by dimerization of the indoxyl by atmospheric oxygen to form the indigo molecule (2-(1,3-dihydro-3-oxo-2H-indol-2-ylidene)-1,2-dihydro-3H-indol-3-one, **2**) [58–62]. In addition, the molecule isatin (**5**) is formed from indoxyl as a side reaction in an oxygen-rich environment. Condensation of isatin with indoxyl forms the red molecule indirubin (3-(1,3-dihydro-3-oxo-2H-indol-2-ylidene)-1,3-dihydro-2H-indol-2-one **3**) [57].

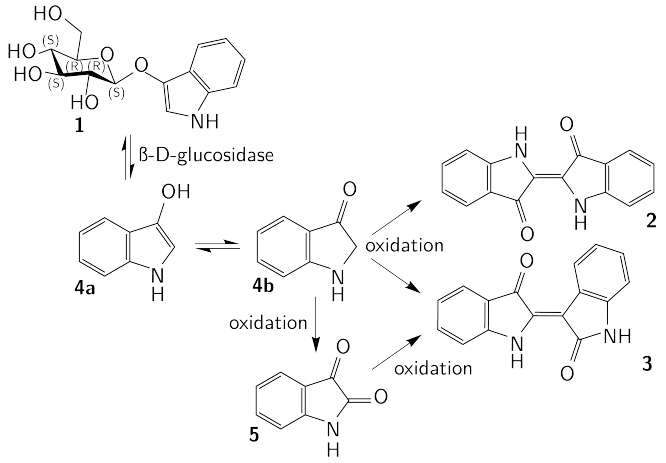



**Figure 3.7: Biosynthesis Pathway from Indican to Either Natural Indigo or Indirubin. 1**: indican, **2**: indigo molecule, **3**: indirubin, **4a/4b**: indoxyl tautomers, **5**: isatin. Adapted from Maugard *et al.* (2002). [6, 59, 63–65]

As a structural isomer of nonpolar indigo, indirubin absorbs shorter wavelengths and therefore appears in a reddish hue. While both indigo and indirubin exhibit pronounced intermolecular hydrogen bonds in their crystal structure, indirubin shows a more restricted intramolecular hydrogen bond and is a polar molecule [63, 66–68]. Their precursor molecule, the glycosylated indican, in turn has a higher polarity. Indirubin is well-known in traditional chinese medicine for the treatment of leukemia or skin diseases [69–71].

By the $L_D$ value mentioned in chapter 3.1.1, eq. 3.1, the lateral diameter of molecules involved in the indigo pathway can be determined. They are listed in the following (cf. table 3.1) [33].

**Table 3.1: Lateral Diameter $L_D$ of Molecules Involved in Indigo Synthesis.** Theoretical calculation of $L_D$ of indican, indigo molecule, indirubin, indoxyl and isatin. Own calculations based on Morel *et al.* (2008) [33].

| **Molecule** | **Molecule Span [Å]** | | | **$L_D$ [Å]** |
|---|---|---|---|---|
| | $S_7$ | $S_8$ | $S_9$ | |
| Indican | 11.12 | 7.37 | 4.11 | 8.44 |
| Indigo molecule | 7.35 | 6.27 | 2.26 | 6.67 |
| Indirubin | 11.0 | 6.96 | 3.93 | 8.00 |
| Indoxyl | 6.57 | 5.29 | 0.00 | 5.92 |
| Isatin | 5.76 | 4.32 | 2.40 | 4.94 |

#### 3.2.2.2 Influence on *Indigofera tinctoria* L. Pathway

As known from literature, this pathway can be manipulated and the selectivity of biological indirubin synthesis promoted. Until now, the influence on the indigo biosynthesis pathway is only documented by the addition of L-cysteine under mild, aqueous conditions in a microbial whole cell or enzyme system (e.g. Escherichia coli expressing the flavin-dependent monooxygenase (MaFMO)).

This enables the targeted oxidation of indole to indoxyl and its derivatives. This produces the stable intermediate 2-cysteinylindoleninone, which contributes to indirubin synthesis (cf. figure 3.8) [64, 72].

In particular, cysteine intervenes in the following processes: The major product shifts from indigo molecule to indirubin when cysteine is added. To explain the points of action of cysteine in biosynthesis pathway, Kim *et al.* (2019) firstly introduce prevention of oxidized indoxyl from forming indigo molecule but developing an intermediate product called 2-cysteinylindoleninone (**6**) in a non-enzymatic, oxidative way. The intermediate product reacts with isatin (**5**) spontaneously to indirubin (**3**) unless isatin as a reactant is a limiting factor for indirubin synthesis. The isatin synthesis might take place by hydrolysis of 2-cysteinylindoleninone (**6**) or oxidation of indoxyl (**4**) at C2. Beyond that, Kim *et al.* (2019) assume that cysteine

promotes reaction of isatin with 2-cysteinylindoleninone which results in indirubin formation. [64, 72, 73] The slower formation of isatin than of indoxyl is decribed by Sánchez-Viesca *et al.* (2021) [74].

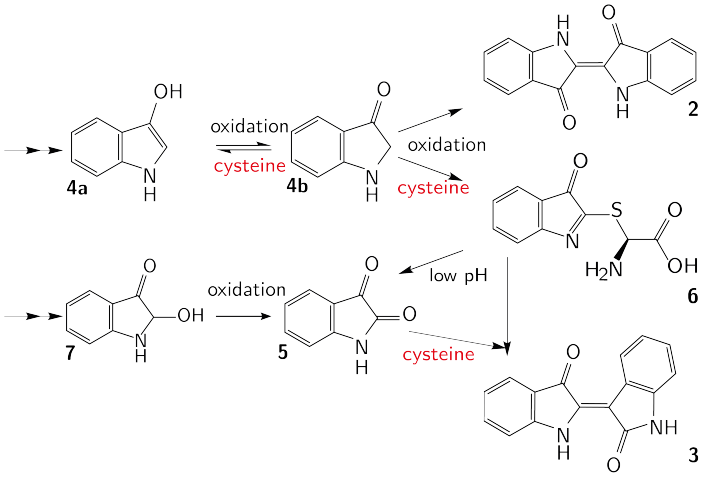



**Figure 3.8: Biosynthesis Pathway from Indican to Either Natural Indigo or Indirubin in Microbiological Environment with Supplementation of Cysteine.** **2**: indigo molecule, **3**: indirubin, **4a**/**4b**: indoxyl tautomers, **5**: isatin, **6**: 2-cysteinylindoleninone, **7**: dioxindole. Adapted from Kim *et al.* (2019). [64, 72]

The previously introduced $L_D$ value (cf. chapter 3.1.1, eq. 3.1) for cysteine can be calculated as follows (cf. table 3.2) [33]:

**Table 3.2: Lateral Diameter $L_D$ of L-Cysteine.** Own calculation based on Morel *et al.* (2008) [33].

| **Molecule** | **Molecule Span [Å]** | | | **$L_D$ [Å]** |
|---|---|---|---|---|
| | $S_7$ | $S_8$ | $S_9$ | |
| L-Cysteine | 5.21 | 4.15 | 3.12 | 5.20 |

### 3.2.2.3 Definition of Terms Concerning *Indigofera tinctoria* L.

The term *indigo* is used for various applications both in different languages as well as in scientific literature. The meaning can only be understood up by the context in which the term is used. Therefore, in accordance with literature [75], the following naming is consulted:

**Table 3.3: Definition of Terms concerning *Indigofera tinctoria* L..**

| Number | Term | Definition |
|---|---|---|
| 1 | *Indigofera tinctoria* L. | Indigo Plant |
| 2 | natural Indigo | Indigo molecule, naturally derived from plant |
| 3 | synthetic Indigo | Indigo molecule, synthetically produced |
| 4 | Indigo molecule | Indigo molecule regardless of origin |
| 5 | Indigo Crushed Leaves (InCL) | Dried, threshed and crushed leaves of *Indigofera tinctoria* L. |
| 6 | Indigo | Generic term for plant and molecules |

#### 3.2.2.4 Chlorophyll in Plants

Chlorophylls, the most abundant pigments on earth, are present in plants in large quantities and have an influence on plant physiological processes. They should therefore be considered when looking into a plant composition. Two types of chlorophyll are present in higher plants: chlorophyll a (cf. figure 3.9) and chlorophyll b.

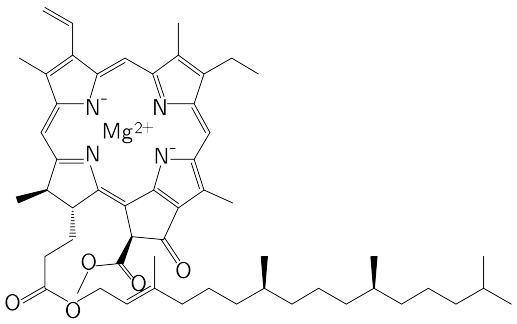



**Figure 3.9: Structural Formula of Chlorophyll a.** Consisting of four entangled pyrrole rings forming a ring-shaped macrocycle with methyl groups, a central chlorin-ring coordinating the magnesium and a long, lipophilic phytol side chain. Adapted from Hörtensteiner *et al.* (2011). [76]

The lipophylic phytol side chain anchors the chlorophyll in the membranes of the chloroplasts. The degradation of chlorophyll takes place primarily during aging

(senescence), in fall or under stress. To break down chlorophyll b, it is first converted into chlorophyll a by a reductase. Chlorophyll a, in turn, is degraded by the enzymatically removal of the central magnesium atom resulting in the molecule pheophytin a and subsequently in pheophorbide a. The subsequent opening of the macrocycle by another enzyme leads to the formation of a colorless, linearized tetrapyrrole called red chlorophyll catabolite. [76]

### 3.2.3 Coloration on Other Substrates than Hair

The coloration with natural or synthetic indigo is also common in textile industry [2, 77, 78]. Various substances are relevant in this sector, which are discussed in more detail below.

One type of textile fibers are natural fibers, which can be processed directly into yarn as they are produced by nature in order to produce individual fabrics. These include, for example plant fibers which all have the same basic structure made of cellulose. Cellulose consists of the natural monomer glucose. Depending on the bond between the glucose molecules, starch and glycogen can also be formed as polymers. Three representatives of this group are listed below.

**Cotton** is the most commonly used plant fiber from which textiles can be made. The cotton bush (*Gossypium*) is a mallow plant and grows to a height of between 25 cm and two meters. The part that humans need for the textile industry are the hairs in the cotton bolls. The cotton fiber is divided into different layers and consists mainly of cellulose. The fibers are pure white and can be easily dyed. Cotton can be processed into many different textiles. [79–81]

However, for centuries, flax was the plant from which people in Europe obtained fibers for making clothes. **Linen** is still used today, but on a much smaller scale, as it has been displaced by cotton. Linen is obtained from the bast plant *Linum usitatissimum* (common flax) and is also largely composed of cellulose. Through various processing steps, it is turned into fibers and then into spinnable bundles in order to produce yarn. In clothing, it is used for blouses, blazers, shirts, pants, dresses, skirts and shoes. [79, 80]

**Hemp** is obtained from the bast fiber (*cannabis sativa*) and its processing is similar to that of linen. Since hemp is a plant-based substance, its main component is cellulose. Hemp is used, for example, as tarpaulin, rope and underlay fabric for carpets. [80, 82]

**Silk** is also a natural fiber produced by insects like the silk moth and spiders. Fibroins, sericins and spidroins are fibrillar proteins that serve as building blocks of silk. [83] The best known type of silk is the cocoon silk of the silkworm moth larva (*Bombyx mori*), which consists of fibroin and sericin in a ratio of 7:3. [84, 85] The fibers can be easily dyed. [80, 81, 86]

In contrast to the natural fibers above, **viscose** is a regenerated fiber. These are based on a renewable raw material, but are still heavily chemically modified. In the case of viscose, the renewable raw material is cellulose, which is derived from cellulose fibers from wood. The contained lignin, which is responsible for the lignification of these fibers, must be extracted. Viscose is then produced in various steps and finally using a wet spinning process. Viscose can and is used in all three textile sectors: Clothing, upholstery and decorative fabrics, technical fabrics. [79, 80, 87]

## 3.3 Role of Enzymes in Dye Release for Hair Coloration

As mentioned in chapter 3.2.2.1, enzyme activity plays a crucial role in dye release from *Indigofera tinctoria* L.. For this reason, the structure and function as well as the influence of environmental factors on enzymes and the effect on the indigo pathway of plant's own $\beta$-D-glucosidase are discussed below. Normally, in plants enzymes are located in the chloroplasts. [6]

### 3.3.1 Structure and Function of Enzymes

Enzymes are proteins and act as catalysts in biochemical reactions. They convert specific or non-specific substrates without being consumed themselves – they are therefore available again after the reaction. The basic building blocks of an enzyme

are amino acids, which combine to form polypeptide chains of different lengths. The diversity and functionality of proteins results from the sequence and combination of the incorporated amino acids. These amino acid chains interact with each other and fold into complex structures which can be divided into the following four levels. [88, 89]

1. Primary structure: the sequence of amino acids in the linear polypeptide chain.

2. Secondary structure: Structural elements such as $\alpha$-helices and $\beta$-sheets, which are stabilized by hydrogen bonds between atoms of the polypeptide chain.

3. Tertiary structure: The overall three-dimensional arrangement of the polypeptide chain. It is stabilized by covalent bonds (e.g. disulfide bonds), ionic interactions, hydrophobic effects and hydrogen bonds. The active center, which interacts with the substrate, is also formed in this structure.

4. Quaternary structure (if present): Exists when an enzyme consists of several polypeptide chains that join together to form a functional complex with several subunits. [88, 89]

The properties of a protein are closely linked to its structure on all four levels. [88, 89] Enzymes accelerate and catalyze reactions highly specified. Normally, a single reaction or a group of very similar reactions is catalyzed. The first step in an enzymatic reaction is the binding of a substrate to the active site of the enzyme, resulting in an enzyme-substrate complex. The allosteric center on the enzyme regulates the binding of effectors that influence enzyme activity. [88]

### 3.3.2 Enzyme Inhibition

Enzyme activity can be modulated by inhibitors – chemical substances that reduce reaction rates or restrict the functionality by binding to the enzyme. Inhibitions are classified as:

**Reversible inhibition**, meaning that the inhibitor reversibly binds to the enzyme and thus restricts its function. Full enzyme activity returns after the inhibitor is removed. Reversible inhibition can be divided into competitive, uncompetitive and non-competitive. In *competitive inhibition*, the inhibitor and substrate compete for

enzyme binding at active site. In contrast, in the case of *uncompetitive inhibition*, an uncompetitive inhibitor only binds to the enzyme-substrate complex, which ensures that its function is restricted. A special type of this inhibition is substrate excess inhibition. Here, inhibition is observed by additional substrate molecules, which results in a catalytically inactive enzyme-substrate complex. The effect is more pronounced at higher substrate concentrations, where a decrease in the reaction rate is observed. The *non-competitive inhibition* results in a substance binding to the allosteric site and changing the conformation of the enzyme resulting in less activity [90, 91].

Beside the reversible inhibition, there are **irreversible inhibitions**. They are caused by reactive molecules which bind to the enzyme and result in inactivation. Even after separation of the substance and enzyme, the enzyme activity does not return. [88, 90, 92]

### 3.3.3 Influence of Environmental Factors on Enzyme Activity

In addition to structural factors, external conditions as pH value and temperature also influence protein folding and subsequently function. Changes in these parameters can lead to conformational changes or to (partial) unfolding (denaturation), which can be reversible or irreversible. Depending on their structure and folding, proteins differ in their physical properties and biological functions, which is also used for classification. [93, 94] The amino acids of a protein consist of a carboxyl group, an amino group, a hydrogen atom, and a characteristic side chain. The carboxyl group is negatively charged at a neutral pH value, as it releases a proton. In an acidic environment, it remains protonated and therefore uncharged, whereas in a basic environment, it is deprotonated and negatively charged. The amino group behaves oppositely: In an acidic environment, it is protonated and carries a positive charge. As the pH value rises, it releases its proton and becomes neutral. The side chains of the amino acids, especially the ionizable ones, also influence the charge of the enzyme as they can be protonated depending on the pH value. They are determined by the individual composition of amino acids of a protein [95]. All in all, the total charge of an enzyme is strongly pH-dependent [88, 96].

As with any chemical reaction, the temperature has an influence on the activation energy. As the temperature rises, the speed of enzyme-catalyzed reactions also increases, as more kinetic energy is supplied to the molecules. Above the optimum temperature point, the activity decreases again because the enzymes are denatured by heat or their flexibility reduces the promotion of the reaction. The optimum temperature and the maximum temperature of an enzyme for activity depends strongly on its structure. [97–99]

### 3.3.4 Significance of $\beta$-D-Glucosidase in *Indigofera tinctoria* L. Dye Release

The $\beta$-D-glucosidase catalyzes the hydrolytic cleavage of the $\beta$-D-glycosidic bond in the *Indigofera tinctoria* L. glycoside indican. Subsequently, a glucose and an indigo color precursor, the aglycone indoxyl, are formed. [100] This step initiates the formation of the indigo dye from the glycolised precursor stored in *Indigofera tinctoria* L. plant's leaves. In addition to this function, $\beta$-D-glucosidase also plays a central role in cell metabolism. It is involved in the regulation of hormone balance, in stress and defense reactions and in the breakdown of toxic metabolic products. Due to these diverse tasks, it is one of the most important enzymes in plant cells. [101]

# 4 Thesis Aim

The demand for natural and sustainable hair dyes is steadily increasing due to ecological and health reasons. The conventional hair dyes that have been widely used to date are based on fossil raw materials and, in addition to being poorly biodegradable, also have toxic and allergenic properties. The usage poses significant risks to consumers and the environment. At the same time, plant-based hair dyes currently consist of plant powders that are mixed with water and applied to the hair as a paste. They lack dye intensity, color spectrum, wash stability, and ease of use. Therefore, they do not yet represent an alternative to oxidative hair dyes.

*Indigofera tinctoria* L. is one representative of promising plant-based dyes, whose color formation mechanism on hair is not fully understood. The aim of this dissertation is therefore to systematically investigate the suitability of *Indigofera tinctoria* L. as a natural hair dye and to gain new insights into the underlying dyeing mechanisms. This should contribute to the scientific foundation of plant-based hair dye systems and support the development of environmentally friendly, health-safe, high-performing, and convenient alternatives to synthetic hair dyes.

The focus is on the conversion of the dye precursor indican to indigo and indirubin on the hair. The role of the substrate to be dyed in the color formation of indigo is to be clarified, as well as the advantages of natural indigo sources compared to synthetic ones. By identifying key components for controlled dye release in extract production and evaluating harvest and processing conditions for *Indigofera tinctoria* L. plant material, this work contributes to the development of effective, biodegradable hair dyes that meet both cosmetic and ecological requirements.

# 5 Material and Methods

The materials and methods on which this work is based are set out below.

## 5.1 Statistical Analysis

Statistical analysis of experiments was carried out with IBM SPSS Statistics Version 29.0 (IBM Corp.). Normal distribution of the data was assumed. The descriptive statistics including mean value (MV) and standard deviation (SD) were carried out ans presented with MV ± SD.

## 5.2 Hair Material Treatment and Examination Methods

Untreated, white, selected yak hair strands from Kerling Haarfabrik GmbH (Backnang, Germany) were used for all experiments. The total length of a strand incl. plastic bonding was 11.8 cm and the length of free hair fibres amounted to approx. 7.5 cm with a weight of 0.7 g.

### 5.2.1 Hair Treatment

The hair treatment could be subdivided into the preparation including pre-washing and drying as well as the coloration itself.

#### 5.2.1.1 Preparation of Hair Strands for Coloration

Before coloration application, the strands were washed following a standardized protocol. First, the strands were rinsed with water while combing with a tap water flow of 25 mL/s. After that, they were placed in 20 mL/strand of an aqueous 12.5% sodium laureth sulfate (SLES) solution adjusted to pH 4.5 (Hydrogen Chloride

(HCl), 10%) for 30 min at room temperature (RT). Next, the strands were rinsed with tap water at a flow of 25 mL/s and combed approx. 10 times until no further SLES bubbles were visible on strands. Last, the strands were air-dried overnight and ready for further treatment. [102]

#### 5.2.1.2 Coloration of Hair Strands and Substrates

Dyeing experiments were carried out with *Indigofera tinctoria* L. crushed leaves powder (InCL) supplied by Kremer Pigmente GmbH & Co. KG. The plant is cultivated yearly between December and April, and there are three harvests in a single cultivation. The first harvest period is three months from the time of cultivation, the second harvest occurs 50 days after the first and the third harvest another 50 days later. The region of the cultivation area is Viluppuram, India [103]. For harvest examinations, *Indigofera tinctoria* L. plants were used. They were cultivated in Tindivanam, Tamil Nadu, India on a farm run by KMA Exports which is specialized in cultivation of indigo for coloring. Plant material was supplied by Indfrag Biosciences Pvt Ltd (Bangalore, India). [1, 104]

Further chemicals used in this work for hair coloration is listed below (cf. table 5.1).

**Table 5.1: Overview of the Substances Used for Hair Treatment.**

| Designation | CAS Number | Manufacturer |
|---|---|---|
| (L)-(+)-Cysteine for synthesis | 52-90-4 | Merck KGaA (Darmstadt, Germany) |
| Ascorbic Acid | 50-81-7 | Merck KGaA (Darmstadt, Germany) |
| Isatin | 91-56-5 | ThermoFisher GmbH (Kandel, Germany) |
| Hydrochloric Acid | 7647-01-0 | VWR International GmbH (Darmstadt, Germany) |
| Sodium Hydroxide | 1310-73-2 | VWR International GmbH (Darmstadt, Germany) |
| Synthetic Indigo (C.I.73000) | 482-89-3 | ThermoFisher GmbH (Kandel, Germany) |

Hydrochloric acid and sodium hydroxide (NaOH) aqueous dilution to 10% (w/v) were freshly prepared in the laboratory. Non-commercial liquid processed fermentation product of fungal origin with a $\beta$-D-glucosidase activity between 5 and 10% supplied by AB Enzymes GmbH (Darmstadt, Germany) was used.

Coloration with InCL was carried out by adding 5% (w/v) plant powder into 50 mL of de-ionized water and stirring it with the strand for 30 min following the protocol of Sargsyan et al. [105]. The natural pH of this suspension was 6.8.

Moreover, 1% (w/v) cysteine was added to the dyeing suspension with the same natural pH. Another coloring approach was the addition of 1% (w/v) isatin and cysteine to InCL suspension at RT or at 37 °C with a natural pH of 6.1 or 5.8, respectively. The addition of each 1% (w/v) isatin and ascorbic acid lead to a natural pH of InCL suspension of 2.7 at 37 °C. Coloration with synthetic indigo was carried out by adding various concentrations to 10 mL of ammonium thioglycolate, dissolved, 40 mL water added, pH adjusted with HCl to pH 3 and strand added for 30 min. Further, a coloration approach was adding indican at different concentrations and 50 µL $\beta$-D-glucosidase to 50 mL water, including a strand and color according to the known protocol.

After dyeing, the strands were rinsed with tap water at RT with a total tap water flow of 25 mL/s and combed until the dyeing mixture had been removed. Subsequently, the strands were blow dried with a commercially available blow-dryer at approx. 50 °C. Strands are evaluated freshly after coloration and 14 days thereafter. In between, they were maintained at RT, without shielding from UV radiation and without any intermediate washing.

Same applied to dyeing of textiles cotton, viscose, silk, hemp and linen provided by Anita Pavani Stoffe OHG (Heuchelheim, Germany). 0.7 g of each substrate was added to a suspension of 5% (w/v) InCL in 50 mL de-ionized water, stirred for 30 min at RT and rinsed under tap water with a flow of 25 mL/s. The textiles were blow-dried an color evaluated on day 0 and day 14 at RT without shielding from UV radiation or any intermediate washing in between.

#### 5.2.1.3 Wash Fastness Tests of Colored Strands

The wash stability test included a manual wash of the freshly InCL dyed strand with a conventional shampoo (Schauma 7-herbal shampoo, Schwarzkopf, pH 4.5 ± 0.2, 12 ± 0.5% (v/v) SLES). For this purpose, 0.5 g shampoo was used per wash. Each washing process consisted of the 30-second pre-cleaning of the test strand under running deionized water (V = 40 ± 20 ml/s, T = approx. 20 °C), manual incorporation of the shampoo ten times in circular movements (from the brush body to the tip of the hair) with subsequent rinsing and combing. This washing process was repeated five times to recreate six washes. Afterwards, the strands were blow dried and color fastness evaluated. The wash cycle is repetated five times to imitate a total of 30 washes. After each wash cycle comprising six washes, color is assessed (cf. figure 5.1). [102]

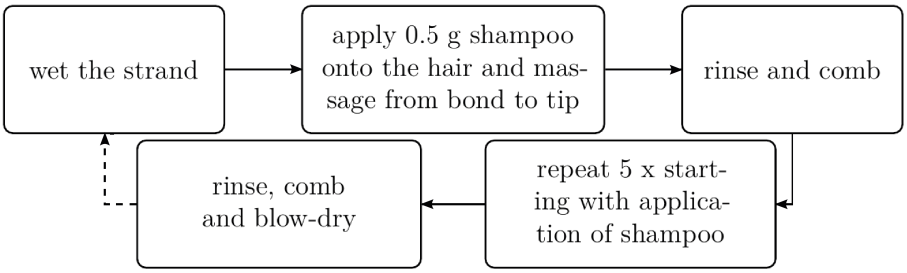



**Figure 5.1: Procedure of the Wash Fastness Test, which Comprises Five Successive Steps.** After wetting of the strands, the next two steps are carried out six times each. This is followed by final rinsing, combing and blow-drying. The cycle is carried out a total of five times to recreate 30 hair washes. The color is evaluated after every six washes (one cycle). Adapted from Hachmann *et al.* (2025). [106].

The wash resistance of the hair dye can then be classified as temporary, semi-permanent, demi-permanent or permanent, depending on the measured color removal. [10, 39]

### 5.2.2 Measurement Method for Assessment of Hair Colors

The color of the hair strands was recorded using images thereof, among other methods. To do this, the strands are placed on a white background and scanned using a flatbed A4 scanner (Canon, Tokyo, Japan). The recordings were taken directly after strand treatments and repeated 14 day later.

To quantify the color change values, color intensities, color directions and washing stabilities of the dyed hair strands are to be measured using a spectrophotometer called Spectraflash SF600X (Datacolor, Risch, Switzerland). The range of the electromagnetic spectrum visible to humans extends from 380 nm to 720 nm. The chromatic color ultimately perceived by the optic nerve in the brain depends on the different degrees of absorbed and reflected light. Spectrophotometric measurements can be used to measure absorption behavior, which can be used to draw conclusions about colors. The three-dimensional color space CIELAB (Commission Internationale de l'Éclairage L*a*b*) is shown in figure 5.2, whose coordinate system corresponds to a color model, is shown in the following figure [102].

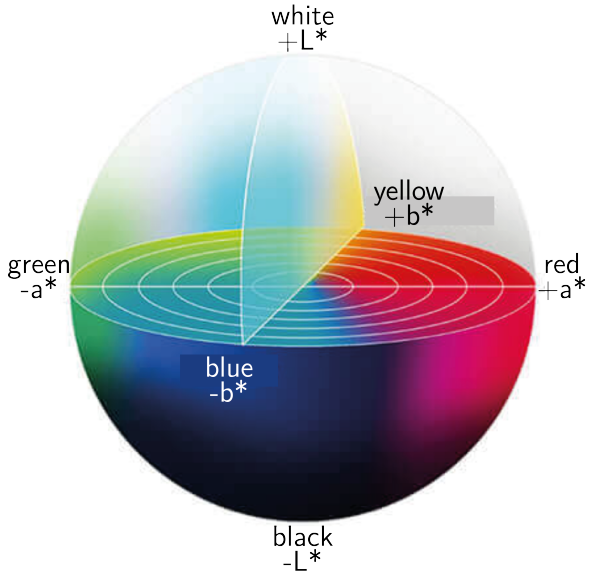



**Figure 5.2: CIELAB Color Space.** Three-dimensional color coordinate system with L*, a* and b* axis. Adapted from Agudo *et al.* (2014). [107].

The color definition is based on three axes. The L* axis defines the brightness from 0 (black) to 100 (white), the a* axis gives a value from -100 (green) to +100 (red) and the b* axis from -100 (blue) to +100 (yellow). All axes are orthogonal to each other. The L*a*b* values can be used to define any perceptible color. According to DIN 6174 (2007), the differences in absolute colors can be defined as ΔE and is determined using equation 5.1. [108]

$$\Delta E = \sqrt{((\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2)} \tag{5.1}$$

A $\Delta$E value below 1 is not visible by human color perception. Upon closer inspection, values of 1-2 are visible, whereby the range from 2-10 can be perceived more accurately. From 11 to 49, the colors compared are more similar than different. Above that, they tend to be more different, up to a value of 100 when the colors are opposites. [109]

For application in this work, measurements were taken after the respective color treatment and repeated 14 days later. For the spectrophotometer measurements, a D65 illuminant and an 8° diffuse optical configuration were used. The spectral reflectance data for each sample from 380 nm to 700 nm were converted to colorimetric data using DCI Color software. The reflectance measurements were performed four times for each strand, each time rotating the strand by 90°, and the mean value of the measurements was automatically calculated by the software. However, it was recommended to evaluate color of hair strands not only ba L*a*b* values but also through visual assessment. [110]

### 5.2.3 Hair Microtome Cuts for Microscopy and Determination of Color Penetration Depth

The penetration depth of the dye inside the hair fiber can be determined by microtome cuts of the colored yak belly hair fibres carried out with following tools (cf. table 5.2).

**Table 5.2: Overview of the Tools Used for Microtome Cuts.**

| Designation | Specification | Manufacturer |
|---|---|---|
| Kryostat | Epredia CryoStar NX70 with Vakutom | ThermoFisher GmbH (Kandel, Germany) |
| Tissue Freezing Medium | Embedding Medium | Leica Biosystems (Deer Park, USA) |
| Light Microscope | Olympus DP71 Digital Microscope | Olympus Europa SE & Co. KG (Hamburg, Germany) |

Cuts were carried out at Henkel AG & Co KGaA site (Swenja Kalischke, Scalp Tissue Engineering, Düsseldorf, Germany) using a kryostat. Hair preparation was done by embedding fibers in Tissue Freezing Medium and freezing to -50 °C. Afterwards, the frozen hair fibers were cut at -16 °C and a knife temperature of -23 °C to a

thickness of 3 µm. The cuts were then put on a microscope slide and fixed with a cover glass. To measure the penetration depth, the microscope slides were placed under a microscope for measurements.

Relative penetration depth was calculated by relating the penetration of hair dye to the diameter of the corresponding hair (cf. eq. 5.2). It is defined as:

$$Relative\,penetration\,depth\,(\%) = \frac{abs.\,penetration\,depth\,(\mu\mathrm{m})}{hair\,radius\,(\mu\mathrm{m})} x100 \qquad (5.2)$$

Since the hair diameter is not perfectly spherical shape, the relative penetration depth is determined with the mean values of the smallest and largest radius of the fiber. [106]

## 5.3 Plant Material Extraction Methods

The following describes two extraction methods that were used to extract *Indigofera tinctoria* L..

### 5.3.1 Soxhlet Extraction of Plant Material

Soxhlet extraction is a process for the continuous extraction of substances from solids, whereby the solvent is used in a cycle of evaporation, condensation and reflux. In the process, the sample is repeatedly in contact with fresh solvent, because of continuous recycling and circulation within the system. Thermally unstable substances can also be extracted, as the temperature needed for extraction set up is relatively low due to the pressure applied. The efficient extraction of soluble components from a solid matrix is therefore enabled. [111] The main goal in the context of this application of Soxhlet extraction is to efficiently dissolve and extract ethanol-soluble components from InCL (Kremer Pigmente GmbH).

**Table 5.3: Overview of the Substances Used for Soxhlet Extraction.**

| Designation | CAS Number | Manufacturer |
|---|---|---|
| Pure Ethanol | 64-17-5 | Fisher Scientific GmbH (Schwerte, Germany) |
| Technical Ethanol | 64-17-5 | Own filling, exchange container |
| Cooling Liquid | Water-Glycol-Mixture, Kryo 30 | LAUDA GmbH (Lauda-Königshofen, Germany) |

**Table 5.4: Overview of the Materials Used for Soxhlet Extraction.**

| Designation | Specification | Manufacturer |
|---|---|---|
| Soxhlet Extractor | 250 mL DURAN® Borosilicate Glass | Lenz Laborglas GmbH & Co.KG (Wertheim, Germany) |
| Tea Bag Filter | Holderless | REWE eG (Köln, Germany) |
| Extraction Sleeve | 250 mL DURAN® Borosilicate Glass | Lenz Laborglas GmbH & Co.KG (Wertheim, Germany) |
| Solvent Flask | 500 mL DURAN® Borosilicate Glass | Lenz Laborglas GmbH & Co.KG (Wertheim, Germany) |
| Condenser | Double-Walled Coil Capacitor | Lenz Laborglas GmbH & Co.KG (Wertheim, Germany) |
| Vacuum Pump | Hei-VAC Vario Station | Heidolph Instruments GmbH & Co. KG (Schwabach, Germany) |
| Rotary Evaporator | Hei-VAP Core | Heidolph Instruments GmbH & Co. KG (Schwabach, Germany) |

The Soxhlet extraction set up contains a Soxhlet extractor itself in which the sample of 5 g InCL in a tea bag filter to prevent fine distribution of the plant material is placed inside an extraction sleeve. Underneath, there is the solvent flask with 250 mL either technical or pure ethanol (EtOH) which is in contact with a water bath at 60 °C. A condenser is located at the top of the extractor and filled with cooling liquid which circulates at -5 °C. The EtOH in the flask is heated in the water bath, evaporates early through applied pressure of approx. 180 mbar by vacuum pump and rises upwards in the ascending arm of the extractor. At the top of the extractor, the vapor condenses and the solvent drops into the upper part surrounding the extraction tube and flows through the sample material (cf. figure 5.3).

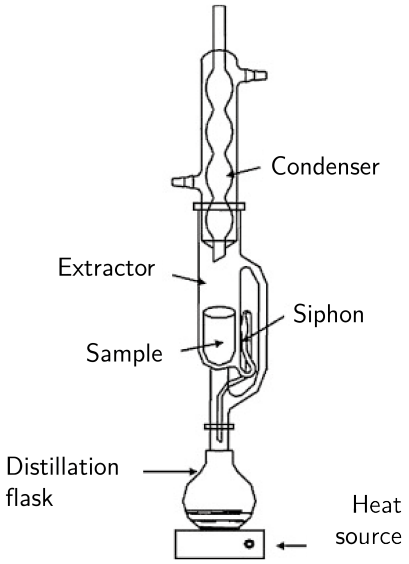



**Figure 5.3: Schematic Structure of a Soxhlet Extraction.** Adapted from Luque de Castro *et al.* (2010). [111]

The extractor is designed in such a way that it constricts the upper part of the extractor so that the solution can circulate in the lower part until the siphon level is reached and the solvent inside the extractor empties into the solvent flask. Thus, the accumulated solvent returns to the solvent flask at regular intervals, starting the cycle all over again. This design ensures that the solvent continuously wets the sample material in condensation and overflow cycles, maximizing extraction efficiency. The system remains closed and is carried out without direct manipulation of the solvent. Due to the high pressure in the system, the boiling point of solvents used is lowered the time required and solvent consumption are reduced. [111]
After a sufficient amount of Soxhlet cycles and after removal of the solvent, Indigo Ethanolic Extract (InEE) and Indigo Ethanolic Extraction Residue (InEER) were produced. Removal of solvent from indigo ethanolic extract was done by a rotary evaporator system including a vacuum pump at 60 °C water bath and approx. 180 mbar pressure until completely dry. InEER was dried under fume hood for 12 hours until fully dried.

### 5.3.2 Liquid-Liquid Extraction of Plant Material

For investigations of the ethanolic Indigo extract, an separation funnel extraction was performed. The aim was to selectively transfer components from the extract into

different liquid phases to separate them from interfering substances. The fact that components dissolve differently in two immiscible liquids (e.g. water and an organic solvent) was exploited. A 500 mL separating funnel (Lenz Laborglas GmbH & Co. KG, Wertheim, Germany) was used. The dry InEE with a concentration of 2% (w/v) was resuspended in 100 mL de-ionized water, and mixed with 80 mL petroleum ether (PE, 50-70, own bottling). Phase seperation was improved through addition of sodium chloride (NaCl, Carl Roth GmbH & Co. KG, Karlsruhe, Germany). Through three shaking cycles, in which PE was repeatedly removed and 50 mL fresh PE was added twice, nonpolar or weakly polar substances should dissolve in this nonpolar solvent. From InEE, among others, fats, oils and chlorophylls were expected to move into the PE phase. The polar indican should remain in the aqueous phase. The phases can then be checked for the desired substances using thin layer chromatography and colored after addition of 50 µL $\beta$-glucosidase (cf. chapter 6.6).

## 5.4 Chromatography

Thin layer chromatography (TLC), column chromatography (CC) and high performance liquid chromatography (HPLC) applied in this work are described below. Different chemicals and material were used for chromatographies (cf. tables 5.5 and 5.6).

Further, non-commercial liquid processed fermentation product of fungal origin with a $\beta$-D-glucosidase activity between 5 and 10% supplied by AB Enzymes GmbH (Darmstadt, Germany) was used.

**Table 5.5: Overview of the Substances Used for Chromatographies.**

| **Designation** | **CAS Number** | **Manufacturer** |
|---|---|---|
| Pure Ethanol | 64-17-5 | Fisher Scientific GmbH (Schwerte, Germany) |
| EtOAc | 141-78-6 | VWR International (Radnor, USA) |
| Synthetic Indigo (C.I.73000; 94%) | 482-89-3 | ThermoFisher GmbH (Kandel, Germany) |
| Silica Gel 60 | 7631-86-9 | Merck KGaA (Darmstadt, Germany) |
| Sea Sand | 14808-60-7 | Carl Roth GmbH + Co. KG (Karlsruhe, Germany) |
| Indirubin (98%) | 479-41-4 | Cayman Chemical (Ann Arbor, USA) |
| Synthetic Indican | 487-60-5 | ThermoFisher GmbH (Kandel, Germany) |
| Isatin (98%) | 91-56-5 | ThermoFisher GmbH (Kandel, Germany) |
| Acetonitrile (ACN) HPLC Grade | 75-05-8 | CARLO ERBA Reagents GmbH (Emmendingen, Germany) |
| Dimethyl Sulfoxide (DMSO) HPLC Grade | 67-68-5 | ThermoFisher GmbH (Kandel, Germany) |
| Formic Acid (FA) HPLC Grade | 64-18-6 | Merck KGaA (Darmstadt, Germany) |

**Table 5.6: Overview of the Materials Used for Chromatographies.**

| Designation | Specification | Manufacturer |
|---|---|---|
| TLC Plate | Silica Gel 60 F254 | Merck KGaA (Darmstadt, Germany) |
| UV Lamp | Wavelengths: 254 & 365 nm | VILBER LOURMAT GmbH (Eberhardzell, Germany) |
| Pump LABOPORT | Mini-Membran-Vakuumpumpe N 96 | KNF DAC GmbH (Hamburg, Germany) |
| Absorbent Cotton | Cotton | Paul Hartmann AG (Heidenheim, Germany) |
| Water Purification System | Arium Pro Ultrapure Water System | Sartorius AG (Göttingen, Germany) |
| Analytical HPLC | AZURA® | Knauer GmbH (Berlin, Germany) |
| Autosampler | AS 6.1 L | Knauer GmbH (Berlin, Germany) |
| RP Analytical Column | Eurospher II 100-5 C18, 250 x 4.6 mm, 5 µm, 100 Å | Knauer GmbH (Berlin, Germany) |
| Diode Array Detector | AZURA® DAD 2.1 L | Knauer GmbH (Berlin, Germany) |
| Thermostat | AZURA® CT 2.1 | Knauer GmbH (Berlin, Germany) |
| Chromatography Data System | ClarityChrom®9 | Knauer GmbH (Berlin, Germany) |
| Ball Mill | Pulverisette 23 | Fritsch GmbH (Idar-Oberstein, Germany) |
| Grinding Bowl | Zirconium Oxide, 30 mL | Fritsch GmbH (Idar-Oberstein, Germany) |
| Grinding Balls | Zirconium Oxide, 15 mm | Fritsch GmbH (Idar-Oberstein, Germany) |
| Thermal Shaker | ThermoMixer® C | Eppendorf SE (Hamburg, Germany) |
| Centrifuge | 5425 | Eppendorf SE (Hamburg, Germany) |
| Rotor | FA-24x2 | Eppendorf SE (Hamburg, Germany) |
| Spice Mill | EGK 200, 200W | Rommelsbacher GmbH (Dinkelsbühl, Germany) |

### 5.4.1 Thin Layer Chromatography

Compounds should be distributed on the stationary phase through a liquid mobile phase and thus individual identification should be enabled. 10 µL of each sample was applied on silica plate and left to run in the developing chamber with a filling level of ethyl acetate (EtOAc) below the application point of the samples on the plate. The mobile phase is considered to move up the plate due to capillary action. Because of the different interactions of the analytes with the silica plate and different levels of solubility in the running solvent, separation is achieved along the running track of the respective sample. The running could be stopped by removing the plate from the chamber, whereby the running front must be drawn in directly. The less polar compound is considered to move higher up the plate which results in higher $R_f$ values calculated as follows (cf. eq. 5.3).

$R_f$ values were calculated with

$$R_f = \frac{A}{B} \tag{5.3}$$

With

A = path length of the solvent from the start line to the center of the stain
B = path length of the solvent from the starting line to the solvent front [112].

Since not all substances are colored and can therefore be detected directly, there is the possibility of staining the plate on the one hand and irradiate with UV-light at 245 and 316 nm for fluorescent substances on the other hand. Staining method used was moisten the plate with a 1.2% (v/v) $\beta$-D-glucosidase solution to check whether indigo precursors form the typical color.

### 5.4.2 Column Chromatography

Column chromatography was conducted to isolated the dye precursor indican from InCL. Findings from the TLC concerning running behavior could be used for preparation of the the CC. However, for CC, silica gel was used which results in same separation behavior of samples compared to TLC.

A cylinder shaped glass column with the dimensions of 30 x 3 cm amounting to a volume of 635.85 L was packed with first a small piece of absorbent cotton, then a layer of sea sand, subsequently EtOAc pre-moistened silica, afterwards a dried mixture of sample and silica and finally a layer of sea sand again to prevent stirring up of the sample. EtOAc was added as a running solvent which flows from top to bottom of the column. It has been ensured that there is always solvent available so that the column did not run dry. A pump was connected to the system to pump the solvent through the column using air pressure (cf. figure 5.4).

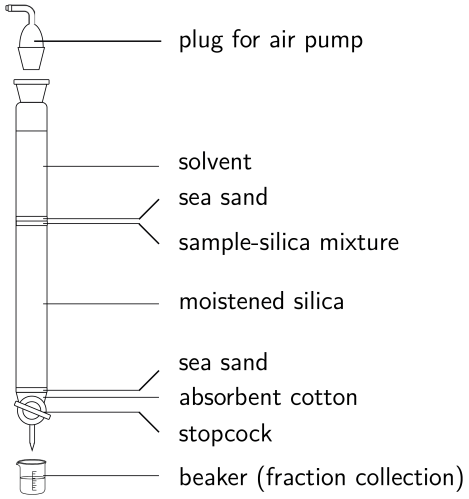



**Figure 5.4: Schematic Structure of a Column Chromatography.** Adapted from Bajpai *et al.* (2016). [113]

The compounds in the applied sample, similar to TLC, have different interaction abilities with the stationary and the mobile phase and therefore run along with the mobile phase at different times. [113] In parallel, after one column volume has run through, fractions were collected in beakers underneath the column. Each fraction was analyzed by TLC for detectable ingredients, in particular indican by staining with a 1.2% (v/v) $\beta$-D-glucosidase solution (cf. Thin Layer Chromatography 6.6). Fractions that appeared blue after staining were combined, solvent evaporated with rotary evaporator set up and analyzed with Nuclear Magnetic Resonance (NMR, cf. chapter 5.5).

### 5.4.3 High Performance Liquid Chromatography

A stock solution of 100 mg/L of standard substances indican and isatin was prepared in a mixture of ultra pure water (UPW) and acetonitrile (ACN) (volume ratio 55:45), synthetic indigo and indirubin were prepared in dimethyl sulfoxide (DMSO). Dilutions of 20, 15, 10, 7,5, 6, 5, 3, 2,5, 1,5 1 and 0,5 mg/L with respective solvents were analysed via HPLC system using ACN (solvent A) and UPW with 0.1% formic acid (solvent B) gradually (flow rate 0.6 mL/min) (cf. table 5.7).

**Table 5.7: Solvent Gradient of HPLC Method.**

| **Time** [min] | **Solvent A** [%] | **Solvent B** [%] |
|---|---|---|
| 0-1 | 5 | 95 |
| 1-20 | 40 | 60 |
| 20-25 | 100 | 0 |
| 25-32 | 100 | 0 |
| 32-35 | 100 | 0 |
| 35-40 | 5 | 95 |

Column thermostat was heated to 40 °C. The injection volume for all standards was 100 µL. Compounds were detected by diode array detector (DAD) at wavelengths of 240 and 300 nm. Quantification was carried out by the area below peaks at 240 nm for indican, isatin and indirubin and at 300 nm for indigo molecule. Collected data were analysed with ClarityChrom® 9 (Knauer GmbH, Berlin, Germany).

#### 5.4.3.1 HPLC Method Validation

To calculate the concentrations of indigo molecule, indirubin, indican and isatin in µg/mL, the response of the corresponding peak areas in mAU · s were divided by their slope ($S_{PA}$) in mAU · s µ g/mL resulting in $R_{IndigoPA}$, $R_{IndirubinPA}$, $R_{IndicanPA}$ and $R_{IsatinPA}$ as the response molecule peak area. The coefficient for the calculations ($r^2$) as well as slopes ($S_{PA}$) are listed below (cf. table 5.8) [65, 114, 114, 115].

**Table 5.8: Slope ($S_{PA}$) in mAU · s µ g/mL and Coefficient for the Calculations ($r^2$) of $R_{IndigoPA}$, $R_{IndirubinPA}$, $R_{IndicanPA}$ and $R_{IsatinPA}$.**

| **Molecule** | **Slope**($S_{PA}$) [mAU · s µg/mL] | **Coefficient**($r^2$) |
|---|---|---|
| Indigo molecule | 892.60313 | 0.9985322 |
| Indirubin | 601.64354 | 0.9999246 |
| Indican | 27.94484 | 0.9987543 |
| Isatin | 1761.99746 | 0.999503 |

These values are used in the equations below to calculate response peak areas (cf. eq. 5.4, 5.5, 5.6 and 5.7).

$$R_{IndigoPA}\ (mAU{\cdot}s) = 892.60313\ (mAU{\cdot}s\ \mu\text{g/mL}) \times conc_{Indigo}\ (\mu\text{g/mL}),\ r^2 = 0.9985322 \tag{5.4}$$

$$R_{IndirubinPA}\ (mAU{\cdot}s) = 601.64354\ (mAU{\cdot}s\ \mu\text{g/mL}) \times conc_{Indirubin}\ (\mu\text{g/mL}),\ r^2 = 0.9999246 \tag{5.5}$$

$$R_{IndicanPA}\ (mAU{\cdot}s) = 27.94484\ (mAU{\cdot}s\ \mu\text{g/mL}) \times conc_{Indican}\ (\mu\text{g/mL}),\ r^2 = 0.9987543 \tag{5.6}$$

$$R_{IsatinPA}\ (mAU{\cdot}s) = 1761.99746\ (mAU{\cdot}s\ \mu\text{g/mL}) \times conc_{Insatin}\ (\mu\text{g/mL}),\ r^2 = 0.999503 \tag{5.7}$$

The linearity of the standard substance calibration curves was evaluated over the concentration range of 0.5 to 20 mg/L plotting the data and performing linear

regression analysis. A linear response was confirmed as described in eq. 5.4, 5.5, 5.6 as well as 5.7.

Using the calibration curves based on peak heights as the response variable ($R_{XPH}$ = response molecule peak height in mAU and $conc_X$ = molecule concentration in mg/L), the Limit of Detection (LOD) was calculated by multiplying the SD ($\sigma$) by 3.3 and dividing by the peak height slopes ($S_{PH}$) of the calibration curves. Similarly, the Limit of Qantification (LOQ) was calculated by multiplying the SD by 10 and dividing by the same peak height slopes. The coefficients of determinations ($r^2$) for this regression were determined, indicating excellent linearity of the method (cf. table 5.9).

**Table 5.9: Slope ($S_{PH}$) in mAU · s µ g/mL and Coefficient for the Calculations ($r^2$) of $R_{IndigoPA}$, $R_{IndirubinPA}$, $R_{IndicanPA}$ and $R_{IsatinPA}$.**

| **Molecule** | **Slope**($S_{PH}$) [mAU · s mg/L] | **Coefficient**($r^2$) |
|---|---|---|
| Indigo molecule | 58.58885 | 0.9999321 |
| Indirubin | 72.35949 | 0.9999246 |
| Indican | 2.79841 | 0.9990136 |
| Isatin | 63.537 | 0.9988613 |

LOD and LOQ in accordance with ICH guideline Q2(R2) (eq. 5.8 and 5.9) [116] were calculated (cf. eq. 5.8 and 5.9).

$$LOD = 3.3 \times \sigma \times {S_{\mathrm{PH}}}^{-1} \tag{5.8}$$

$$LOQ = 10 \times \sigma \times {S_{\mathrm{PH}}}^{-1} \tag{5.9}$$

The SD ($\sigma$) of the response at 300 nm for indigo molecule and 240 nm for indirubin, calculated from solvent blanks DMSO (n = 3) between minutes 28.5 and 29.7 (indigo molecule) as well as minutes 28.875 and 29.45 nm (indirubin) are listed below. Further, the SD ($\sigma$) of the response at 240 nm for indican and isatin, calculated from

solvent blanks UPW/ACN (55:45) (n = 3) between minutes 14.8 and 15.7 (indican) as well as minutes 18 and 19.7 nm (isatin) are shown (cf. table 5.10).

**Table 5.10: SD ($\sigma$) of the Responses Calculated from Solvent Blanks UPW/ACN (55:45) and DMSO at Elution Times of Indigo Molecule, Indirubin, Indican and Isatin.**

| **Molecule** | **Solvent** | **Wavelength** [nm] | **SD** ($\sigma$) |
|---|---|---|---|
| Indigo molecule | DMSO | 300 | 1.270810567 |
| Indirubin | DMSO | 240 | 3.19132647 |
| Indican | UPW/ACN | 240 | 0.269382987 |
| Isatin | UPW/ACN | 240 | 0.340728593 |

The above shown data resulted in the HPLC method validation parameters LOD and LOQ for standard quantification with the linear range between 20, 15, 10, 7,5, 6, 5, 3, 2,5, 1,5 1 and 0,5 mg/L (cf. table 5.11).

**Table 5.11: HPLC Method Validation Parameters for Indican, Isatin, Indigo Molecule and Indirubin. Range of Linearity (mg/L), LOD (mg/L) and LOQ (mg/L).**

| **Molecule** | **Range of Linearity** [mg/L] | **LOD** [mg/L] | **LOQ** [mg/L] |
|---|---|---|---|
| Indigo molecule | 0.5-20 | 0.07157804 | 0.21690314 |
| Indirubin | 0.5-20 | 0.145542449 | 0.441037724 |
| Indican | 1-20 | 0.317667481 | 0.962628731 |
| Isatin | 0.5-20 | 0.017696844 | 0.053626799 |

#### 5.4.3.2 Processing of Hair Strands for HPLC Analysis

Examinations of colored hair strands analogously to Mantzouris *et al.* (2014) differently treated strands were stirred in 50 mL UPW/strand for 60 min, whereby the water was changed twice in total. Further, dried strands were ground with the help of a ball mill which was equipped with a zirconium oxide grinding bowl and

three balls. The runtime was 2 x 5 min with a cool-down time of 2-3 min in between and a frequency of 50/s. [117] Parallel to that, strands treated in the same way but without stirring in water were left for 14 days so that the color development could be examined without being influenced during this time.

8 mg of powdered every strand to be analyzed was mixed with 2 mL of either UPW/ACN or DMSO in a tube and subsequently inserted into a thermal shaker at 80 °C and 1000 rpm for 30 min. The samples were then transferred into a centrifuge for 20 min at RT and 21300 x g [75]. The supernatant was used for HPLC analysis [117]. The quantification of indigo molecule and indirubin from DMSO, as well as indican and isatin from UPW/ACN, was carried out with injection of 100 µL into the HPLC by autosampler. Method as described above (cf. chapter 5.4.3.1) was applied. The chromatographic peaks were identified and quantified by the retention time and integrals of standards. The HPLC data were converted from mg/L into µg/g hair strand so that every result could be evaluated in the context of hair coloration as well as compared with each other. The experiments for molecule determination with HPLC were carried out five times.

#### 5.4.3.3 Processing of *Indigofera tinctoria* L. Samples for HPLC Analysis

Preparation of *Indigofera tinctoria* L. samples for quantification of indigo molecule, indirubin, indican and isatin was carried out analogous to processing of the hair strands (cf. chapter 5.4.3.2) with minor deviations. The plant material was ground with a commercially available spice mill before continuing with the same treatment of samples as for hair analyses. Each experiment for molecule determination with HPLC was carried out three times.

The data from the HPLC analyses of the *Indigofera tinctoria* L. samples were converted from mg/L into mg/g plant material sample in order to ensure the best possible comparability of the results.

## 5.5 Nuclear Magnetic Resonance Analysis

Nuclear Magnetic Resonance (NMR) applied in this work was used to verify the molecular structure of isolated compounds from column chromatography. Measure-

ments were carried out with a device from University Hamburg, department of chemistry (AK Hackl). The aim was to verify and identify the isolated compound after purification using CC with $_1$H-NMR.

The device used was a Fourier 300 NMR spectrometer operated with TopSpin® software (Bruker Corporation, Billerica, USA). The measurement frequency was 300.13 MHz (7.05 Tesla). Used solvent was deuterated acetone (acetone-$d_6$ ($CD_3COCD_3$), Merck KGaA (Darmstadt, Germany). The measurements were conducted with a concentration of 10 mg/mL at RT. It had a 5 mm BroadBand Fluorine-Optimized probe (BBFO) optimized for, among others, $^1$H nuclei with automatic tuning and matching (ATM) and a integrated magnetic field gradient in z-direction (z-gradient). To evaluate the spectra, MestReNova 15.0.1 (Mestre-Lab Research, Santiago de Compostela, Spain) was used for comparison with the literature.

## 5.6 Use of Artificial Intelligence in the Work Process

The artifical intelligence (AI) models ChatGPT 4.0 (OpenAI, San Francisco, USA), Microsoft 365 Copilot (Microsoft Corporation, Redmond, USA), Perplexity AI (Perplexity AI Inc., San Francisco, USA) and ChatPDF (ChatPDF GmbH, Laboe, Germany) were used in the preparation of this dissertation. Their use was limited to brainstorming ideas for structuring the outline, optimizing individual formulations with regard to readability, identifying relevant literature, and addressing specific formatting issues.

The experiments and evaluation were carried out entirely without the help of AI. The final outline and content of the thesis were developed independently. The efficiency of the literature research was increased by AI support, although all sections were validated through manual review. Human evaluation remained essential, particularly regarding the methodological quality, citability, and relevance of the sources used. The aim was not to replace human data processing or interpretation, but to supplement it responsibly.

# 6 Results

The following chapter contains the presentation of the results. It deals with the investigation of the coloring properties of *Indigofera tinctoria* L. crushed leaves (InCL) on hair. In addition, the development of extracts from InCL is examined and the influence of the harvesting and processing of the plant material on its composition with regard to hair coloration is determined. Finally, a proposal for the color formation pathway from *Indigofera tinctoria* L. is made through induction.

## 6.1 Investigation of the Dyeing Properties of Components from *Indigofera tinctoria* L.

To evaluate the fundamental coloration behavior of InCL on hair, strands were treated with various concentrations in water for 30 min at RT according to the standard protocol (cf. figure 6.1). This aimed to assess both the initial coloration result and its stability under ambient conditions. The natural pH of 5% (w/v) InCL suspension was 6.8. The visual assessment of hair strands colored with different concentrations of InCL (cf. figure 6.1) was supplemented by a quantitative color analysis in the CIELAB color space. MV of measured CIELAB values as well as their SD are displayed. The resulting $\Delta$E and $\Delta$a* values comparing colorations of a strand on day 0 and day 14 are shown in a function of the InCL concentration below (cf. figure 6.2).

Initially, the color on strands treated with InCL at each concentration were shown in a blue-turquoise tone. They all differed from the untreated strand by $\Delta E_1$ between 23.4 to 36.6 freshly after application. The lowest $\Delta E_1$ value and highest L* and a* values were measured at 0.5% (w/v) InCL. The $\Delta E_1$ increased and the L* and a* values decreased towards 3%, 4% and 5% (w/v). However, the coloration was not linear to the application concentration, as the 3% strand showed the lowest L* value and highest $\Delta E_1$ value of all strands on day 0. The lowest a* value, on the other hand, was measured for the 5% InCL strand. The reduction of the L* and a* values indicated the color becoming darker redder color among increasing InCL concentrations. At the same time, the b* value, which depicts the blue to

yellow axis in the L*a*b*-color space, changed overall in a range from |4.5| (between b* = -7.5 to -3.0).

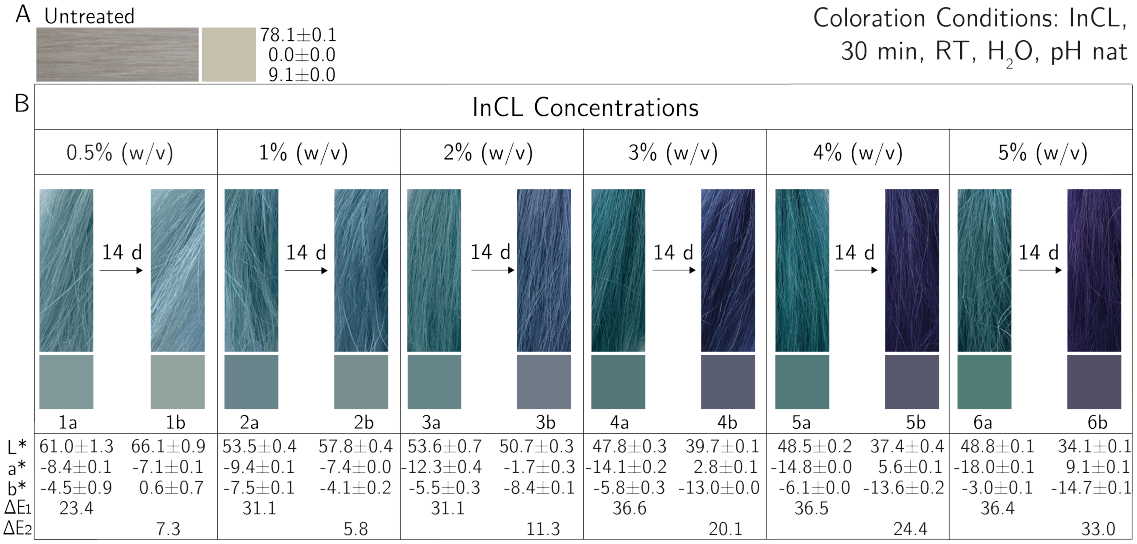



**Figure 6.1: Hair Colorations with InCL at Different Concentrations.** A: Untreated strand. B: Colored strands. Dried and crushed plant leaves added to water at concentrations from 0.5% (w/v) to 5% (w/v). Strand was added in dye suspension for 30 min at RT. Color assessment took place directly after application and freshly dyed and 14 days after that. $\Delta E_1$ represents the color difference between the freshly treated strand and the untreated reference strand (day 0). $\Delta E_2$ indicates the color change of the treated strand after 14 days, relative to its initial color on the respective day 0.

It was found that from of a concentration of 2% (w/v) of InCL, a color change of the strands from initial blue to purple took place within 14 days. Throughout this time, the colored strands were stored at RT, without UV protection or intermediate washing. The change was lowest at a concentration of 0.5% (w/v) InCL in water ($\Delta E_2 = 7.3$) or 1% (w/v) InCL in water ($\Delta E_2 = 5.8$). Concentration of InCL and $\Delta E_2$ are positively correlated. At the concentration of 5% (w/v) InCL, the $\Delta E_2$ value is highest at 33.0. There was a strikingly large increase in the difference between a* values (by 430%) of the concentrations of 1% and 2% (w/v) within 14 days ($\Delta$a* values of 1%: 2.0 and of 2%: 10.6). The increase in the a* values was also evident at the higher InCL concentrations (3%, 4% and 5%). These values showed that the color change of a dyed strand within 14 days from a concentration of 2% InCL onward was clearly into the red direction of the red-green axis.

This development can also be seen in figure 6.2. The $\Delta E_2$ values increased with increasing InCL concentration and reached a maximum of 33.0 at 5% (w/v) InCL. At the same time, the a* value also increased, indicating a color shift towards red on the green-red axis of CIELAB color space. The increases in both values ($\Delta E_2$ and a*) ran almost parallel, which indicated the strong influence of the a* value on the $\Delta E_2$ value.

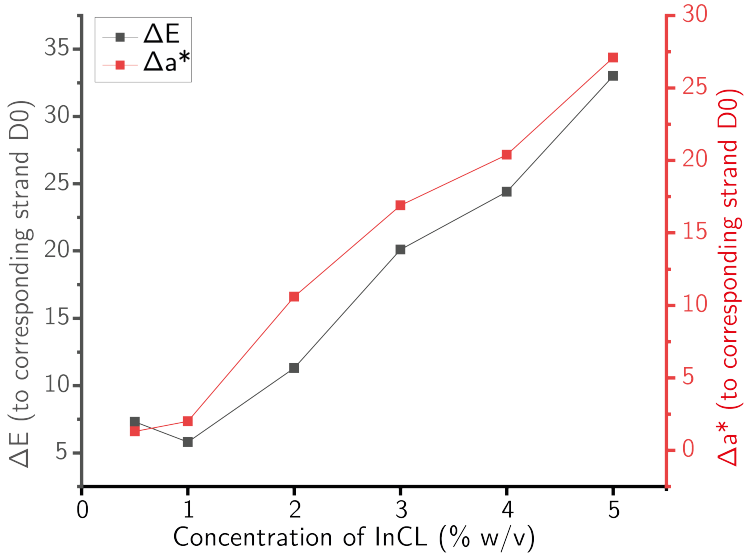



**Figure 6.2: Diagram of ΔE and Δa* Values of Hair Coloration at Different InCL Concentrations.** Color development (ΔE) and red shift (Δa*) of hair strands depending on the InCL amount used. Color assessment took place directly after application and freshly dyed and 14 days after that. Color change of the treated strand after 14 days, relative to the initial color on the respective day 0 is indicated.

After more than 14 days, the color does not change significantly. This strand shows an example of the color of the strand after one year without special intermediate treatments (cf. figure 6.3). The $\Delta E_3$ comparing the 5% (w/v) InCL colored strands after 14 days and after one year was 6.1. The strand measured after one year became slightly darker and more yellow. This, compared with the result between fresh dyeing and after 14 days as indicated by $\Delta E_2$ of 33.0, illustrated the relevance of the first 14 days after staining. Therefore, the color change of all strands was only included up to day 14 in the following.

The observations described above demonstrate that InCL-based dyeing exhibits a concentration-dependent color shift, with notable changes occurring within the first 14 days after application. Thereafter, the coloration remains largely stable. These findings provide opportunities and limitations for natural hair coloring with InCL, which will be explored further in the subsequent section.

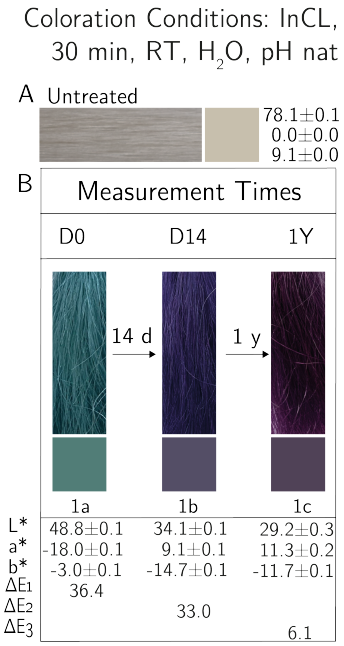



**Figure 6.3: Hair Coloration with 5% (w/v) *Indigofera tinctoria* L. One Year After Application.** A: Untreated strand. B: Colored strands. Color assessment of strands took place directly after application, 14 days after and one year after that.$\Delta E_1$ represents the color difference between the freshly treated strand and the untreated reference strand (day 0). $\Delta E_2$ indicates the color change of the treated strand after 14 days, relative to its initial color on the respective day 0. $\Delta E_3$ reflects the color difference between 14 days and one year after application.

### 6.1.1 Coloration with Synthetic Indigo on Hair

In order to get more insights into the coloration result and the associated color change after hair dyeing with InCL, dyeing with the synthetic indigo molecule was first examined in more detail. This is the main coloring component from the indigo plant and serves as a reference for subsequent plant-based experiments. It is a widely used raw material for dyeing textiles. The dyeing protocol is therefore inspired by the application technique used in the textile industry [118].

Indigo molecule can be purchased from various retailers with different purities. The central process for dyeing with synthetic indigo is vat dyeing, in which the indigo molecule is converted into a water-soluble white yellowish leuco-form by reduction, dyeing the fabric blue after oxidation in air. Sodium dithionite ($Na_2S_2O_4$) is the most used reducing agent in industrial and experimental indigo vat dyeing. [118] The coloring properties were tested in the laboratory on strands of hair as reference coloration for the subsequent experiments with the plant material. Determining the exact amount of indigo molecule product from the plant is dependend on both the origin and the extraction method used. There are several different approaches to produce and extract the molecule using different solvents, temperatures, pHs and extraction duration [119–122]. For the purposes of the performed colorations, quantities of produced indigo from various cultivation regions of *Indigofera tinctoria* L. extracted with water were used as the required quantity of the indigo for the coloration (cf. figure 6.4) [1]. Teangulum *et al.* (2012) found that average quantity of extracted indigo molecule from *Indigofera tinctoria* L. amounts to 105 mg per 100 g fresh leaves. The exact water content in the fresh indigo leaves used is unknown. On average, this is around 80% in fresh plant leaves [123]. Assuming that the average water content is appropriate for *Indigofera tinctoria* L. leaves, the indigo molecule content would therefore be 105 mg/20 g of dried leaves. The coloration introduced in chapter 6.1 was repeated once with equivalent amounts of indigo molecule. The indigo molecule quantities were calculated based on the coloring of a strand in 50 mL of water and therefore 2.5 g of InCL used for a 5% (w/v) InCL dyeing (other quantities analogously). Strands are evaluated freshly after coloration and 14 days thereafter (cf. figure 6.4). Due to more suitable application possibilities on the hair, ammonium thioglycolate was used as a reducing agent for these coloration experiments. The pH was adjusted to 3 an color assessment was carried out on day 0 and 14 days after application (cf. figure 6.4).

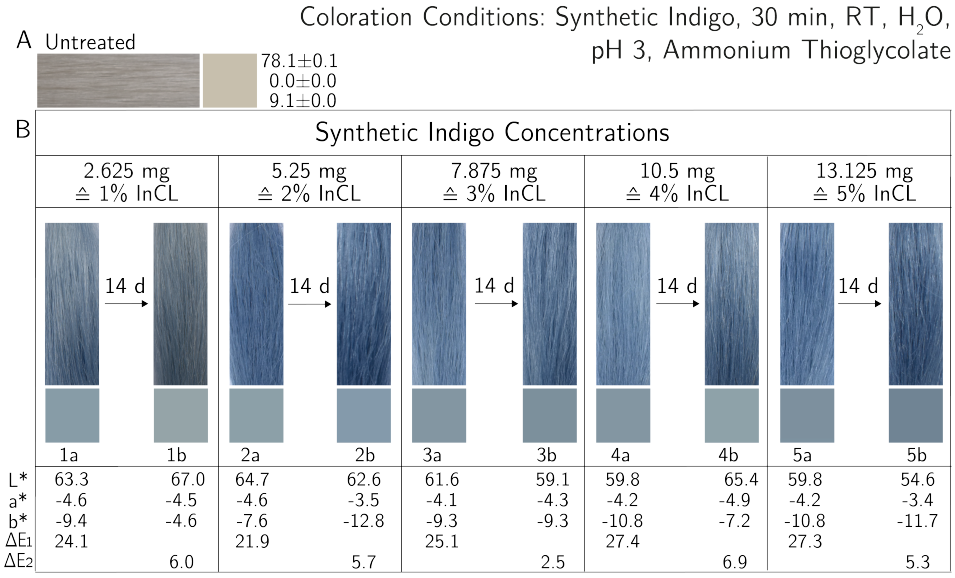



**Figure 6.4: Hair Colorations with Synthetic Indigo at Different Concentrations.** A: Untreated strand. B: Colored strands. Equivalent synthetic indigo amounts of concentrations from 1% (w/v) to 5% (w/v) InCL were dissolved in 5 mL Ammonium thioglycolate, 40 mL water were added, pH adjusted to 3 and strand was stirred for 30 min at RT. Color assessment took place directly after application and freshly dyed and 14 days after that. $\Delta E_1$ represents the color difference between the freshly treated strand and the untreated reference strand (day 0). $\Delta E_2$ indicates the color change of the treated strand after 14 days, relative to its initial color on the respective day 0.

The assessment of color after dyeing with synthetic indigo was done spectrophotometric, indicated with the CIELAB color space. The resulting $\Delta E_2$ and $\Delta a^*$ values comparing colorations of a strand on day 0 and day 14 are shown in a bar chart diagram of the different synthetic indigo concentration below (cf. figure 6.5).

The uneven distribution of color on the hair was striking. This could also be seen in the visible difference between the color tile and the strand as the color measurement took place on a small section of the strand. The greatest difference between strands on day 0 and day 14 after application shown in $\Delta E_2$ value amounted to 5.3 for the 5% (w/v) InCL equivalent. This is also reflected in the bar chart 6.5 which shows the hair color difference $\Delta E_2$ and $\Delta a^*$ between day 0 and day 14 as a function of the amount of synthetic indigo used. The influence of different quantities of synthetic indigo was not consistent – both $\Delta E_2$ and $\Delta a^*$ fluctuated. The color difference $\Delta E_2$ ranged from 2.5 to 6.9. At the same time, the $\Delta a^*$ value hardly changed.

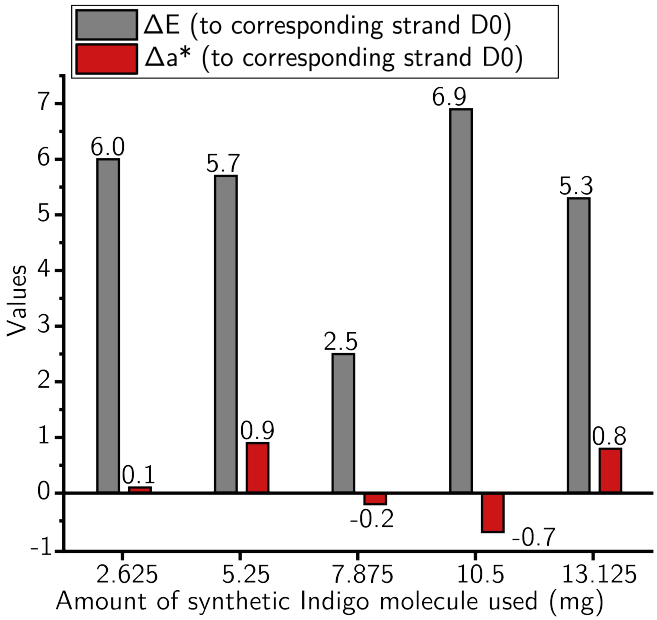



**Figure 6.5: Bar Chart of ΔE and Δa* Values of Hair Coloration at Different Synthetic Indigo Concentrations.** Color development (ΔE) and red shift (Δa*) of hair strands depending on the synthetic indigo amount used. Color assessment took place directly after application and freshly dyed and 14 days after that. Color change of the treated strand after 14 days, relative to the initial color on the respective day 0 is indicated.

Overall, the $\Delta E_2$ values did not increase analogously to the concentration of indigo molecule used. Furthermore, there was no color development trend within 14 days in only one direction on the L* axis for all strands. The a* value on the red-green axis in particular changed imperceptibly. The results showed that dyeing with pure synthetic indigo molecule under selected conditions did not exhibited any significant color change comparable to the one observed with InCL over 14 days. This suggested that other plant, than only the indigo molecule formed, play a significant role in color development. Based on the results, another component involved in color formation from *Indigofera tinctoria* L., the glycolyzed dye precursor indican, was investigated.

### 6.1.2 Isolation of Indican from InCL and Its Application in Hair Dyeing

In order to generate a deeper understanding of the coloration properties of the indigo molecule and indican, its precursor and storage form in plants, the latter is extracted from InCL and subsequently applied to hair dyeing. This approach allows for the assessment of the practical feasibility of pure indican in potential hair coloration applications. Furthermore, isolating the precursor enables the exclusion of other plant-derived constituents that could influence the final color outcome, thereby allowing a more accurate evaluation of the dyeing effect attributable solely to indican. The plant material used for this purpose was an heat denatured, powdered extract of InCL expected to be rich in indican, hereinafter referred to as *InHD p.*. The extract production, aiming for denaturation of the plant-own enzyme, which is responsible for glucose cleavage from indican in order to stabilize it, is described during this work in chapter 6.3.1.

#### 6.1.2.1 Column Chromatography of Indigo Plant Material for Indican Isolation

Column chromatography was performed to specifically isolate the dye precursor indican from the plant material. This method enabled the separation of indican from other plant components and supported the assessment of the feasibility of pure indican in potential hair coloration applications.

The starting material for indican isolation was heat-denatured InCL powder (InHD p., cf. chapter 6.3.1). The specialty about this extract was the denatured plant's own enzyme activity. This was favorable because the enzymatic hydrolysis was prevented not only by the choice of solvent, but also by the lack of enzyme activity. 2% (w/v) InHD p. was dissolved in ethanol (EtOH) and stirred overnight. The InHD and EtOH mixture was filtrated, and the filter cake was taken up again in EtOH and stirred. The procedure was repeated twice. In parallel, 100 parts per billion (ppb) synthetic indigo was absorbed in EtOH and filtered due to its poor solubility. First, both dissolved substances (InHD p. and synthetic indigo) were examined for their running behavior using normal-phase thin-layer chromatography (TLC) with silica. 10 µL was applied to each TLC-plates and left to run in ethyl acetate (EtOAc). The indigo molecule could be traced on the plate by its own color.

Indican, as the expected main component in InHD p., was made visible by staining with a 1.2% (v/v) $\beta$-D-glucosidase solution. The cleavage of the sugar moiety and the formation of the resulting indigo molecule could take place on the plate and the running behavior of the substance could thus be made visible (cf. figure 6.6). The resulting $R_f$ values are listed below (cf. table 6.1).

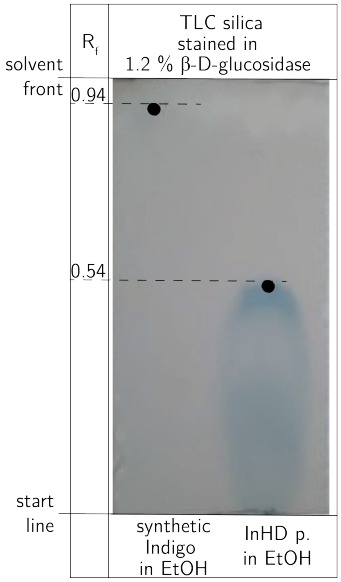



**Figure 6.6: TLC of Synthetic Indigo and InHD p..** Both substances were solved/-suspended in EtOH and applied to a silica plate. The running solvent was EtOAc and staining took place through a 1.2% (v/v) $\beta$-D-glucosidase solution.

**Table 6.1: $R_f$ Values from TLC of Synthetic Indigo and InHD p..**

| Number | Substance | $R_f$ value |
|---|---|---|
| 1 | synthetic Indigo | 0.94 |
| 2 | InHD p. | 0.54 |

A component in InHD p. (2) has actually turned blue after enzyme treatment. Furthermore, the TLC analysis showed that both applied samples exhibited different running behaviors. Indigo molecule (1) formed a clear stain with an $R_f$ value of 0.94, while the main component in InHD p. (2) showed a more dispersed, greasier stain at a lower $R_f$ value of 0.54 indicating more intense interaction with the material of the silica plate. Overall, this demonstrating that it had a higher polarity than

the indigo molecules in the solvent system used, as it ran less far. These results indicated that the visible substance on silica plate in the InHD p. sample was in fact indican. Firstly, the enzyme treatment turned it into blue indigo and secondly, it had a higher polarity than synthetic indigo, which matches the structure of the molecule. This preliminary experiment provided information on the interactions and retention time of the target substance indican for column chromatography.

The TLC analysis confirmed the presence of indican in InHD p. through enzymatic conversion and characteristic running behavior. These preliminary investigations provided important information about the polarity and retention time of the target molecule and served as the basis for subsequent column chromatography for targeted isolation. Column chromatography was subsequently performed to isolate indican from InHD p.. This method enabled the separation of indican from other plant compounds and thus representing a crucial step in obtaining the pure dye precursor on a laboratory scale.

The same ethanolic solution as described above was used for column chromatography. 6.5 g of InHD p. was mixed with 100 mL of EtOH, stirred for one hour, then the solvent was set aside and 100 mL EtOH was added again. The procedure was then repeated once more, so that a total of 300 mL EtOH was used and subsequently mixed with 8 g of silica. The mixture was dried in rotary evaporator. The column was prepared with silica, the described dried silica sample and EtOAc for chromatography. EtOAc fractions were collected in 25 mL beakers and tested individually via TLC for the target substance indican by light at 245 nm and $\beta$-D-glucosidase staining.

A total of 80 fractions were collected. The first 8 fractions showed the range of the plant's variety of colors (cf. 6.7).

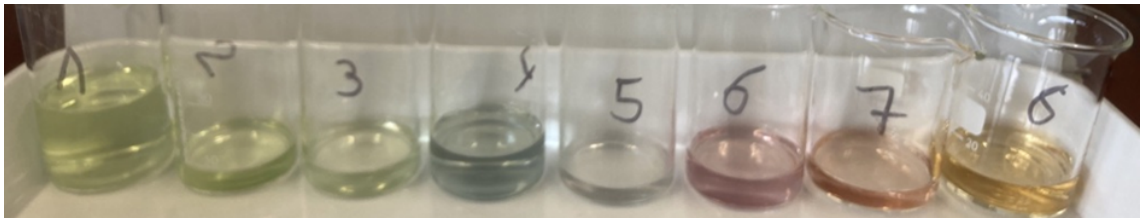



**Figure 6.7: Fractions 1-8 of Column Chromatography** from InHD p.. The running solvent was EtOAc.

Through color perception it was assumed that fractions 4 and 6 contained mainly early released natural indigo and indirubin. The less polar indigo molecule was first

detached from the column again, indirubin followed shortly after. Fractions 35 to 75 showed absorption at 245 nm under UV light. Further, they showed blue color on TLC-plate after staining with $\beta$-D-glucosidase. All these fractions were collected and dried by rotary evaporator. Afterwards, indican purity was confirmed by $^1$H-NMR in acetone in accordance with literature [124].

Fractions 35-75: $^1$H-NMR (400 MHz, Acetone) $\delta$ 7.53 (*d*, $J$ = 8.0 Hz, 1H), $\delta$ 7.19 (*d*, $J$ = 8.2 Hz, 1H), $\delta$ 6.98 (*dd*, $J$ = 8.0, 1.9 Hz, 1H), $\delta$ 6.95 (*s*, 1H), $\delta$ 6.85 (*dd*, $J$ = 8.0, 6.9 Hz, 1H), $\delta$ 4.61 (*d*, $J$ = 7.6 Hz, 1H), $\delta$ 4.26 - 4.16 (*m*, 2H), $\delta$ 3.92 (*q*, $J$ = 7.1 Hz, 1H), $\delta$ 3.77 (*d*, $J$ = 14.0 Hz, 1H), $\delta$ 3.62 - 3.58 (*m*, 2H), $\delta$ 3.56 (*d*, $J$ = 5.8 Hz, 1H).

The successful isolation of 0.105 g indican from 6.5 g InHD p. (yield: 1.62%) confirmed the suitability of the chosen chromatographic method. The purity of the product was verified using $^1$H-NMR. After successful separation of indican from other plant substances, it was subsequently examined with regard to its hair dyeing properties. However, it should be noted that quantification of indican content in InCL (Kremer Pigmente GmbH & Co. KG) via HPLC resulted in 33.6 mg indican/g InCL.

#### 6.1.2.2 Coloration with Indican on Hair

After dyeing the hair with pure synthetic indigo molecule and successfully isolating the color precursor on the plant material, the next step was the investigation of hair dyeing with commercially available indican (purity: 98%). The aim was to analyze the concentration dependence of color development under controlled conditions and to evaluate indican as the sole dye carrier. Various amounts of indican were dissolved in water, color formation was initiated with adding $\beta$-D-glucosidase formulation to the dye bath. Strands were stirred in the solution for 30 min at RT. The indican concentrations selected for application on hair are shown below (cf. figure 6.2). Furthermore, color was assessed visually and in CIELAB color space (cf. figure 6.8).

**Table 6.2: Amounts of Indican in Water Mixed with $\beta$-D-Glucosidase Formulation for Hair Coloration.**

| Strand | Amount of Indican | Corresponding Concentration |
|---|---|---|
| | [mg/strand] | (w/v) [%] |
| 1 | 1 | 0.004 |
| 2 | 2 | 0.008 |
| 3 | 3 | 0.012 |
| 4 | 4.4 | 0.0176 |
| 5 | 5.5 | 0.022 |
| 6 | 7.4 | 0.0296 |
| 7 | 30 | 0.12 |
| 8 | 50 | 0.2 |
| 9 | 100 | 0.4 |

A difference between the coloration on day 0 and the color 14 days after application could be measured on each strand. However, the degree of change between the strands differed greatly, with $\Delta E_2$ between 2.2 and 42.3 being calculated. It is noticeable that the color change increases with increasing concentrations of indican. The concentration of 0.004% (w/v) indican in water resulted in a change of $\Delta E_2 = 2.9$ from day 0 to 14, whereas the hundredfold concentration of 0.4% resulted in $\Delta E_2 = 42.3$.

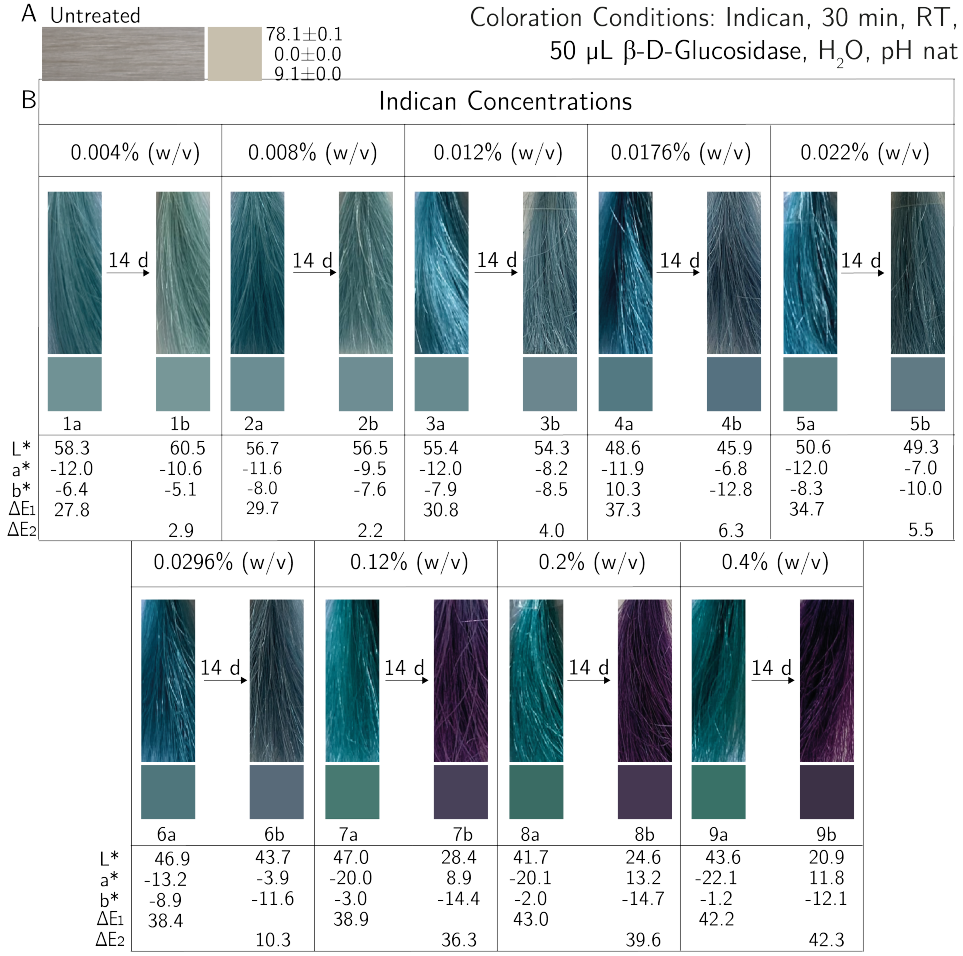



**Figure 6.8: Hair Colorations with Indican at Different Concentrations.** A: Untreated strand. B: Colored strands. Concentrations from 0.004% (w/v) to 0.4% (w/v) mixed with 50 µL $\beta$-D-glucosidase formulation each for 30 min at RT. Color assessment took place directly after application and freshly dyed and 14 days after that. $\Delta E_1$ represents the color difference between the freshly treated strand and the untreated reference strand (day 0). $\Delta E_2$ indicates the color change of the treated strand after 14 days, relative to its initial color on the respective day 0.

In particular, the difference in L* and a* value could be observed when comparing these strands. The strand with a higher concentration showed a significantly darker ($L^*_{0.004\%} - L^*_{0.4\%} = $ -39.6) and redder ($a^*_{0.004\%} - a^*_{0.4\%} = 22.4$) coloration when comparing these values on day 14. The trend of lower L* and higher a* values was observed overall within each individual strand over the course of 14 days after application, albeit to a lesser extent. The only exception here was the 0.004% (w/v) indican strand, which L* value increased by 2.2.

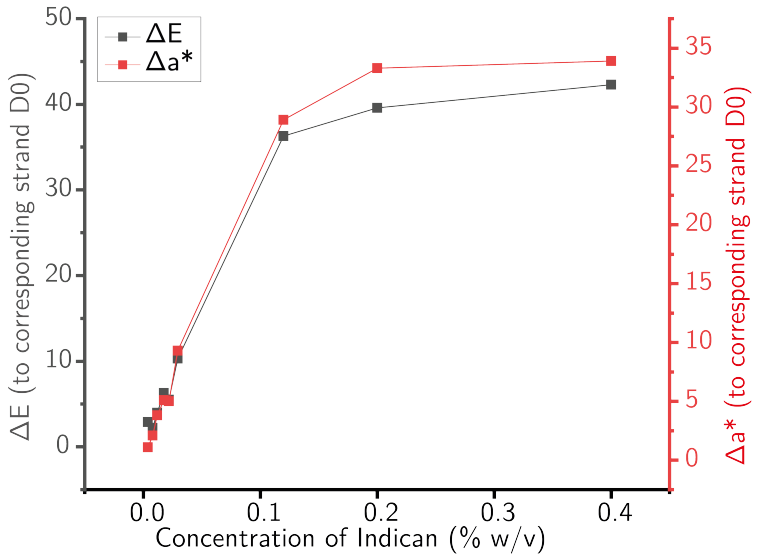



**Figure 6.9: Diagram of ΔE and Δa* Values of Hair Coloration at Different Indican Concentrations.** Color development (ΔE) and red shift (Δa*) of hair strands depending on the indican amount used. Color assessment took place directly after application and freshly dyed and 14 days after that. Color change of the treated strand after 14 days, relative to the initial color on the respective day 0 is indicated.

As displayed in figure 6.9, the highest increase in $\Delta E_2$ values within the colored strand on day 0 and day 14 was observed between the strands treated with 0.0296% (w/v) and 0.12% (w/v) indican. Parallel to the strongest increase in the concentration used, the ΔE value also multiplied from 10.3 to 36.3. Looking at the individual color space values in particular, the Δa* value was particularly striking as it increased by 28.9 in the strand with 0.12% (w/v), whereas it only increased by 9.3 within with 0.0296% (w/v) indican treated strand. Overall, the $\Delta E_2$ values increased with increasing concentration of indican used in dye solution. The maximum $\Delta E_2$ value was measured at 0.4% (w/v) indican with 42.3. At the same time, the Δa* value also increased, showing a color shift on green-red axis towards red.

These findings showed that, starting at a concentration of 0.12% (w/v) pure indican in the dye suspension, a color shift from the initial blue to purple could be observed on the hair over the course of 14 days. Below this concentration, the color result remained more stable. This underscored the importance of indican as a dye precursor in plant-based hair dyes and provided important insights for the optimization of natural formulations.

## 6.2 Blue Coloration with *Indigofera tinctoria* L.

Hair coloration with InCL leads to an initial blue hue on strand (cf. chapter 6.1). The characteristics of the coloration in terms of substrate specificity, color stability influence of pH on coloration and the possibility of preserving this blue color, also at higher InCL concentrations, are discussed below. The investigations are underpinned by microtome cuts of *Indigofera tinctoria* L. colored hair.

### 6.2.1 Influence of Dyeing Substrate on Blue Coloration with InCL

After investigating the color behavior of the individual InCL components, it was essential to clarify whether this development was specific to hair as a substrate or if it also occurs on other dyeing substrates. In order to investigate this, five other materials were treated the same way as hair strands. The textiles cotton, viscose, silk, hemp, and linen were selected for the best manageability, comparability and allowed coloration application on a white base. 5% (w/v) InCL in 50 mL water were used to dye 0.7 g of each fabric which corresponds to the usual amount of hair used for this dye preparation (cf. figure 6.10).

The lowest L*-value was measured on the hair strands treated with 5% (w/v) InCL in water at RT, both freshly after coloration and 14 days later. Beyond that, the $\Delta$E value of these strands (33.0), which compared the coloration on day 0 and day 14, was significantly higher for hair than for all other substrates used (cotton: 4.6, viscose: 6.6, silk: 6.5, hemp: 5.5, linen: 4.9). In addition, the change in the a* value in particular within 14 days was not as pronounced with any substrate coloration as that on the hair with |24.2| (cotton: |1.9|, viscose: |3.1|, silk: |5.1|, hemp: |1.7|, linen: |3.9|).

The results showed that the color change within 14 days after InCL application was clearly substrate-specific. In particular, the hair material exhibited a stronger color shift and red color development than all other substrates tested. This suggests that the hair structure and its components were actively involved in color formation and may possess properties that favor reaction with InCL components. This finding was central to the development of convenient plant-based dyes with *Indigofera tinctoria* L..

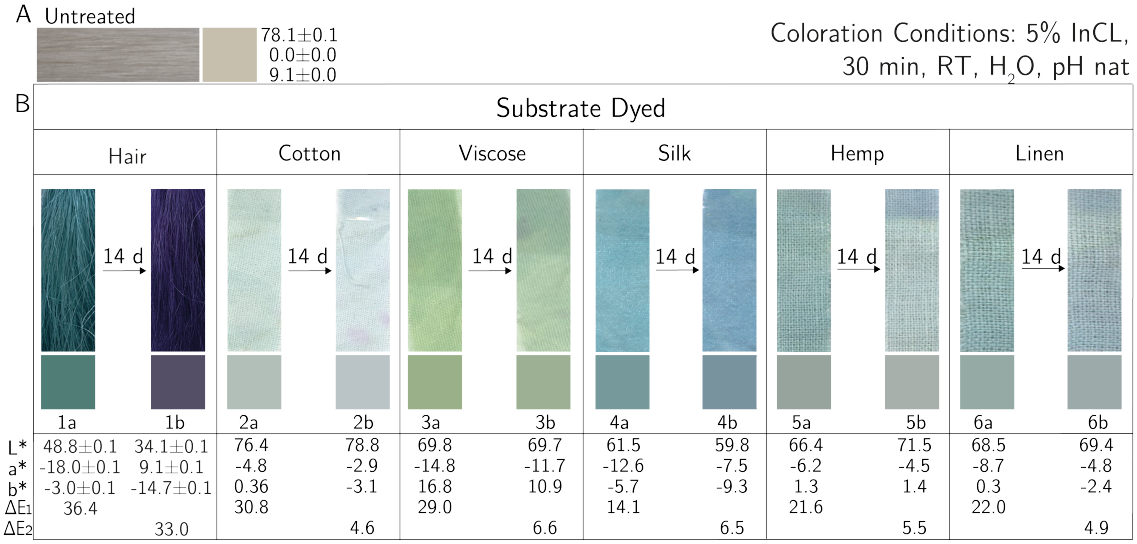



**Figure 6.10: Colorations with 5% (w/v) InCL on Different Substrates.** A: Untreated strand. B: Colored substrates. In addition to hair, substrates tested were cotton, viscose, silk, hemp and linen each for 30 min at RT. Color assessment took place directly after application and freshly dyed and 14 days after that. $\Delta E_1$ represents the color difference between the freshly treated strand and the untreated reference strand (day 0). $\Delta E_2$ indicates the color change of the treated material after 14 days, relative to its initial color on the respective day 0.

### 6.2.2 Influence of Aqueous Post-Treatment on the Color Stability of InCL-Dyed Hair

Since previous experiments showed a color change in hair strands dyed with InCL, it was tested whether post-treatment with water could improve color stability. The aim was to preserve the original blue color for longer and prevent the undesired red shift within 14 days.

Strands were prepared with 5% (w/v) InCL in water for 30 min at RT and blow-dried afterwards. The strands were subsequently placed in 50 ml de-ionized water and stirred for 90 min. After that they were combed and dried again (cf. figure 6.11).

The stirring in water resulted in noticeable shift in hue compared to the reference strands. The treatment immediately led to slightly less green and more blue shade with a $\Delta E$ value of 12.9. After 14 days, the water-treated strands retained a clearer blue coloration and showed a markedly reduced red/purple shift compared to the

control. This was particularly evident in the $\Delta E$ value – while the non-post-treated strands were at $\Delta E = 33.0$, the post-treated ones showed a $\Delta E_2$ of 1.7.

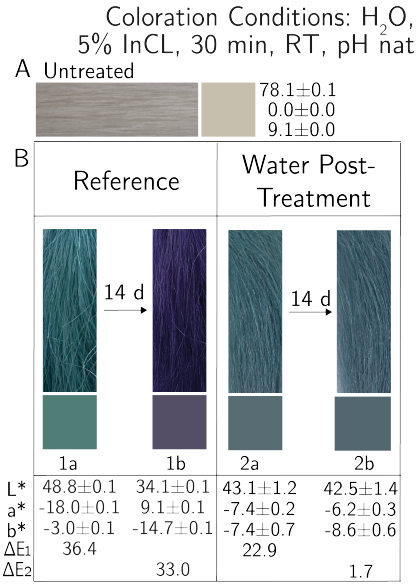



**Figure 6.11: Water Post-Treatment of Freshly 5% (w/v) InCL Dyed Strands.** A: Untreated strand. B: Colored strands. Dyed strands were placed in 50 ml de-ionized water and stirred for 90 min at RT. Color assessment took place directly after application and freshly dyed and 14 days after that. $\Delta E_1$ represents the color difference between the freshly treated strand and the untreated reference strand (day 0). $\Delta E_2$ indicates the color change of the treated strand after 14 days, relative to its initial color on the respective day 0.

These results showed that post-treatment with water led to significantly improved color stability. The $\Delta E_2$ of 1.7 which was measured 14 days after water post-treatment, would only be visible to the human eye upon very close inspection. The red color shift which was observed in non-post-treated strands, was almost completely prevented. This suggest that water-soluble compounds were removed by rinsing, thus preventing further reactions taking place on hair. Water post-treatment contributed to improved color stability on hair.

### 6.2.3 Influence of Wash Fastness Test on Color Stability of InCL-Dyed Hair

Wash stability is a key quality feature for practical application of hair dyes. A wash fastness test of freshly 5% (w/v) InCL dyed hair was conducted to assess color stability od hair dyed with InCL under realistic conditions. The test examined how repeated wash cycles influenced color intensity and direction (cf. figure 6.12).

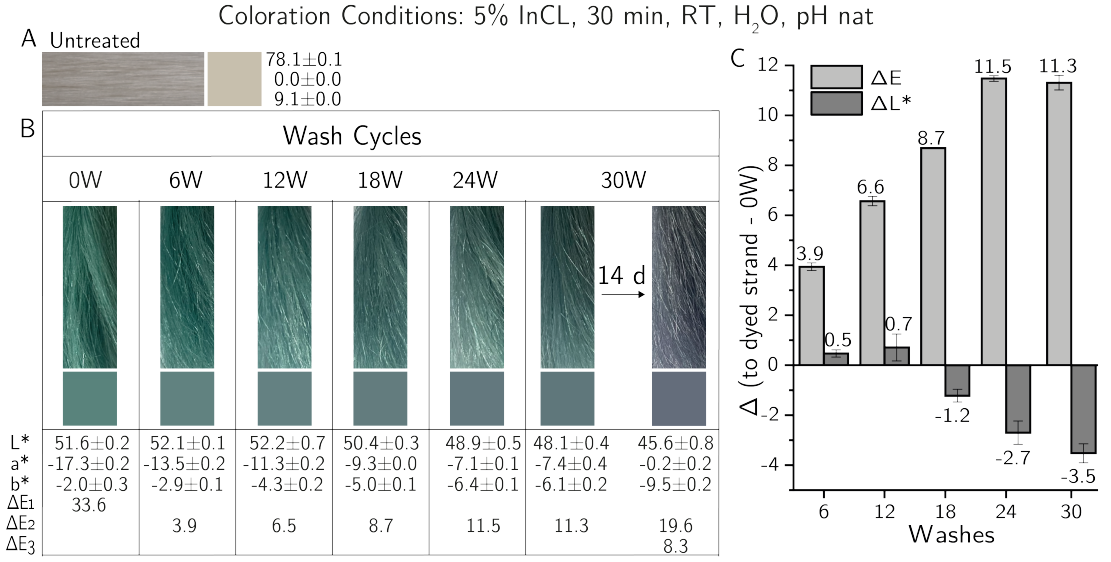



**Figure 6.12: Wash Fastness Test of Freshly 5% (w/v) InCL Dyed Strands.** A: Untreated strand. B: Dyed strands were subjected to 6, 12, 18, 24, and 30 wash cycles. Unwashed strand was compared to untreated strand ($\Delta E_1$). Color differences ($\Delta E_2$) were measured relative to the unwashed dyed strand. The 14-day aged strand was compared to the freshly 30-times washed strand (day 0) to assess color stability over time, expressed with $\Delta E_3$. C: Bar chart with $\Delta E$ and $\Delta L^*$ values of every wash cycle, each in relation to the unwashed strands (0W).

The color difference between a freshly dyed strand and those that have been subjected to the wash stability test increased with each wash cycle until 24 washes. The coloration after 24 and 30 washes differed in $\Delta E_2$ value by 0.2, in $\Delta L^*$ value by 0.8, whereby the more often washed strands had a lower $\Delta E_2$ value. The $\Delta E_2$ overall change ranged between 3.9 and 11.5. It should be noted that the color did only become darker (-L*), but that the a* and b* values changed. The color developed in the less green (+a*, max. |10.1|) and more blue direction (-b*, max. |4.1|), whereby the brightness (L*) was in the range of 52.1 to 48.1 in all strands. Overall, the SD

was low for all values. 14 days after wash fastness test, the 30 times washed strand differed from the same on day 0 by $\Delta E_3$ of 8.3.

These results showed that the dyeing process described exhibited high wash stability. Despite multiple wash cycles, the color intensity remained almost unchanged, and the color shift was significantly lower than in untreated strands. The slight change in $\Delta E_2$ and $\Delta L^*$ values underlined the robustness of the coloration. Thus, InCL fulfills a key feature for use as a competitive hair dye, it could be classified as a permanent dye.

### 6.2.4 Influence of pH on Color Outcome of InCL-Dyed Hair

In addition to color stability after washing tests, it was also of interest for the investigations of the InCL dyeing properties to determine the influence of the pH value of the dye suspension on the dyeing result and its stability. For this purpose, the pH of the 5% (w/v) InCL color suspension was adjusted to 3, 5, 7, 9 and 11 with either HCl or NaOH. The natural pH of 5% (w/v) InCL suspension was 6.8 (cf. figure 6.13).

It was observed that the pH value of the dye preparation did not cause any advantage in the stabilization of the blue coloration, but had an influence on the overall coloration. Application of coloration suspension at pH 11 led the slightest color outcome on the strand ($\Delta E$ to untreated strand = 16.3) and the least pronounced color shift within within 14 days ($\Delta E_{D0toD14} = 4.6$). Furthermore, the color suspension at pH 3 showed an intensified, yet reduced intensity compared to the usual application, but also to the color change within the 14 days ($\Delta E$ to untreated strand = 27.3; $\Delta E_{D0toD14} = 11.2$).

As the natural pH value of the water and InCL suspension approached 6.8, the intensity of the coloration and its change over the course of 14 days also increased. The most pronounced color change during the measurement period was observed at the natural pH ($\Delta E = 33.0$). The range of $\Delta E$ values of the strands colored at pH 5, 7 and 9 over the period was from 16.8 to 19.5, with pH 7 showing the highest value among them. At the same time, the darkest coloration was measured on day 0 in the strand treated with pH 5 ($L^* = 45.6$).

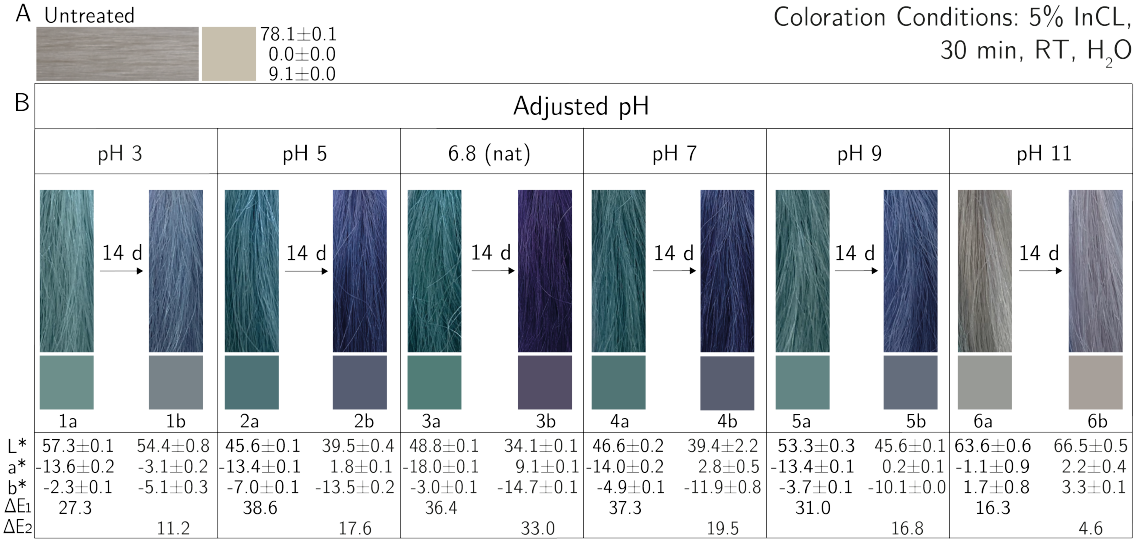



**Figure 6.13: Hair Colorations with 5% (w/v) InCL at Different pH.** A: Untreated strand. B: Colored strands. Colorations at pH 3, 5, 7, 9 and 11 on hair for 30 min at RT. Color assessment took place directly after application and freshly dyed and 14 days after that. $\Delta E_1$ represents the color difference between the freshly treated strand and the untreated reference strand (day 0). $\Delta E_2$ indicates the color change of the treated strand after 14 days, relative to its initial color on the respective day 0.

The results showed that the pH value of dyeing suspension had a significant influence on color intensity and color change over time. While extremely acidic or basic conditions led to lower color intensity, pH 6.8 (nat) showed the most intense color result and the strongest color shift in the course of 14 days. This underlined the importance of targeted pH adjustment for the optimization of plant-based hair dyes.

### 6.2.5 Influence of Temperature on Color Outcome of InCL-Dyed Hair

In addition to pH, the influence of dye bath temperature on the color result with 5% (w/v) InCL was a potential factor influencing color outcome. Since hair dyeing often takes place in applications close to the human body, the effect on increasing the temperature to 37 °C, corresponding to the average human body temperature, compared to RT on the color result was investigated (cf. figure 6.14).

Initially, the colors at different temperatures differed by ΔE of 4.7. The strand treated at elevated temperature showed a slightly less green and blue tint. After 14 days, the color development of the strands were similar (RT: ΔE = 33.0; 37 °C: ΔE = 32.3). Comparing the strands on day 14, a difference of ΔE = 3.8 was measured. The strand dyed at higher temperatures appears darker (L* = |2.8|) and slightly less yellowish (b* = |2.5|).

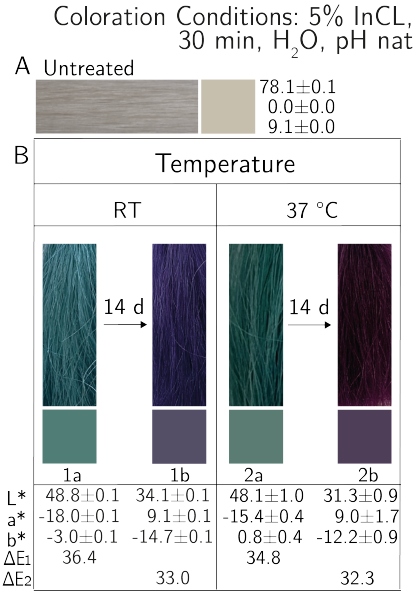



**Figure 6.14: Hair Colorations with 5% (w/v) InCL at Different Temperatures.** A: Untreated strand. B: Colored strands. Colorations at RT and 37 °C on hair for 30 min at natural pH measured both freshly dyed and 14 days after application. $\Delta E_1$ represents the color difference between the freshly treated strand and the untreated reference strand (day 0). $\Delta E_2$ indicates the color change of the treated strand after 14 days, relative to its initial color on the respective day 0.

Overall, however, the results showed an increased temperature of 37 °C led to a slightly more intense and darker color without significantly affecting long-term color stability. Even though the color appeared slightly more intense due to the increased temperature and the color shift was slightly smaller, further tests were carried out primarily at RT due to the improved handling. Nevertheless, it could be concluded that temperature has a moderate influence on color intensity when dyeing with InCL on hair, and should be taken into account in practical applications.

### 6.2.6 Evaluation of InCL Dye Penetration in Hair via Microtome Cuts

In order to understand more precisely what happens inside the hair structure when InCL was applied, microtome cuts of three dyed hair fibers were made and the penetration depth of the dye was measured microscopically. The aim was to draw conclusions about the localization of the color components in the hair. Due to the experimental setup, strands that had completed their color development to purple were cut (cf. figure 6.15). The work was carried out by Swenja Kalischke (Scalp Tissue Engineering, Henkel AG & Co. KGaA).

In the strands treated with RT, a penetration depth of 8.8-24.8% of the total mean hair diameter was observed. Even if the color of the hair appears purple after complete color development, only a blue color was clearly visible in a circular pattern of the outer cortex. The inner area of the cortex was slightly reddish, although it was not possible to determine the exact color using light microscopy. However, it can be concluded that the color penetrates beyond the outermost layer of the hair, the flat cuticle, into the interior of the hair, the cortex, after treatment with 5% (w/v) InCL at RT for 30 min.

The colorations at 37 °C resulted in relative penetration depth of 37.7-59.3% which proved that large parts of the cortex had been stained. The blue color, analogous to the coloration at RT, could be detected more clearly than the red color components of the purple strand. The microtome cuts with subsequent microscopic examination showed that color molecules were located beyond the cuticle in the cortex, where it exhibited an uneven, grainy color distribution. Coloring at elevated temperatures resulted in a deeper penetration, indicating color dependency in diffusion of dyes. The visible blue color in the outer cortex as well as the presumed reddish component in the inner cortex correspond to the observed color development to purple. The red component remained diffuse and difficult to identify.

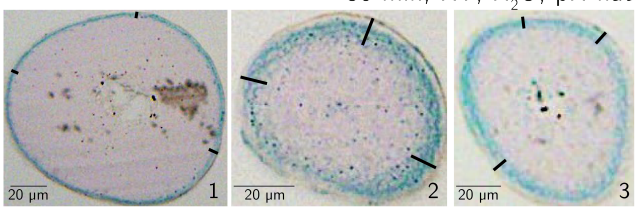
This could indicate different diffusion behaviors of the dyes. The sharper limitation of dye distribution at RT suggests that the cuticle and outer cortex have a stronger barrier effect against dyes, which was partially overcome at higher temperatures. Although the penetration depth increased at 37 °C, the absolute amount of the penetrated substance appears to increase proportionally according to visual perception.

Overall, the result provided important insights into the mode of action of plant-based dyes in hair.

Coloration Conditions: 5% InCL, 30 min, RT, $H_2O$, pH nat

A

B



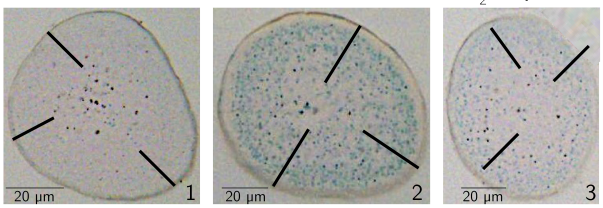

C

| | Penetration of Dye into the Hair Fiber | | |
|---|---|---|---|
| | 1 | 2 | 3 |
| Penetration Depth (µm) | 4.2<br>3.6<br>2.9<br>3.6±0.7 | 8.0<br>12.2<br>7.9<br>9.3±2.5 | 3.9<br>3.4<br>2.5<br>3.3±0.7 |
| Relative Penetration Depth (%) | 10.5 | 24.8 | 8.8 |

Coloration Conditions: 5% InCL, 30 min, 37 °C, $H_2O$, pH nat

A

B




C

| | Penetration of Dye into the Hair Fiber | | |
|---|---|---|---|
| | 1 | 2 | 3 |
| Penetration Depth (µm) | 20.2<br>27.1<br>13.6<br>20.3±6.8 | 25.8<br>28.7<br>31.3<br>28.6±2.7 | 24.5<br>18.8<br>20.3<br>21.2±3.0 |
| Relative Penetration Depth (%) | 37.7 | 59.3 | 46.6 |

**Figure 6.15: Microscopic Images of Microtome Cuts from Strands Dyed with 5% (w/v) InCL at RT and at 37 °C.** A: Colored strand. B: Three different hair fibers of one colored strand cut and examined. C: Penetration depth (µm) depicts the absolute penetration and SD of the dye into the hair without appreciating the hair's irregular shape. The relative penetration depth in % describes the penetration in relation to the radius of the hair.

## 6.3 Production and Evaluation of *Indigofera tinctoria* L. Extracts

The objective was to extract the key coloration components of indigo raw material and concentrate them to higher levels, enhancing the extract's effectiveness for dyeing applications. Parts of the plant that are useless or a hindrance to coloration applications should be left out. A preparation method of the plant material should be developed that allows safe incorporation in cosmetic formulas.

### 6.3.1 Heat-Denatured Indigo Extract Production

An objective in the development of convenient plant-based hair dyes from *Indigofera tinctoria* L. is the production of stable, storable, and controllably activatable dye formulations. In this context, a method for heat denaturation on InCL was investigated, in which enzymatic activity was excluded by an aqueous heat-treatment as described in patent specification US 2004/0055096 A1 [125], the raw material could be introduced to water without any enzymatic degradation taking place. By adding a $\beta$-D-glucosidase formulation shortly before application, the color formation on the hair fiber can be reactivated. This chapter contains the production, application and effect of heat-denatured indigo extract in liquid and powdered form.

For production of heat-denatured Indigo (InHD), 80 ml de-ionized water was heated to approx. 95 °C. Then 20 g of InCL was added (20% (w/v)) and stirred at the same temperature for 5 min. The solution was then cooled down to RT. The next step was filtration of this dispersion (filter: Whatman 10340810, viscose, pore size 100 µL, approx. 5 to 10 min, vacuum pump at approx. 500 mbar). The filtrate was a brown, opaque solution without precipitate, with a natural pH value of approx. 8.2, which was adjusted to 6.2 using HCl. This solution is referred to below as *InHD liq.* and could be used directly for hair treatment. However, dyeing with only InHD liq. showed no color on the hair strand (2) under usual conditions (50 ml on 0.7 g hair strand, dyeing for 30 min at RT with stirring) so the addition of another component to the solution was needed (cf. figure 6.16).

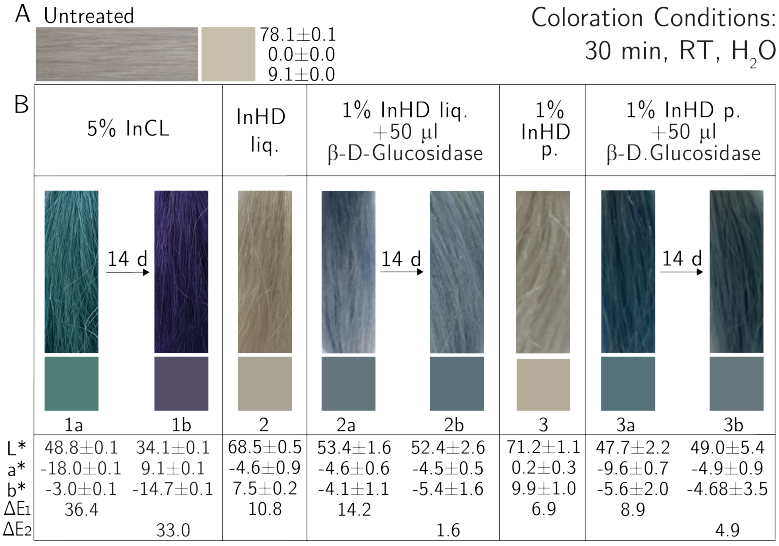



**Figure 6.16: Hair Colorations with InHD (Liquid and Powdered) Colorations Mixed with 50 µL $\beta$-D-Glucosidase Formulation.** A: Untreated strand. B: Colored strands. Colorations were carried out for 30 min at RT. Color assessment took place directly after application and freshly dyed and 14 days after that. $\Delta E_1$ represents the color difference between the freshly treated strand and the untreated reference strand (day 0). $\Delta E_2$ indicates the color change of the treated strand after 14 days, relative to its initial color on the respective day 0.

The treatment of a strand with 50 µL of $\beta$-D-glucosidase formulation added to 50 ml of the InHD liq. for 30 min at RT resulted in a blue shade being visible on the hair fiber after dyeing (2a). Initially, the color difference between strands treated with 5% (w/v) InCL (1a) and treated with InHD liq. and $\beta$-D-glucosidase formulation addition (2a) amounted to $\Delta E = 13.0$. The color change of the reactivated InHD liq. from day 0 to day 14 on the hair (2a to 2b) was measured with a $\Delta E$ value of 1.6. By initially inactivating the enzyme activity present in the indigo and adding an enzyme activity in the form of a $\beta$-D-glucosidase formulation shortly before application, a coloring performance of the initially non-staining component InHD could be achieved again.

For reasons of shelf life and uncomplicated handling, it was desirable to obtain the enzyme-deactivated indigo in dry form and to reactivate it with enzyme preparation. The test series was conducted by the intern Stephanie Schönbeck as part of the promotion project. It was possible to transfer the the protocol described above to the Indfrag Biosciences Pvt Ltd pilot production plant to produce this powder on a large scale (cf. figure 6.17).

The same procedure as for InHD liq. was carried out until cooling denatured suspension down to RT. It was then centrifuged at 20,133 rcf using a fixed-angle rotor for 20 min at RT. The supernatant was removed (optionally with a filter) and collected – a brown, opaque solution without precipitate remained, with a pH value of approx. 7.7-8.2. The pH value of the solution was adjusted to approx. 6.8 to 7 using HCl. Most of the water contained was removed using a rotary evaporator at 55-60 °C, 200 rpm and 20 mbar. The sticky solution was completely dried for at least twelve hours in a crystallization tray in a heating cabinet (45 °C). The resulting product was crushed using a conventional spice mill at laboratory level. An enzyme-denatured indigo powder that could be absorbed in water was obtained (cf. figure 6.17).

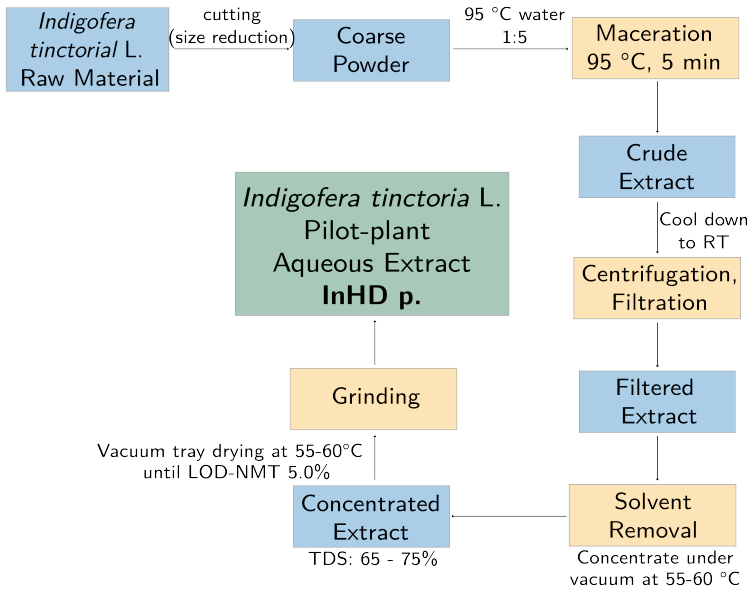



**Figure 6.17: *Indigofera tinctoria* L. Aqueous Extraction Protocol Carried out by Indfrag Biosciences Pvt Ltd at Pilot Production Plant Level.** Extraction protocol to produce InHD p. at pilot plant. TDS: Total Dissolved Solids, LOD-NMT: Loss on Drying - not more than. Adapted from Indfrag Biosciences Pvt Ltd (2024).

Deviations for the pilot plant level were during drying and grinding. The final drying step of the sticky InHD solution was carried out in a vacuum tray drying chamber at 55 - 60 °C until the Loss on Drying (LOD) was not more than 5%. Afterwards, the extract was ground to obtain an even powder. Hereinafter the dry extract is referred

to as *InHD p.*. The examination of the indican content in the InHD p. revealed 103.1 mg indican/g InHD p..

Staining with the InHD p. in water under usual conditions (50 ml, 1% in water to 0.7 g hair strand, staining for 30 min at RT with stirring) showed no color on the hair strand (cf. figure 6.16, strands 3a/b). Coloration could be done analogous to InHD liq. with addition of 50 µL $\beta$-D-glucosidase formulation to 50 mL solution of 1% (w/v) InHD p. and dyeing the strand in this solution for 30 min at RT with stirring (3a). After two weeks, the change of color within the reactivated InHD p. strands could be represented by the $\Delta$E value of 4.9.

By initially heat-inactivating the enzyme activity present in InCL and adding an enzyme activity in the form of $\beta$-D-glucosidase formulation shortly before application, a coloring performance of the initially non-staining component InHD liq. or p. could be achieved again. On the other hand, the color change from an initially turquoise-blue tone to a reddish and darker shade was prevented to a certain extent (1: $\Delta$E = 33.0; 2: $\Delta$E = 1.6; 3: $\Delta$E = 4.9).

The InHD p. was used for all further experiments with this type of extract for the advantages it brings. The production of a storable, enzyme-inactivated powder with subsequent activation thus represented a promising approach for the development of a stable, plant-based hair dye, which was even feasible for large scale production.

#### 6.3.1.1 Influence of InHD p. Concentration on Color Outcome on Hair

After successfully producing a powdered heat-denatured indigo extract (InHD p.), the most suitable concentration of InHD p. for hair coloration should be determined. The aim was to analyze the color intensity and stability depending on the InHD p. concentration used and to define a suitable application amount for further dyeing experiments. For this purpose, the color results of 0.5%, 1%, 2%, 3%, 5% and 10% (w/v) InCL on hair were evaluated. In each case 50 µL $\beta$-D-glucosidase formulation was added to 50 mL InHD p. solution shortly before adding a strand (cf. figure 6.18).

It can be observed that the color intensity decreases with increasing concentration of InHD p. In contrast, no clear regularity in the $\Delta$E value could be observed depending

on the concentration used. The color differences between the hair strands treated with InHD p. and the untreated strand show a clear dependence on the concentration used. When using 0.5% and 1% (w/v) InHD p., the color difference to the untreated hair was $\Delta E = 38.9$ and $\Delta E = 38.3$, respectively. These values indicate an intense color change, although no noticeable individual deviations within the L*a*b* color space were observed. Both strands showed a relatively stable color result in the course of 14 days with $\Delta E_2 = 5.2$ and $\Delta E_2 = 5.3$.

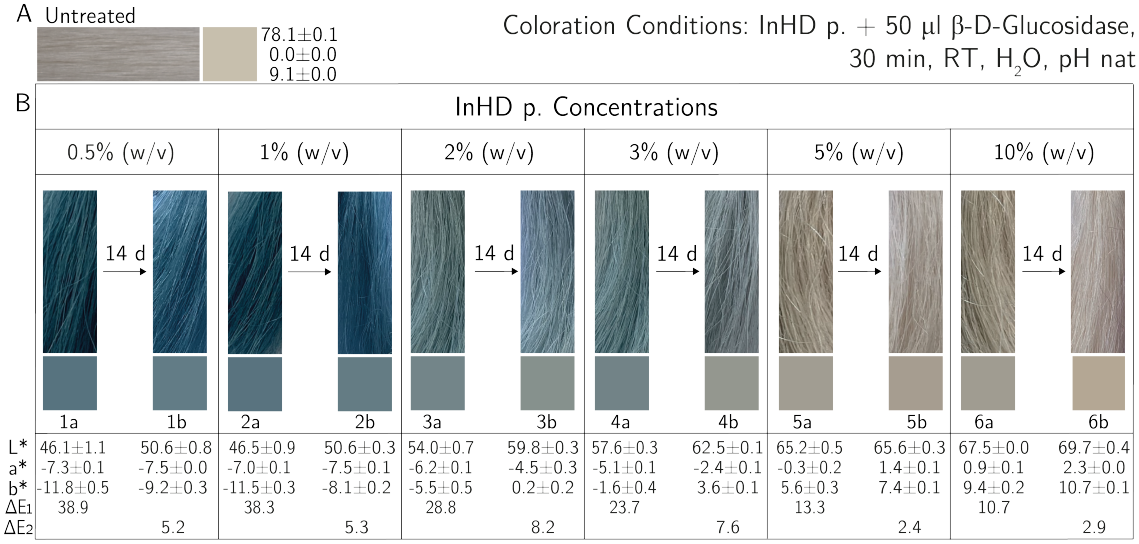



**Figure 6.18: Hair Colorations with InHD p. at Different Concentrations Mixed with 50 µL $\beta$-D-Glucosidase Formulation.** A: Untreated strand. B: Colored strands. InHD p. added to water at concentrations of 0.5%, 1%, 2%, 3%, 5% and 10% (w/v) mixed with 50 µL $\beta$-D-glucosidase formulation. Strand was added in dye suspension for 30 min at RT. Color assessment took place directly after application and freshly dyed and 14 days after that. $\Delta E_1$ represents the color difference between the freshly treated strand and the untreated reference strand (day 0). $\Delta E_2$ indicates the color change of the treated strand after 14 days, relative to its initial color on the respective day 0.

However, the color outcome of 2% (w/v) InHD p. and $\beta$-D-glucosidase formulation in water deviated with $\Delta E = 28.8$ from the untreated strand and with $\Delta E = 8.2$ between day 0 and day 14. This indicated a less intense and less stable color result compared to the usage of 0.5% and 1% (w/v) InHD p.. The application concentration of 3% (w/v) InHD p. led to a difference of $\Delta E_1 = 23.7$ compared to the untreated strand and $\Delta E_2 = 7.6$ related to the same strand in the course of 14 days after application. This trend also continued with the concentrations of

5% and 10% (w/v) InHD p., with the exception that 10% with a ΔE of 2.9 describing the color shift between 14 days showed a higher difference in the L*a*b* values than 5% with ΔE = 2.4. It can be summarized that no blue coloration of the hair could be detected above 5% (w/v) InHD p. concentration.

The CIELAB measurements of InHD p. treated strands are displayed in the bar chart 6.19 below. The color parameters ΔE and Δa* comparing hair dye on day 0 and day 14 as a function of the concentration of InHD p. are shown.

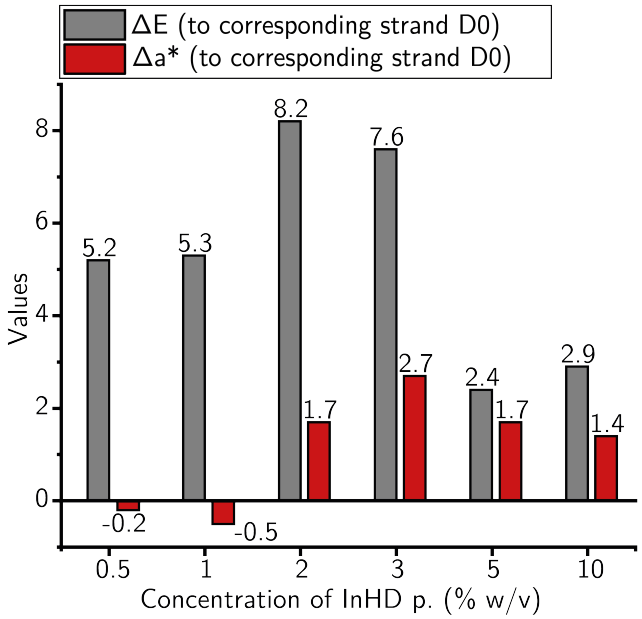



**Figure 6.19: Bar Chart of ΔE and Δa* Values of Hair Coloration at Different InHD p. Concentrations.** Color development (ΔE) and red shift (Δa*) of hair strands depending on the InHD p. amount used. Color assessment took place directly after application and freshly dyed and 14 days after that. Color change of the treated strand after 14 days, relative to the initial color on the respective day 0 is indicated.

The ΔE initially increased with increasing concentration, reached a maximum at 2% (w/v) and 3% (w/v) InHD p. (ΔE = 8.2 and 7.6, respectively), dropped significantly at 5% (w/v) ΔE = 2.4), and rose sharply at 10% (w/v) ΔE = 2.9). Δa* values were slightly negative or positive ranging between -0.5 to 2.7 at low concentrations and reached a peak at 3% (w/v) with 2.7, indicating the strongest red shift within the different InHD p. concentrations applied. It was shown that the color effect of InHD p. did not increase linearly with concentration.

In conclusion, the results showed that low concentrations of 0.5% and 1% (w/v) InHD p. in particular led to intense and stable blue color on hair. Higher concentrations, on the other hand, showed lower color intensity and concentrations of 2% (w/v) and 3% (w/v) exhibited greater color change over 14 days. For further investigations, 1% (w/v) InHD p. was therefore chosen as the optimal concentration, as it offers a balanced ratio between color intensity and stability.

#### 6.3.1.2 Influence of pH on Color Outcome of InHD p.-Dyed Hair

Since it was already shown with InCL dyeing, that pH value of the staining suspension had an influence on color intensity and stability, this parameter was also investigated for the application of the powedered, heat-dentaured indigo extract (InHD p.). The aim was to identify the optimal pH conditions of 1% InHD p. coloration on hair. The dye solution was adjusted to 3, 5, 9 and 11 with either HCl or NaOH, whereby the pH adjusted during production was 7. The heat denatured extract was reactivated with 50 µL $\beta$-D-glucosidase formulation to 50 mL solution of InHD p. shortly before application (cf. figure 6.20).

Analogous to the influence of the coloration with InCL, a high pH value also had a negative effect on the color outcome. A coloration at pH 9 and pH 11 was barely discernible as the $\Delta$E compared to the untreated strand were 4.3 (pH 9) and 3.1 (pH 11). The strands that were dyed at a more acidic pH than the natural one (pH 3 and 5) did show a coloration – but it was less intense compared to pH 7. The coloration at pH 3 differed by $\Delta E = 14.7$ and the one at pH 5 by $\Delta E = 11.5$ from the coloration at pH 7 freshly after application. In addition, a color change towards a redder shade was observed: With changes of $\Delta E = 31.4$ (pH 3) and $\Delta E = 28.3$ (pH 5) between the strands directly after coloration and on day 14, there was no stable result achieved. This was in contrast to the coloration at pH 7.

The results showed that a neutral pH value of around 7 provided the best conditions for stable and intense coloring with 1% InHD p. and 50 µL $\beta$-glucosidase formulation in water. Both acidic and basic conditions led to significantly lower changes over 14 days but at the same time lower intensity of dye. This confirmed the importance of pH adjustment for application of powdered indigo extract and provided an important basis for the formulation of stable plant-based hair dyes from *Indigofera tinctoria* L..

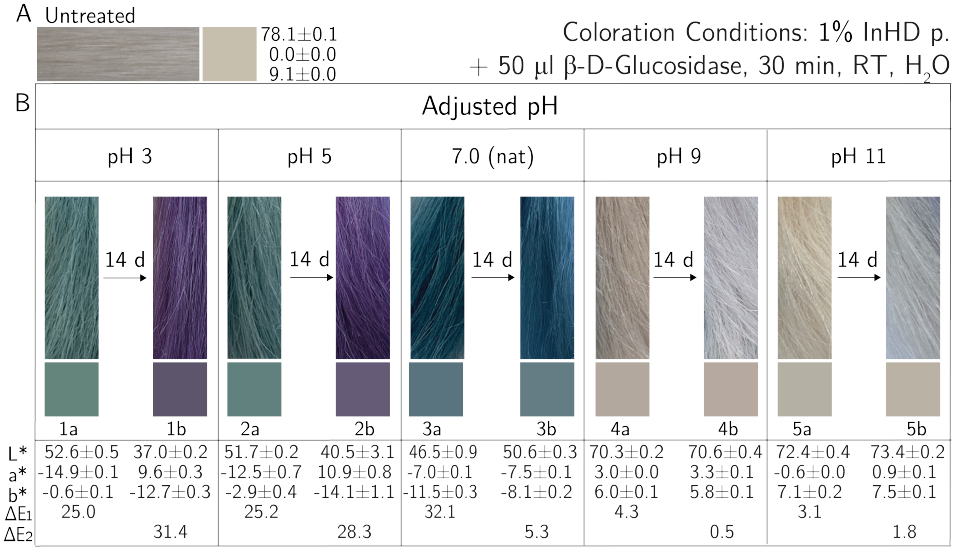



**Figure 6.20: Hair Colorations with 1% (w/v) InHD p. Mixed with 50 µL $\beta$-D-Glucosidase Formulation at Different pH.** A: Untreated strand. B: Colored strands. Colorations at pH 3, 5, 7, 9 and 11 on hair mixed with 50 µL $\beta$-D-glucosidase formulation each for 30 min at RT measured both freshly dyed and 14 days after application.$\Delta E_1$ represents the color difference between the freshly treated strand and the untreated reference strand (day 0). $\Delta E_2$ indicates the color change of the treated strand after 14 days, relative to its initial color on the respective day 0.

### 6.3.1.3 Influence of Temperature on Color Outcome of InHD p.-Dyed Hair

As the appropriate concentration and pH environment for InHD p. coloration on hair was determined, the staining should be assessed at an elevated temperature of 37 °C corresponding to human body temperature. This was compared to RT during application (cf. figure 6.21). The aim was to analyze how color intensity and stability was affected.

On day 0, the strand dyed at RT appeared slightly darker ($\Delta L^* = -1.5$), greener ($\Delta a^* = -0.5$), and more yellow ($\Delta b^* = +2.9$) than the strand dyed at RT indicated by a $\Delta E$ value of 3.3. Within 14 days, the difference increased to $\Delta E = 4.5$. The color shift was more pronounced in the strand dyed at a lower temperature with a $\Delta E$ value of 5.3 compared to 3.9 between the strands dyed at 37 °C. Primarily, the final color result of the higher-temperature strand appeared slightly lighter ($\Delta L^* = -1.5$).

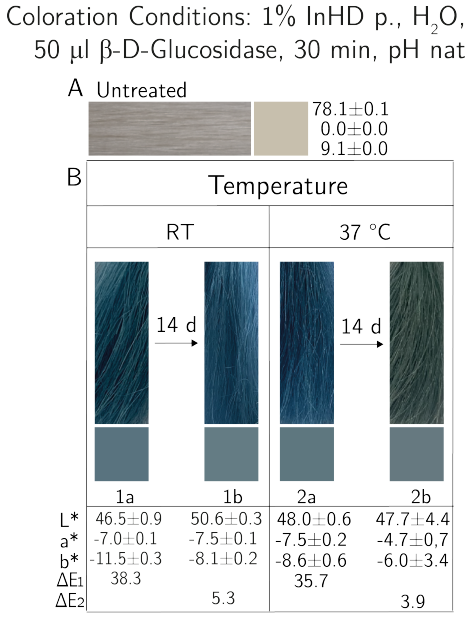



**Figure 6.21: Hair Colorations with 1% (w/v) InHD p. Mixed with 50 µL $\beta$-D-Glucosidase Formulation at Different Temperatures.** A: Untreated strand. B: Colored strands. Colorations were carried out at RT and 37 °C on hair for 30 min at natural pH measured both freshly dyed and 14 days after application. $\Delta E_1$ represents the color difference between the freshly treated strand and the untreated reference strand (day 0). $\Delta E_2$ indicates the color change of the treated strand after 14 days, relative to its initial color on the respective day 0.

The results showed that staining at an elevated temperature of 37 °C compared to RT led to a slightly less intense but more stable color outcome. For practical reasons, further experiments will be carried out at RT.

#### 6.3.1.4 Influence of Wash Fastness Test on Color Stability of InHD p.-Dyed Hair

After determination of the appropriate concentration, pH environment, and temperature for hair coloration with InHD p., its color stability was tested in a wash fastness test under realistic conditions. Color stability of strands dyed with 1% (w/v) InHD p. at pH 7 and RT with addition of 50 µL $\beta$-D-glucosidase formulation was evaluated by a wash fastness test (cf. figure 6.22).

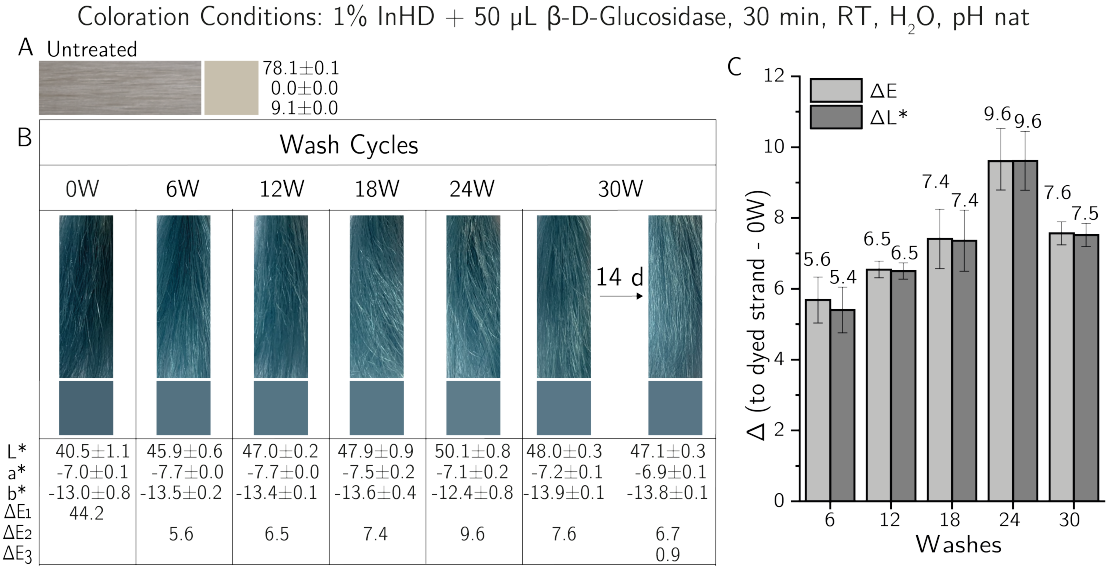



**Figure 6.22: Wash Fastness Test Freshly 1% (w/v) InHD p. and $\beta$-D-Glucosidase Formulation Dyed Strands.** A: Untreated strand. B: Dyed strands were subjected to 6, 12, 18, 24, and 30 wash cycles. Unwashed strand was compared to untreated strand ($\Delta E_1$). Color differences ($\Delta E_2$) were measured relative to the unwashed dyed strand. The 14-day aged strand was compared to the freshly 30-times washed strand (day 0) to assess color stability over time, expressed with $\Delta E_3$. C: Bar chart with $\Delta E$ and $\Delta L^*$ values of every wash cycle, each in relation to the unwashed strands (0W).

The highest change of $\Delta E_2$ value was measured between the non-washed strands and strands after six washes ($\Delta E_2 = 6.5$). This value remained stable for the next six washes (12W). Overall, for all hair washes the $\Delta E_2$ value was between 5.6 to 9.6 and

the $\Delta L^*$ value was similar to it in every wash cycle (5.4 to 9.6). At the same time, no trend in the change of one color dimension in particular could be identified. A specialty, however, was that the color after 24 washes showed the greatest difference to the unwashed strand ($\Delta E_2$ = 9.6, $\Delta L^*$ = 9.6) and in particular the $L^*$ value decreased again after 30 washes ($L^*$ = -2.1), indicating a darker color impression. Within all measured strands, the SD was low. 14 days after wash fastness test, the 30 times washed strand differed from the same on day 0 by $\Delta E_3$ of 0.9 indicating a stable color result.

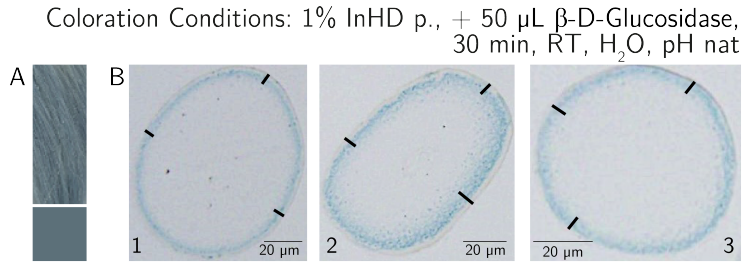
Overall, the wash fastness test showed that the color achieved with InHD p. exhibited high stability against wash cycles. Color changes remained minimal throughout all washes, even 14 days after the strand which was washed 30 times showed only minimal differences from its initial color. InHD p. thus fulfilled crucial requirements for practical application as a convenient and competitive hair dye. It could be classified as permanent.

#### 6.3.1.5 Evaluation of InHD p. Dye Penetration in Hair via Microtome Cuts

In addition to the various experiments concerning the dyeing conditions with InHD p., further insight into the processes inside the hair fiber should be obtained. Microtome cuts of InHD p. dyed hair were prepared and color penetration depth was measured (cf. figure 6.23). The work was carried out by Swenja Kalischke (Scalp Tissue Engineering, Henkel AG & Co. KGaA).

The relative penetration depth of color after application of InHD p. at RT for 30 min ranged between 8.4-17.6%, indicating a diffusion of the dye into the cortex under the applied conditions. A sharp ring was visible inside the cortex, at the cuticle border, which showed similarities to this of InCL at RT in the cross-section view. Further, a grainy color distribution within the hair fiber was visible.

Microscopic evaluation of InHD p.-dyed hair microtome cuts showed that the dye penetrated into the cortex, where it was distributed in a ring-shaped and irregular pattern. The observed granular structure and limited penetration depth compared to the dye resulting from InCL, indicated that the dye uptake is locally limited and probably influenced by hair structure. The findings provided important insights into understanding the color formation process of indigo on hair.




C

| | Penetration of Dye into the Hair Fiber | | |
|---|---|---|---|
| | 1 | 2 | 3 |
| Penetration Depth (µm) | 5.5<br>3.9<br>4.0<br>4.5±0.9 | 10.5<br>8.5<br>7.0<br>8.6±1.8 | 4.0<br>4.1<br>3.5<br>3.9±0.3 |
| Relative Penetration Depth (%) | 11.2 | 17.6 | 8.4 |

**Figure 6.23: Microscopic Images of Microtome Cuts from Strands Dyed with 1% (w/v) InHD p. and 50 µL $\beta$-D-Glucosidase Formulation.** A: Colored strand. B: Three different hair fibers of one colored strand cut and examined. C: Penetration depth (µm) depicts the absolute penetration and SD of the dye into the hair without appreciating the hair's irregular shape. The relative penetration depth in % describes the penetration in relation to the radius of the hair.

### 6.3.2 Indigo Ethanolic Extract and Indigo Ethanolic Extraction Residue

The extraction of indican from plant material was a key challenge in the development of sustainable hair dyes from *Indigofera tinctoria* L.. In addition to InHD, another approach tested for indigo extract production was the separation of both the indigo dye precursor indican as well as the enzyme responsible for glucose cleavage from InCL. This concept was introduced by patent specification US 2004/0055096A1 [125]. A possibility to separate both components from InCL was ethanolic Soxhlet extraction. The solvent was chosen because the ethanol soluble indican should be separated from the remaining plant material in the extraction sleeve, in which the ethanol-insoluble enzyme remains contained. The aim of this study was to characterize the efficiency of extraction, the yield of indican in the ethanolic extract and to evaluate the suitability for further use in the dyeing process and product development.

5 g of InCL was added to a 250 mL Soxhlet extractor and extracted with EtOH. By adding the extraction sleeve and glass beads to the extractor, the total volume of

solvent that fitted inside the extractor amounted to 127 mL in the first cycle and 120 mL every following cycle. After completion of the extraction, a distinction was made between the InCL ethanolic extraction residue left in the extraction sleeve (*InEER*) and InCL ethanolic extract (*InEE*).

In each extraction cycle, the amount of extracted indican in solution was determined. As soon as the proven quantity in a cycle approached zero, the extraction was stopped. All cycles performed together resulted in a quantity of 133.9 mg of indican, which was dissolved out of the 5 g InCL deployed and determined by HPLC. This total value could be used to determine the relative amount of indican extracted during each cycle. This way, it was defined how much solvent must come into contact with the plant material for the indican to be almost completely extracted from InCL (cf. figure 6.24).

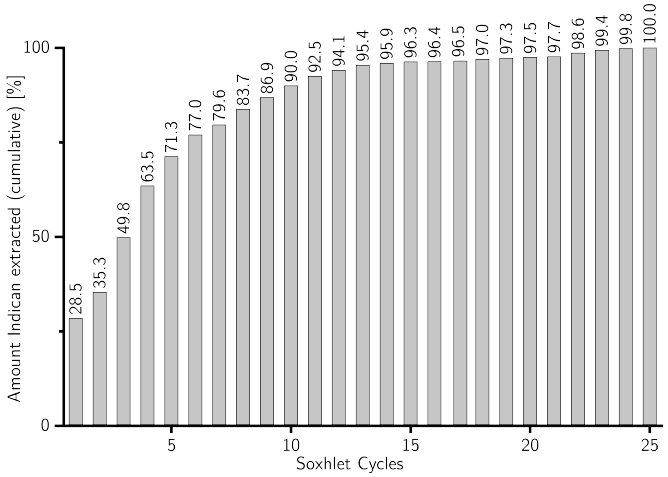



**Figure 6.24: Amount of Cumulative Extracted Indican per Soxhlet Cycle.** Cumulatively extracted Indican per Soxhlet cycle 1-25 in %, the total amount was 133.9 mg Indican/5 g InCL extracted.

Soxhlet extraction with ethanol enabled extensive separation of indican and enzymatic components from the plant source material (InCL). A total of 133.9 mg indican was extracted from 5 g InCL, with 92.5% of this amount already obtained after the first eleven extraction cycles (120.3 mg). The last 4% of the extractable indican was obtained during cycles 15 to 25. A total of 1,327 mL of ethanol had to come into contact with the plant material to enable the separation of ethanol extract (InEE) and residue (InEER).

The yield of dried InEE from 5 g InCL was 1.08 g, corresponding to 21.6% of the amount of InCL used. The residue InEER accounted for 68.0% (3.4 g). HPLC analysis revealed an indican content of 123.6 mg/g InEE and 0.6 mg/g InEER. However, it should be noted that the measured indican content in the InEE does not allow a direct conclusion to be drawn about the total amount of indican in the InCL. While 33.6 mg indican/g InCL was detected via standardized HPLC method for this plant material (cf. chapter 6.1.2.1), only 26.8 mg indican/g of InCL used for Soxhlet extraction was cumulatively detected in InEE cycles and 0.4 mg indican/g of InCL in InEER – a total of 30.2 mg/g. This suggests that the ACN/UPW mixture used for HPLC has a higher extraction efficiency than EtOH. Nevertheless, EtOH was chosen as the preferred solvent for the application due to its suitability for separating indican and enzymes. The data obtained on the extraction yield and the distribution of indican between InEE and InEER provide valuable insights for the optimization of future extraction processes and the development of high-performance, bio-based hair dyes.

#### 6.3.2.1 Evaluation of InEE and InEER Hair Coloration

In order to evaluate the dyeing potential of InEE and InEER from Soxhlet extraction, dyeing test with both were carried out in addition to the analysis of the extracts by HPLC (cf. figure 6.25). The aim was to investigate the whether the separately isolated components – indican and enzyme activity – enable a color outcome after controlled recombination comparable to InCL application on hair.

Both extracts, InEE and InEER individually resuspended in water, showed a slight darkening of the strand. 1% (w/v) InEE in water had a pH of 5.46 and 3% (w/v) InEER in water showed a pH of 7.16. The a* value decreased through treatment into the direction of green. The b* value decreased by 2.9 in the InEE control strand, whereas it was reduced by 9.0 in the InEER control strand. Overall, the controls differed from the untreated strand by a $\Delta E_1$ of 9.1 (InEE) and 17.4 (InEER). It can be concluded that both components alone do not induce staining comparable to the application of 5% (w/v) InCL. Striking about InEE was that it was not completely soluble in water and left a greasy film on the hair, which could only be washed off with the addition of detergents. However, mixing and

stirring 1% (w/v) InEE and 3% (w/v) InEER in water for 5 min exhibited a pH of 6.43.

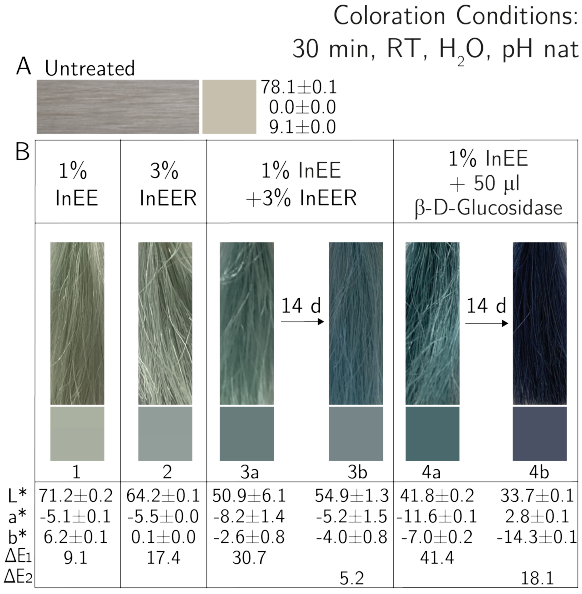



**Figure 6.25: Hair Colorations with 1% (w/v) InEE, 3% (w/v) InEER and Their Combination.** A: Untreated strand. B: Colored strands. Extracts used were 1% (w/v) InEE and 3% (w/v) InEER controls, a mixture of 1% (w/v) InEE and 3% (w/v) InEER as well as 1% InEE with 50 µL $\beta$-D-glucosidase formulation. Colorations were carried out at RT on hair for 30 min at natural pH and measured both freshly dyed and 14 days after application. $\Delta E_1$ represents the color difference between the freshly treated strand and the untreated reference strand (day 0). $\Delta E_2$ indicates the color change of the treated strand after 14 days, relative to its initial color on the respective day 0.

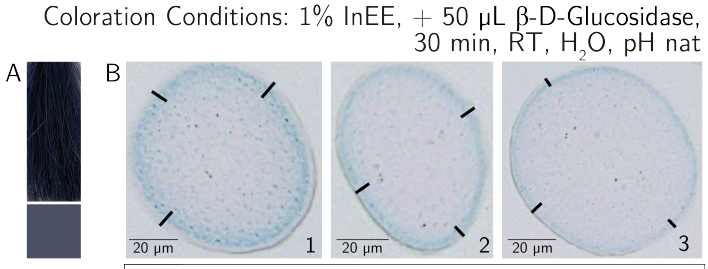
After that, a strand was added for 30 min at RT and a characteristic blue coloration was observed again. The fresh color was best comparable with the coloration result of 4% or 5% (w/v) InCL ($\Delta E = 32.0$ and 32.1) however, deviated strongly. Further, the color shift 14 days after application was minimized to $\Delta E = 5.2$. The $\Delta a^*$ and $\Delta b^*$ value with $|3.0|$ and $|1.4|$ did not change significantly within 14 days. The only striking thing was that after 14 days the strand appears slightly lighter than freshly colored ($\Delta L^* = 4.0$).

To compare those dyeing results with the coloring with InCL, it was shown that neither InEE not InEER alone produced as intense coloration as observed when using InCL. Only through targeted combination of both is was possible to achieve a characteristic blue coloration that was comparable to that of 4% or 5% (w/v) InCL. The stability observed over 14 days underscored the potential of this dyeing approach.

This successfully demonstrated that separation and subsequent recombination of the dye-relevant components from plant material is possible. A promising step towards controllable, plant-based hair dye systems of *Indigofera tinctoria* L. was taken.

#### 6.3.2.2 Evaluation of InEE Dye Penetration in Hair via Microtome Cuts

In order to assess the effect of InEER coloration not merely visually and spectrophotometrically but also inside the hair fiber, microtome cuts were made and used to measure the penetration depth of the color into the hair microscopically (cf. figure 6.26). The aim was to quantify the penetration depth of dye inside the fiber and analyze its distribution behavior to provide information about the contribution of color outcome there. The work was carried out by Swenja Kalischke (Scalp Tissue Engineering, Henkel AG & Co. KGaA).




C

| | Penetration of Dye into the Hair Fiber | | |
|---|---|---|---|
| | 1 | 2 | 3 |
| Penetration Depth (µm) | 6.0<br>7.5<br>7.8<br>7.1±1.0 | 3.3<br>3.2<br>2.5<br>3.0±0.4 | 3.2<br>3.3<br>2.3<br>2.9±0.6 |
| Relative Penetration Depth (%) | 15.9 | 8.1 | 5.4 |

**Figure 6.26: Microscopic Images of Microtome Cuts from Strands Dyed wich 1% (w/v) InEE and 50 µL $\beta$-D-Glucosidase Formulation.** A: Colored strand. B: Three different hair fibers of one colored strand cut and examined. C: Penetration depth (µm) depicts the absolute penetration and SD of the dye into the hair without appreciating the hair's irregular shape. The relative penetration depth in % describes the penetration in relation to the radius of the hair.

The measurement of color penetration depth of InEE into the hair fiber resulted in a penetration depth of 5.4-15.9% relative to the radius of hair cross-section. The absolute penetration depth amounted to 2.9 to 7.1 µm with a SD between

± 0.5 to 1.0 µm. These values indicated a limited but measurable diffusion of dye molecules into the cortex. It could be noted that a blue ring on the outer edge of the hair cortex and a rather greasy color distribution formed, suggesting an aggregation behavior of dye.

Microscopic analysis of microtome cuts after dyeing with InEE showed that the dye compounds penetrate the hair fiber to a limited extent but into the cortex. Important information about the penetration behavior and physico-chemical properties of the extract were provided.

### 6.3.3 Comparison of Hair Coloration with InEE and InHD p. and Their Combination with InEER

To evaluate the applicability of the indigo extracts developed, a direct comparison was made between the ethanolic extract (InEE) and the powdered aqueous heat-dentaured extract (InHD p.). The aim was to identify differences in color stability, handling, and dye intensity and to examine the combination of both with the enzyme-containing ethanolic extraction residue (InEER). The investigations included both direct dyeing tests and their stability as well as the suitability of the respective dyeing systems for suitability of sustainable hair dyeing from *Indigofera tinctoria* L..

First, 0.5 g of both extracts each were mixed with 50 mL water (1% w/v) and 50 µL $\beta$-D-glucosidase formulation and applied to a strand. Here again, InEE was not completely soluble in water and had to be washed off with detergents as it left a greasy film on the hair. This was not the case for InHD p., which was washed off easily. However, both dyeing approaches showed a color on hair (cf. figure 6.27), which differed by $\Delta E = 6.4$ on day 0. The most prominent difference was the $\Delta L^*$ value which diverged by $|5.9|$. Within 14 days the $\Delta E$ value increased to 18.7. The change is also visible within the strands. Whereas InHD p. reactivated with $\beta$-D-glucosidase formulation exhibited a $\Delta E$ of 4.9, the InEE with $\beta$-D-glucosidase formulation showed a $\Delta E$ of 18.1. On the one hand, the latter strand showed a lower $L^*$ value with $|8.1|$ and $b^*$ value with $|7.3|$. On the other hand, the biggest change was visible on red-green axis with a difference in $a^*$ value of $|14.4|$ into the red direction.

Compared to InCL colorations (cf. figure 6.1), on day 0 the lowest difference in coloration between 1% (w/v) InEE with enzyme reactivation was 4% (w/v) InCL ($\Delta E = 21.8$) and 5% (w/v) InCL ($\Delta E = 21.7$). 1% (w/v) InHD p. application with enzyme addition showed lowest differences towards 2%, 4% and 5% (w/v) InCL. The differences amounted to $\Delta E$ of 16.8 (2%) and 17.4 (4% and 5%). Both extract colorations appeared with lower b* values (|9.0| for InEE and |7.6| for InHD p.) than 5% (w/v) InCL. This was visible in extract color results on strands into a more blue and less yellow direction.

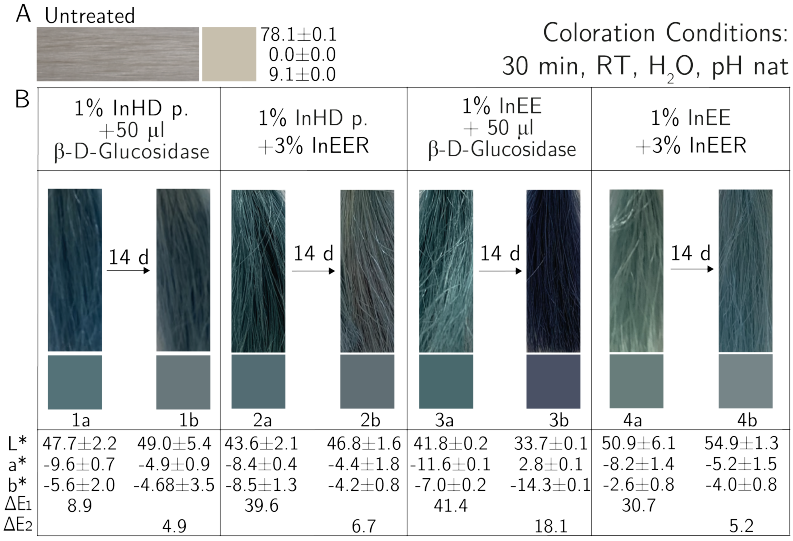



**Figure 6.27: Comparison of Hair Colorations 1% (w/v) InHD p. and 1% (w/v) InEE Reactivated Both with 50 µL $\beta$-D-Glucosidase Formulation and 3% (w/v) InEER.** A: Untreated strand. B: Colored strands. 1% (w/v) InHD p. and 1% (w/v) InEE were both combined with 3% (w/v) InEER controls or 50 µL $\beta$-D-glucosidase formulation each. Colorations were carried out at RT on hair for 30 min at natural pH and measured both freshly dyed and 14 days after application. $\Delta E_1$ represents the color difference between the freshly treated strand and the untreated reference strand (day 0). $\Delta E_2$ indicates the color change of the treated strand after 14 days, relative to its initial color on the respective day 0.

As the InEE extract from Soxhlet extraction featured dyeing results that came close to staining with InHD p., it was tested whether the dyeing feasibility could be approved. Therefore, InEE was resuspended in water and shaked against petroleum ether (*PE*) with the addition of sodium chloride (*NaCl*) for improved phase separation. Both phases were examined with TLC with a solvent mixture of ethyl acetate and isopropanol (4:1). The plate was stained with 1.2% (v/v) $\beta$-D-glucosidase solution and viewed under UV light at 245 nm. The water phase was applied to strand

without enzyme addition and the addition of 50 µL $\beta$-D-glucosidase formulation on day 0 and after 14 days (cf. figure 6.28). It was shown that most of the indican contained in InEE remained in the water phase after liquid-liquid extraction by shaking with PE. This was indicated by lower $R_f$ value of the polar molecule on the polar stationary phase in solvent mixture of ethyl acetate and isopropanol (4:1) than the PE phase which contained less polar natural indigo (with $R_f = 0.72$) and indirubin (with $R_f = 0.67$).

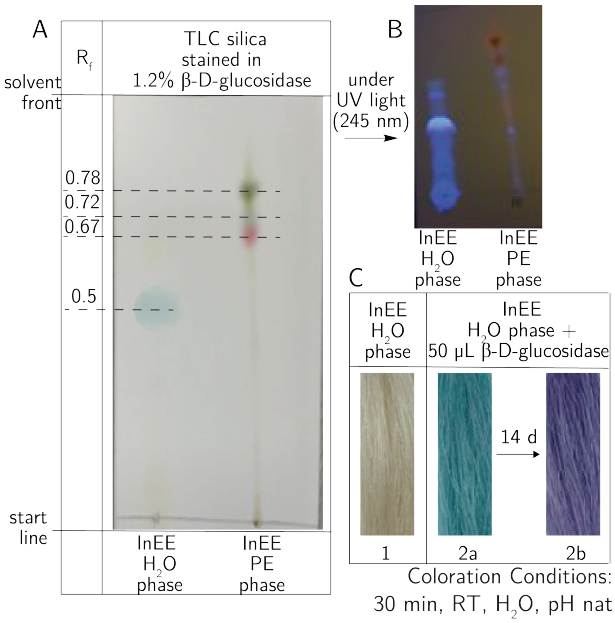



**Figure 6.28: TLC of InEE Phases and Coloration of $H_2O$ Phase with and without Addition of $\beta$-D-Glucosidase Formulation.** Silica plate of InEE water and petroleum ether phases with solvent mixture of EtOAc and isopropanol (4:1) after staining with 1.2% (v/v) $\beta$-D-glucosidase solution (A) and under UV light at 245 nm (B). C: Coloration of water phase with and without addition of 50 µL $\beta$-D-glucosidase formulation on hair measured on day 0 and day 14.

**Table 6.3: $R_f$ Values from TLC of InEE $H_2O$ Phase and InEE Petroleum Ether Phase.**

| Number | Substance | $R_f$ value |
|---|---|---|
| 1 | InEE $H_2O$ phase | 0.5 |
| 2 | InEE PE phase | 0.67, 0.72, 0.78 |

Another indicator for indican content in water phase was the appearance of blue color after TLC plate enzyme staining. However, it was found that the InEE resuspended in water and shaken out with PE still left a sticky, oily film on the hair fiber.

Further, InEER and InHD p. were combined and applied on hair strands to see if the enzyme activity was transferable to another system outside the Soxhlet extraction and if indican remaining from InHD p. could be reactivated with activities besides the commercial $\beta$-D-glucosidase formulation (cf. figure 6.27). For this, 0.5 g of InHD p. was suspended in 50 mL water (1% w/v) and 1.5 g of InEER was added (3% w/v) which resulted in a pH of 6.8. For evaluation, coloration reactivation with InEER and a commercially available $\beta$-D-glucosidase formulation were compared. It could be shown that both reactivation options led to a color impression on hair (cf. figure 6.16 and figure 6.25). However, the coloration results differed from each other in terms of initial staining ($\Delta E = 1.8$) and color development within 14 days ($\Delta E = 2.3$). The InHD p. coloration reactivated with cellulase only changed by $\Delta E = 4.9$ between day 0 and day 14, whereas the reactivation with InEER lead to a change in color of $\Delta E = 6.7$.

All in all, it could be concluded that the application of 1% (w/v) InHD p. reactivated with 50 µL $\beta$-D-glucosidase formulation offered the most suitable handling in terms of hair coloration. For product development, storage of InCL in powder form was more favorable than in liquid form. Further, InHD p. could be washed out easily without leaving a sticky film and a stable color result was achieved over two weeks. The color shift that was observed with 5% (w/v) InCL could be stopped. These findings were crucial for the further development of an environmentally friendly and effective plant-based hair dye.

## 6.4 Red Coloration with *Indigofera tinctoria* L.

During the hair coloration with indican, InCL, and its extracts, a color change from initial blue to purple was repeatedly observed over the course of 14 days after application. This indicated that there was at least one other coloring component in the InCL that is developed within the first two weeks after dyeing indicated by the color shift. This aspect was investigated further. The possibility of forming several color results with one plant material was of interest for various reasons such as flexibility, variation and costs. It is known from literature that a side reaction including isatin can form indirubin from indican. For this reason, it was attempted to create conditions under which indirubin, and less indigo, are initially formed from indican.

### 6.4.1 Preferential Indirubin Synthesis with *Indigofera tinctoria* L. on Hair

Some of the results presented in this chapter have been previously published in the article by Klaas *et al.* (©2025 Wiley), and are reused here with permission. The article was developed as part of the research presented in this dissertation. [126]

The targeted production of a red color from *Indigofera tinctoria* L. represents a promising expansion of the natural color spectrum. Building on findings from Han *et al.* (2012) and Kim *et al.* (2019) regarding the indirubin biosynthesis in microbiological systems influenced by cysteine supplementation, research was conducted to determine whether these mechanisms could be transferred to hair coloration (cf. chapter 3.2.2.1). [64, 72] The focus was on the role of cysteine and isatin as possible dye modulators of dye formation in combination with InCL. The aim was to identify the conditions under which indirubin is preferentially formed instead of natural indigo in order to achive a stable red hair dye.

For this purpose, 5% (w/v) of InCL was mixed with 1% (w/v) (R)-(+)-cysteine (colorless) in water. After stirring the solution for 5 min, a hair strand was placed in the dye dispersion for 30 min and stirred at RT (cf. figure 6.29). The natural pH of the mixture was 6.9. [126]

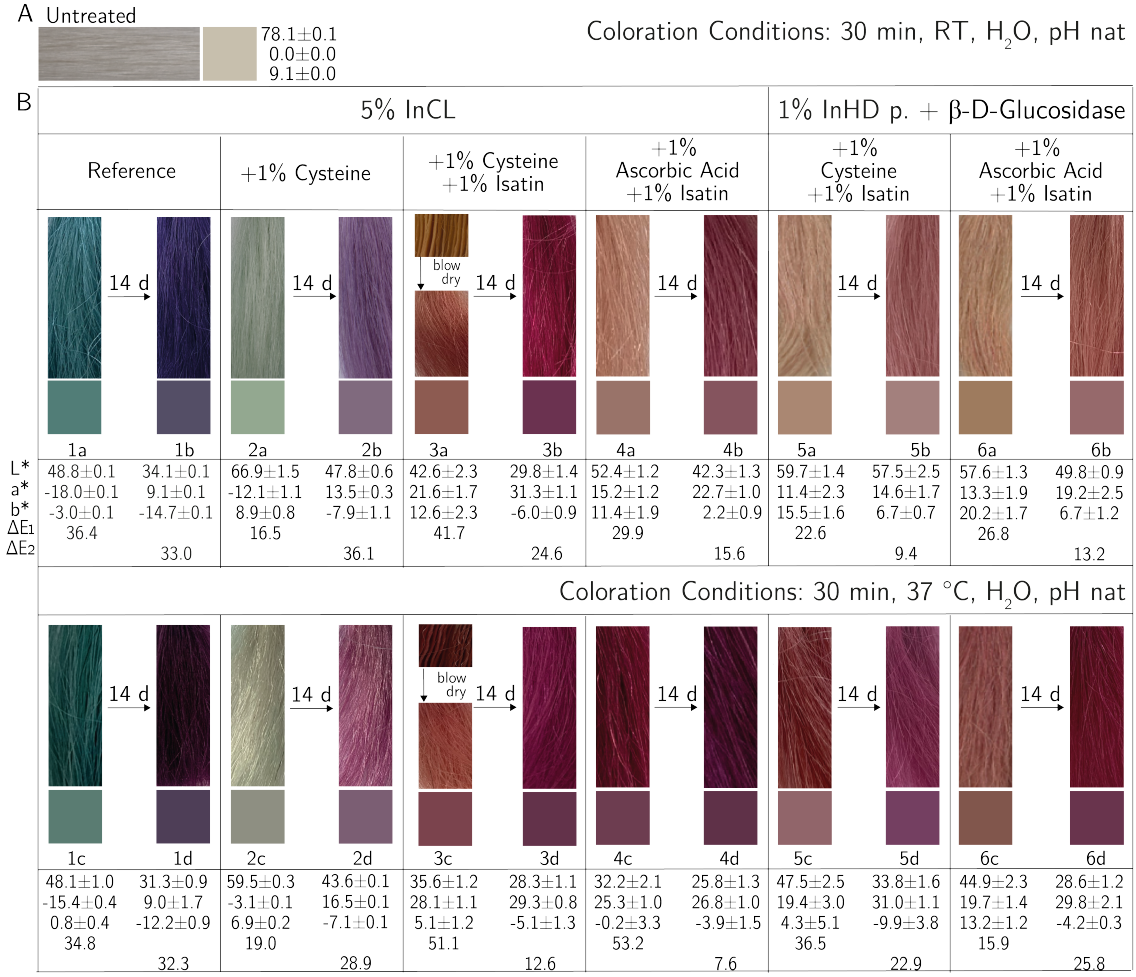



**Figure 6.29: Hair Colorations with Several Combinations of 5% (w/v) InCL or 1% InHD p. and 50 µL $\beta$-Glucosidase Formulation with 1% (w/v) Cysteine, 1% (w/v) Isatin and 1% (w/v) Ascorbic Acid.** A: Untreated strand. B: Colored strands. Each coloration was carried out for 30 min at RT. Color assessment took place directly after application and freshly dyed and 14 days after that. $\Delta E_1$ represents the color difference between the freshly treated strand and the untreated reference strand (day 0). $\Delta E_2$ indicates the color change of the treated strand after 14 days, relative to its initial color on the respective day 0. Adapted with permission from Klaas *et al.* (©2025 Wiley). [126]

Immediately after dyeing, a pale color result with a green tinge could be seen on the hair strand. After 14 days, a red shade on strand was developed. The color difference between these strands was evident in a $\Delta E_2$ of 36.1 (cf. figure 6.29, strands 2a & 2b). The strands colored with only 5% (w/v) InCL (1a & b) differ from the strands, to which cysteine was added, on day 0 by $\Delta E = 12.2$ and on day 14 by $\Delta E = 8.6$ (cf. figure 6.29). The addition of 1% (w/v) cysteine to the solution prevents a strong

turquoise/blue coloration from the beginning. In the course of two weeks after dyeing, a red tone on strand develops primarily. [126]

From previous work [63] it is known that isatin is a by-product formed from indoxyl and plays a crucial role in indirubin synthesis. One molecule indoxyl and one molecule isatin, together in an oxidative environment, form indirubin. In comparison, for indigo formation, no isatin is needed as it is formed from two indoxyl molecules [63]. In order to create the most favorable conditions for indirubin synthesis, in addition to 5% (w/v) InCL and 1% (w/v) cysteine, 1% (w/v) isatin was added to the color suspension. During rinsing of the strand, a slight rose shade was visible on the strand. Only when the hair coloration protocol was continued with the blow-drying step (around 50 °C), a red shade developed (cf. figure 6.29, strands 3a & 3b). Since the temperature treatment had a significant influence on the coloration, the following coloring was carried out at a higher temperature with the same dyeing preparation (cf. figure 6.29, strands 3c & 3d). The increase in color intensity with InCL, cysteine and isatin suspension at 37 °C compared to RT was depicted in a $\Delta E = 12.1$ on day 0. 14 days after application the strands approached each other in terms of color similarity and showed a difference of $\Delta E = 2.7$ between the two application temperatures. The development of color within one strand differed as well: Strands treated at 37 °C changed within 14 days by $\Delta E_2 = 12.6$ which was less than the ones treated at RT with a $\Delta E_2 = 24.6$. [126] Furthermore, the relevance of isatin in the dye bath at an elevated temperature (strands 2c&d) was also demonstrated by the fact that the addition of only cysteine to InCL at an elevated temperature did not lead to a comparable color result.

In order to gain further insight into the underlying mechanisms, cysteine was exchanged in dye bath. Ascorbic acid was tested as another reducing agent for its influence on dyeing with InCL in combination with isatin [72, 127] (cf. figure 6.29, strands 4a - 4d). [126] The color of the strands treated with cysteine and isatin at 37 °C and the ones treated with ascorbic acid and isatin at 37 °C vary by $\Delta E = 6.9$ on day 0, whereas the strands dyed with the addition of ascorbic acid instead of cysteine appear more intense and darker ($L^* = -3.4$). After 14 days the difference between these strands decreases to $\Delta E = 3.7$. However, the tendency of difference in the brightness of the strands remains ($L^* = |2.5|$). The strands treated with ascorbic acid and isatin at 37 °C only differ by $\Delta E_2 = 7.6$ within 14 days, whereas the cysteine with isatin ones differ by $\Delta E_2 = 12.6$. [126]

In addition, a red coloration of strands was also achieved immediately after application with the aqueous, heat-denatured indigo extract InHD in combination with either 1% (w/v) cysteine and 1% (w/v) isatin or 1% (w/v) ascorbic acid and 1% (w/v) isatin both at RT and at 37 °C (5a - d, 6a - d).

As a control, in order to exclude the all-encompassing influence of isatin, cysteine, and ascorbic acid, strands were treated with different combinations of the additives without InCL (cf. figure 6.30, strands 1-3). [126]

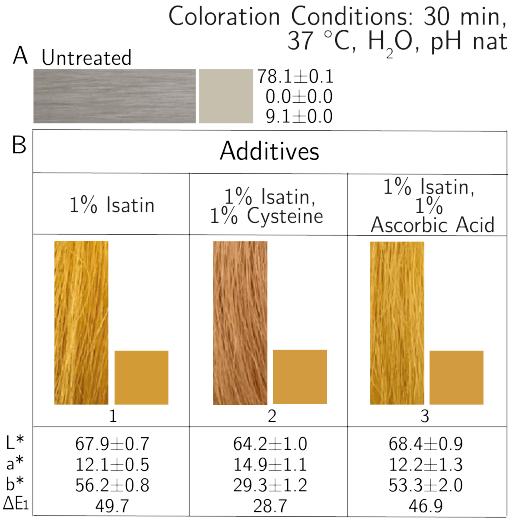



**Figure 6.30: Hair Colorations with 1% (w/v) Isatin, 1% (w/v) Isatin and 1% (w/v) Cysteine, as well as 1% (w/v) Isatin and 1% (w/v) Ascorbic Acid.** A: Untreated strand. B: Colored strands. Each coloration was carried out for 30 min at 37 °C. Adapted with permission from Klaas *et al.* (©2025 Wiley). [126]

These results proved the necessity of InCL plant material for the red dyeing results as the color produced on hair with only isatin, isatin and cysteine, or isatin and ascorbic acid were all yellow or yellow-orange and none of them showed the characterisitc magenta dye which occured with addition of InCL. All in all, it was assumed that the addition of isatin and cysteine or ascorbic acid to InCL dyeing prevented the formation of indigo molecule and stimulated the formation of indirubin, resulting in a stable red coloration immediately after application. [126]

An innovative color shade from InCL in combination with natural additives and at elevated dyeing temperatures that is created immediately after the application, and remains stable for 14 days was developed. The color could be clearly attributed to the presence of InCL, as the additives alone did not produce a comparable color. This

offers revolutionary possibilities in natural hair coloring, as two different, controllable colors - blue and red - could be obtained from one plant. Its properties will be examined further below.

### 6.4.2 Influence of Dyeing Substrate on Red Coloration with InCL

After it was demonstrated that combining InCL with isatin and ascorbic acid produced an immediately visible and stable red hair color, it was to investigate whether the color effect and its stability could be transferred to other substrates. Analogous to the blue coloration (cf. chapter 6.2.1), the role of the hair in the red coloration was to be determined. The aim was to evaluate the substrate specificity of the red dye and to examine whether hair has any special properties concerning dye development.

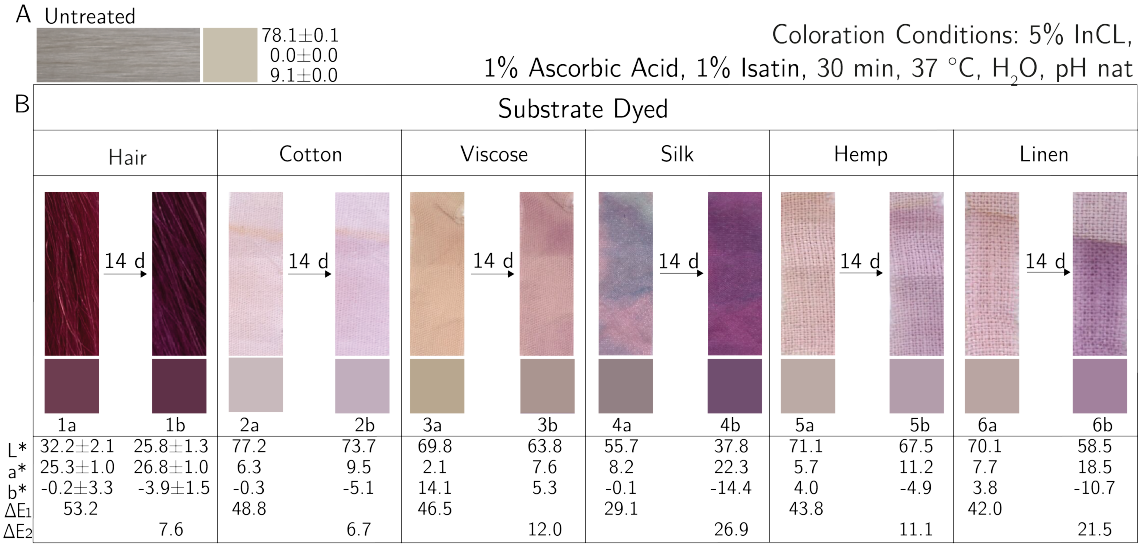



**Figure 6.31: Coloration with 5% (w/v) InCL, 1% (w/v) Isatin and 1% (w/v) Ascorbic Acid on Different Substrates.** A: Untreated strand. B: Colored substrates. In addition to hair, substrates tested were cotton, viscose, silk, hemp and linen each for 30 min at 37 °C. Color assessment took place directly after application and freshly dyed and 14 days after that. $\Delta E_1$ represents the color difference between the freshly treated strand and the untreated reference strand (day 0). $\Delta E_2$ indicates the color change of the treated material after 14 days, relative to its initial color on the respective day 0.

For this purpose, 5% (w/v) InCL, 1% (w/v) isatin and 1% (w/v) ascorbic acid in 50 mL de-ionized water at 37 °C should were used for application on cotton, viscose, silk, hemp and linen. The same dye bath as used for hair strands was used to dye

0.7 g of each fabric (cf. figure 6.31). This corresponded to the usual mass of hair used for the dye preparation (cf. chapter 6.4.1).

The darkest color result (lowest L* value) was measured on the strands treated with 5% (w/v) InCL, 1% (w/v) ascorbic acid and 1% (w/v) isatin in water at 37 °C, both freshly after coloration and 14 days later. Further, the lowest $\Delta E_2$ values were calculated between the coloration from day 0 and day 14 on cotton (6.7), followed by strand (7.6). The color development of hair strand within 14 days showed the lowest change in a* with +1.5 and b* -3.7 value among all substrates, however, with the same development directions – +a* and -b* (cotton: a*+3.2, b*-4.8; viscose: a*+5.5, b*-8.8; silk: a*+14.1, b*-14.3; hemp: a*+5.5, b*-8.9; linen: a*+10.8, b*-14.5).

These results showed that red dyeing with InCL, ascorbic acid and isatin was basically possible on all substrates tested. However, dye stability was higher on hair and on cotton compared to the other substrates. The substrate specificity underscored the particular suitability of hair for indirubin-based dyeing and provided valuable information for the targeted use of plant-based dyes in textile and cosmetic products.

### 6.4.3 Influence of Wash Fastness Test on Color Stability of Red InCL-Dyed Hair

Long-term color stability is a decisive criterion for the application of hair dyes. After the immediate and stable red coloration with InCL, ascorbic acid and isatin at elevated temperatures had been successfully demonstrated, the next step was to evaluate the dye's wash stability. The aim was to examine how repeated washing cycles affect color intensity and quality and whether the innovative red dye retains under everyday conditions. Therefore, a wash fastness test was performed on red dyed strands with 5% (w/v) InCL, 1% (w/v) ascorbic acid and 1% (w/v) isatin at 37 °C (cf. figure 6.32).

With increased washing cycles, the red colored strands respectively became darker, which was reflected by the decreasing L* value (L* 0W = 34.7 to 30W = 29.0). From 18 to 30 washes, the $\Delta L^*$ value ranged between 5.0 and 5.7. Overall, in succession of increasing number of wash cycles, the $\Delta E_2$ value increased from 4.0 to 13.5. The

tendency of the a* value from 0W to 30W moved in the red direction (+6.6), whereby the highest value was measured after 18 washes. At the same time, the b* value developed in the blue direction ($\Delta$b = -11.0) with the lowest value after 30 washes. All in all, the SD for every measured value was low ranging between ± 0.1 and 0.9. After 14 days the 30 times washed strand differed by $\Delta E_3$ of 6.2.

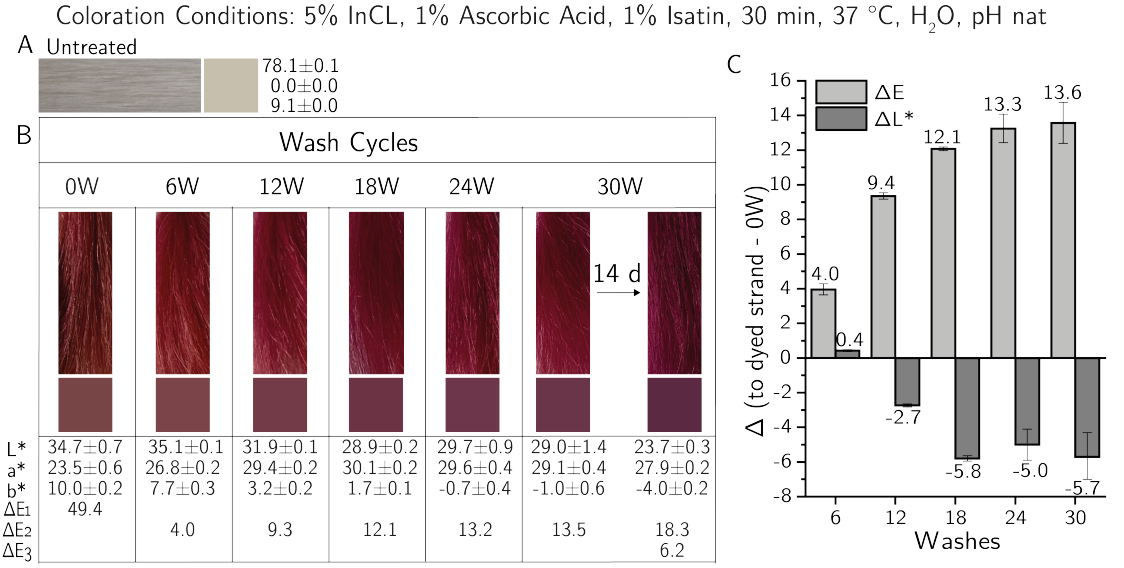
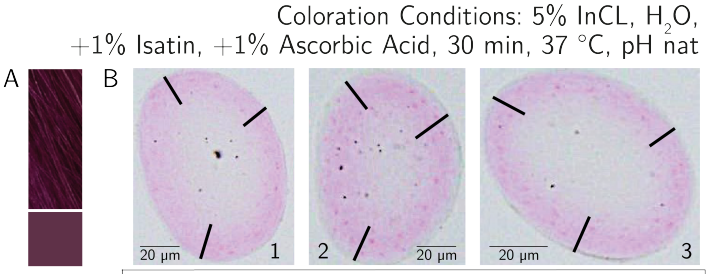



**Figure 6.32: Wash Fastness Test of Freshly 5% (w/v) InCL, 1% (w/v) Ascorbic Acid and 1% (w/v) Isatin at 37 °C Dyed Strands.** A: Untreated strand. B: Dyed strands were subjected to 6, 12, 18, 24, and 30 wash cycles. Unwashed strand was compared to untreated strand ($\Delta E_1$). Color differences ($\Delta E_2$) were measured relative to the unwashed dyed strand. The 14-day aged strand was compared to the freshly 30-times washed strand (day 0) to assess color stability over time, expressed with $\Delta E_3$. C: Bar chart with $\Delta$E and $\Delta$L* values of every wash cycle, each in relation to the unwashed strands (0W).

The wash fastness test carried out showed that the red dye from InCL and natural additives exhibited a remarkable resistance. Despite increasing wash cycles, the color characteristics remained unchanged, with the color depth even increasing, as evidenced by increasing L* value. The slight change in $\Delta$E over 14 days and the low SD underlined the robustness of the color system. This confirmed that the red dye developed is not only immediately visible, but also suitable for hair application under everyday hair wash behavior. The color can therefore be classified as permanent.

### 6.4.4 Evaluation of Red InCL Dye Penetration in Hair via Microtome Cuts

To further characterize the red dye from InCL, ascorbic acid and isatin, the distribution of dye inside the hair fiber was examined microscopically. Microtome cuts were made of the 5% (w/v) InCL, 1% (w/v) isatin and 1% (w/v) ascorbic acid treated red colored strands at 37 °C. The color penetration depth was subsequently measured and the dye inside the hair fiber localized (cf. figure 6.33). The work was carried out by Swenja Kalischke (Scalp Tissue Engineering, Henkel AG & Co. KGaA).




C

| | Penetration of Dye into the Hair Fiber | | |
|---|---|---|---|
| | 1 | 2 | 3 |
| Penetration Depth (µm) | 17.7<br>19.9<br>14.3<br>17.3±2.8 | 12.3<br>15.8<br>13.4<br>13.8±1.8 | 15.2<br>20.8<br>20.4<br>18.8±3.2 |
| Relative Penetration Depth (%) | 42.8 | 34.5 | 47.1 |

**Figure 6.33: Microscopic Images of Microtome Cuts from Strands Dyed with 5% (w/v) InCL, 1% (w/v) Ascorbic Acid and 1% (w/v) Isatin at 37 °C.** A: Colored strand. B: Three different hair fibers of one colored strand cut and examined. C: Penetration depth (µm) depicts the absolute penetration and SD of the dye into the hair without appreciating the hair's irregular shape. The relative penetration depth in % describes the penetration in relation to the radius of the hair.

Microtome cuts of the hair fibers revealed the penetration of a red dye into the hair cortex. The relative penetration depth was 34.5-47.1%. The SD of absolute penetration depths ranged between ± 1.8 to 3.2 µm. The red-colored ring in the cortex appeared as a non-uniform ring, with localized areas of higher intensity. This suggested heterogeneous dye aggregation within the fiber. A substantial diffusion of dye into the cortex was shown.

Clear penetration of the red dye into the cortex was demonstrated. These results confirmed that the red color is not only superficial but penetrates deep into the fiber and is firmly anchored there. The long-lasing color effect of InCL, ascorbic acid and isatin on hair was also structurally verified.

## 6.5 Influence of Harvest and Processing of *Indigofera tinctoria* L. Leaves on Color Outcome and Plant Composition

The quality of raw material *Indigofera tinctoria* L. is dependent on several aspects in cultivation of the plant [1]. This has a direct influence on the composition of dye-relevant ingredients. In order to systematically record these fluctuations, the composition of the plant material from three consecutive harvests was examined at various stages of processing. The aim was to analyze changes in levels of indican, indigo and indirubin, as well as their dyeing potential along the process chain – from the fresh harvest to the dried end product. Samples were taken from two fields and evaluated using HPLC and dyeing tests on hair strands. The source of plant material used in this work for harvest examinations was supplied by Indfrag Biosciences Pvt Ltd (Bangalore, India). It was cultivated in Tindivanam, Tamil Nadu, India by KMA Exports which specialises in cultivation of indigo for coloring.

The plant material examined below was not used for the experiments previously mentioned. Therefore, every sample was again applied on hair and the color outcome was assessed.

### 6.5.1 Cultivation, Harvest and Processing of *Indigofera tinctoria* L.

The cultivation of indigo plant *Indigofera tinctoria* L. starts with the sowing in February each year. After around 90 days, in April/May, the plant's first crop is ready for harvest indicated by first blossom (cf. figure 6.34, A). Harvest starts with farm workers using a sickle to cut off the plant branches and collect them (cf. figure 6.34, B). Then, loaded tractors (cf. figure 6.34, C) drive to drying areas where the branches are spread (cf. figure 6.34, D). As the leaves of the plant are fragile and the leave's structure can be quickly destroyed, care is always taken to ensure that the plant parts remain on the loading area for as short a time as possible and are spread out quickly.

For uniform drying, the harvested plants are turned around after approx. five hours in the sun. The plants which are harvested in the morning are dry the same evening [128]. There is also a harvest in the evening which dries overnight. The dried plant material is collected and threshed so that the leaves are removed from the stems, as these are the parts containing dye precursors. The detached leaves are dried once more in the sun (cf. figure 6.34, E). After thorough drying, the leaves are roughly cleaned by a filtering machine, where the leaves are dropped from above in front of the machine on a conveyor belt and drawn inside the machine by air suction. Heavier parts of the plant such as branches or seeds, as well as small stones that have gotten into the plant material during harvest, are not drawn in and fall down in front of the machine (cf. figure 6.34, E). The plant material is then packed in airtight bags and ready to be shipped (cf. figure 6.34, F). Leaves are subsequently ground so that the dye precursors can be extracted efficiently for hair coloring.

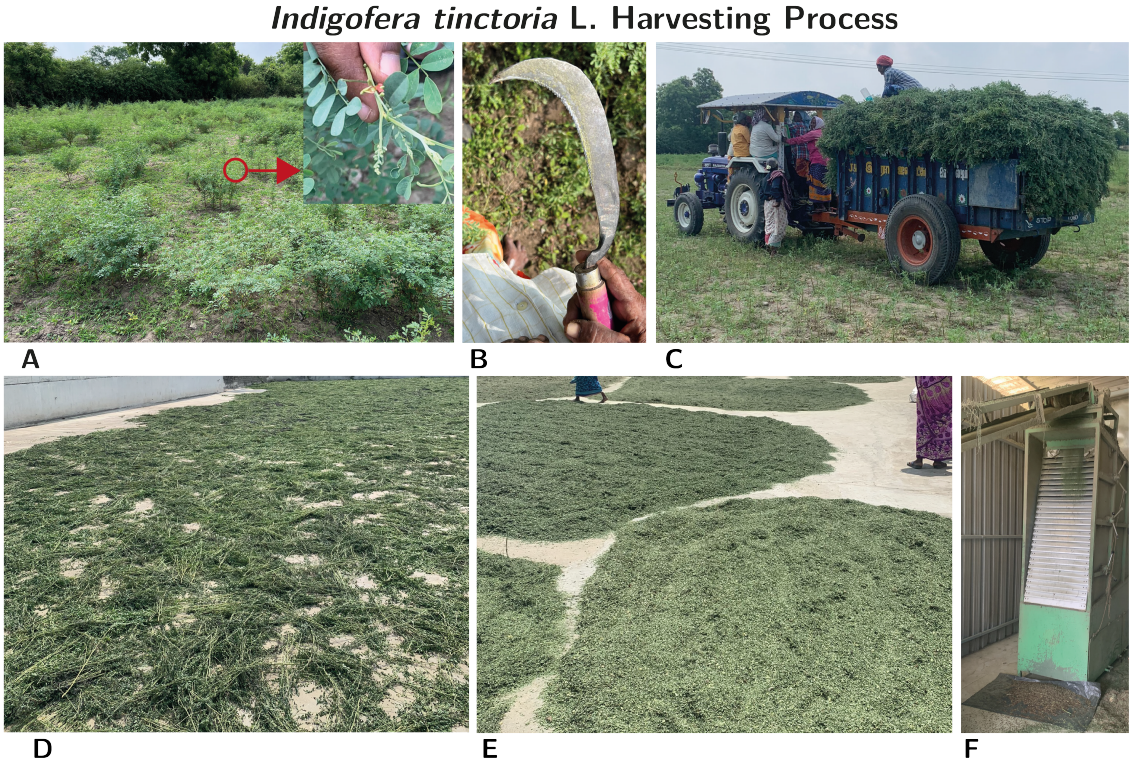



**Figure 6.34: *Indigofera tinctoria* L. Harvesting Process.** Harvesting steps: A - Indigo field, harvest maturity indicated by first blossom. B - Sickle for harvest. C - Fully loaded tractor for transport of harvested branches to drying areas. D - Branches evenly distributed on drying area. E - Detached leaves second time on drying areas. F - Filtering machine. Own recording.

A singular plant can be harvested two more times. The second and third harvest take place 45 days after the previous harvest. This means that the entire harvest cycle of a *Indigofera tinctoria* L. plant is six months, annually recurring. [104]

### 6.5.2 Examination of Plant Material Composition Throughout the Harvest

To determine the different qualities of plant material during the process of harvest until final packaging, each production step from first, second and third harvest is analyzed. The process of each crop's harvest is subdivided into three steps:

1. Collecting fresh plant leaves from field
2. Collecting fresh plant leaves after transport to drying areas before drying
3. Collecting plant leaves which were dried, threshed, cleaned roughly and final packaged

During the above mentioned steps, several samples were taken and collected in the following variants, whereby fields A and B were the same for each crop and each sampling (cf. figure 6.35). The sample designations were derived from sample 1-3, crop 1-3, and, if harvested in the morning, field A or B, and if harvested in the evening (N). This resulted in **sample-nr.crop-nrA/B/N**. For samples 1 and 2, the fresh plant material is crushed with a hand blender in ethanol directly after collection. The usage of ethanol decreased the concentration of water in the system to prevent enzymatic hydrolysis of indican and maintain the composition of dye precursors in plant which subsequently could be evaluated. After that, the ethanol was removed again with the rotary evaporator to produce a dry sample. For both sample groups, every crop was collected from two different fields in the morning. The transportation routes of both fields A and B to the drying areas were approx. 15 km which resulted in a transportation time of around 30 min. Plants from the same fields A and B were used for all harvests.

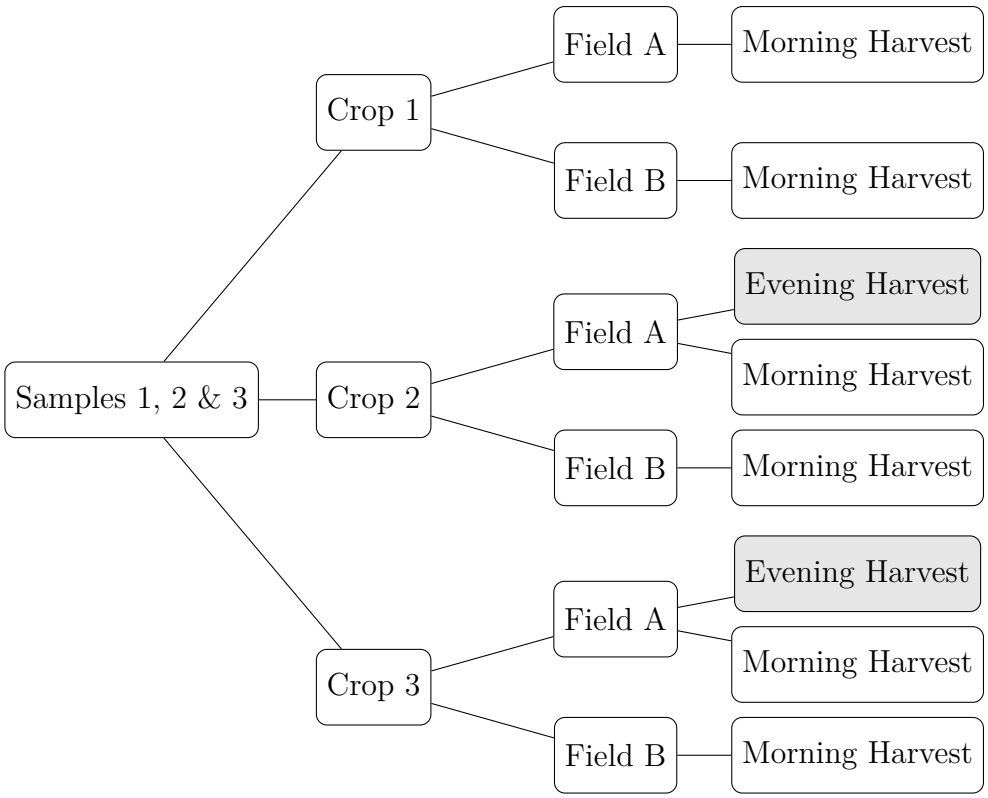



**Figure 6.35: Tree Structure of Taken *Indigofera tinctoria* L. Samples 1, 2 and 3.** Whereby Samples 1 were fresh plant leaves from field, Samples 2 were fresh plant leaves after transport to drying areas before drying and Samples 3 were dried, threshed, roughly cleaned and final packaged. Each sample was divided into crop 1-3, field A or B and harvest time, whereby evening harvests were only analyzes for samples 3 in crop 2 and 3 from field A (gray background).

Samples 3 were already dried when they were taken. They could therefore be used directly without ethanol treatment. In addition to the morning harvest, the evening harvest was also collected from one of the fields, except for crop 1. Each sample was taken three times and then analyzed three times, so that for sample 1 and 2, 54 samples each were evaluated, and 72 evaluations of samples 3 were carried out with regard to their composition of indican, indigo and indirubin content using HPLC. In addition, the samples were reviewed for their respective staining properties using the usual dyeing procedure. The analysis results are shown below grouped by type of sampling steps 1-3.

A specialty during the harvests was, that unlike crop 1 and 2, crop 3 was completely rain-fed. The leaves of both fields A and B appeared greener and denser with more mature stems and foliage.

#### 6.5.2.1 Samples 1: Fresh *Indigofera tinctoria* L. Leaves from Field

In order to determine the composition of the dye-relevant ingredients immediately after harvesting, fresh leaves of *Indigofera tinctoria* L. were homogenized in ethanol immediately after being collected in the field and then dried. These samples (samples 1) allowed the most accurate and implementable analysis possible of the plant source material before any further processing. The aim was to determine the indican, indigo, and indirubin contents via HPLC and to evaluate their influence on the dyeing properties. These samples showed the highest content of indican among indigo and indirubin (cf. figure 6.36). The quantities are given in mg of the respective molecule in one g of the raw material (RM).

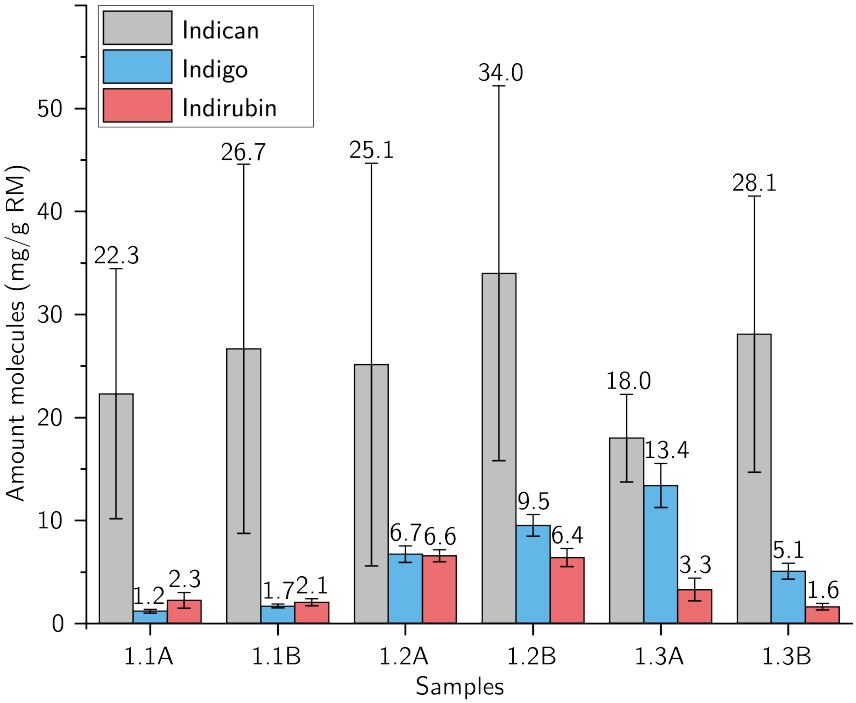



**Figure 6.36: Samples 1: Bar Chart of Fresh Plant Leaves Composition Regarding Amounts of Indican, Natural Indigo and Indirubin.** Leaves were collected from field and directly crushed in ethanol to maintain state. Afterwards, they were dried and resuspended according to analysis protocol. *Indigofera tinctoria* L. leaves of all three crops from two different fields (A and B) and harvest in the morning are shown.

Natural indigo and indirubin were detected in quantities ranging from 1.2 mg/g RM to 13.4 mg/g RM. These quantities were lower than those of indican detected among samples 1, which ranged between 18.0 mg/g RM to 34.0 mg/g RM. At the same time,

the standard deviation was significantly higher for the indican (SD = ±4.3 to ±19.5) than for indigo and indirubin (SD = ±0.2 to ±2.1). When comparing the different harvests with each other, higher amounts of indican was detected in the fresh plant leaves in crop 2 than in crop 1 and 3, whereby samples 1 of crop 1 contained slightly more indican than those of crop 3 (MV crop 1: 24.5 mg/g RM; MV crop 3: 23.1 mg/g RM). In all three crops examined, a larger amount of indican was again detected in the plant leaves harvested in field B than in field A. The amounts of indigo and indirubin are also slightly higher in the leaves of crop 2 than those of crop 1 and 3.

**Table 6.4: Samples 1: Fresh Plant Leaves Composition Regarding Amounts of Indican, Natural Indigo and Indirubin.** Fresh *Indigofera tinctoria* L. leaves of all three crops from two different fields (A and B) and harvest in the morning. MV and SD are displayed.

| Sample | Molecules (mg/g RM) | | |
|---|---|---|---|
| | Indican | Indigo molecule | Indirubin |
| 1.1A | 22.3±12.1 | 1.2±0.2 | 2.3±0.8 |
| 1.1B | 26.7±17.9 | 1.7±0.2 | 2.1±0.3 |
| 1.2A | 25.1±19.5 | 6.7±0.8 | 6.6±0.6 |
| 1.2B | 34.0±18.2 | 9.5±1.1 | 6.4±0.9 |
| 1.3A | 18.0±4.3 | 13.4±2.1 | 3.3±1.1 |
| 1.3B | 28.1±13.4 | 5.1±0.8 | 1.6±0.3 |

Application 5% (w/v) of dried samples 1 on hair led to visible colorations (cf. figure 6.37). The natural pH of these suspensions ranged between 5.9 to 6.2. The initial distinctive green coloration was particularly striking, which had not been observed to this extent in any other color preparation from *Indigofera tinctoria* L. so far. In addition to the visual assessment, this could be identified on the basis of the negative a* values (a* = -5.8 to -11.9) in combination with positive b* values, which indicated a yellow influence (b* = 1.7 to 11.5). The color development after 14 days showed a decline in the green tone in every case (Δa* = 8.7, 5.8, 5.1, 5.9, 0.2 and 0.6). At the same time, the b* value decreased which indicated a color development from yellow into a more blueish direction (Δb* = 13.0, 2.8, 9.6, 11.3, 1.4 and 1.7). However, the L* values from day 0 to day 14 increased in every strand, except for 1.3A (ΔL*: 0.2). The color shift within 14 days in total ranged between ΔE of 1.4 and 12.7.

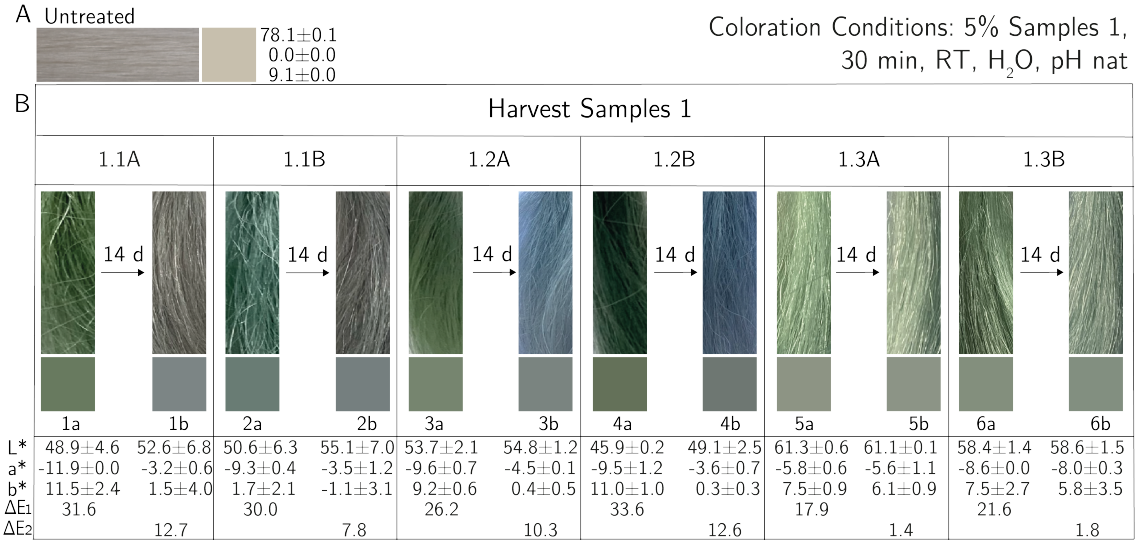



**Figure 6.37: Coloration of Fresh *Indigofera tinctoria* L. Samples 1 on Hair.** A: Untreated strand. B: Colored strands. 5% (w/v) of dry sample were suspended in water, a strand was added for 30 min at RT at natural pH. $\Delta E_1$ represents the color difference between the freshly treated strand and the untreated reference strand (day 0). $\Delta E_2$ indicates the color change of the treated strand after 14 days, relative to its initial color on the respective day 0.

In previous dyeing experiments, e.g. with pure indican (cf. chapter 6.1.2.2), it was observed that the more indican was used, the more intense hair coloration and the corresponding color shift within 14 days observed. A direct correlation between the high indican content in samples 1 and the coloration observed could not be established. The color change within 14 days could not be observed as with indican or 5% (w/v) InCL colorations. Instead of turning purple, the color changed into a gray-blue direction or stayed mainly greenish. The ethanolic processing of fresh plant leaves appeared to have an influence on color behavior.

Analysis of the fresh leaves showed that indican was present in significantly higher concentrations than indigo and indirubin, with values varying between harvests and fields. The striking green coloration of the hair after application of the dried samples was novel and differed from previous color results with *Indigofera tinctoria* L.. Despite the high indican content, no direct correlation to color intensity or development could be established. Rather, the unusual color change within 14 days indicated an influence of sample processing and matrix effects, influencing the dyeing behavior. These findings underscored the importance of targeted raw material treatment for reproducible dyeing results.

#### 6.5.2.2 Samples 2: *Indigofera tinctoria* L. Leaves after Transport to Drying Areas

In the second processing step of the *Indigofera tinctoria* L. harvest, the freshly cut plant material was analyzed after being transported to the drying facilities. The aim was to investigate the effects of transport on the composition of the molecules relevant for dyeing. To preserve molecular integrity, the leaves were homogenized in ethanol immediately upon arrival. Subsequent HPLC analysis enabled quantitative determination of indican, indigo, and indirubin, as well as an assessment of the dyeing potential of the samples. The highest amounts of molecules detected, among the examined samples with the exception of samples 2.1A, was indican (cf. figure 6.38).

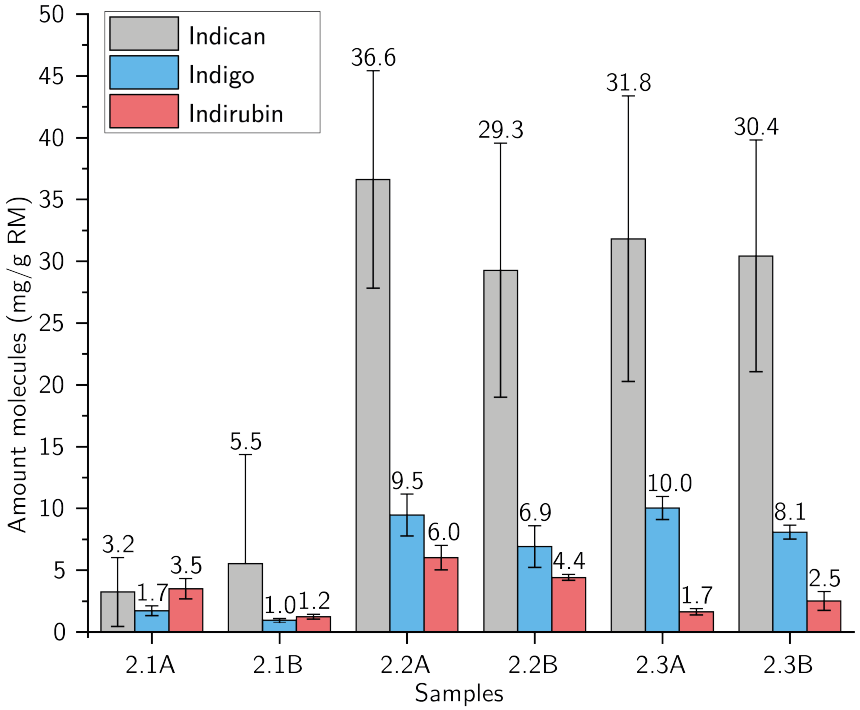



**Figure 6.38: Samples 2: Bar Chart of Transported Fresh Plant Leaves Composition Regarding Amounts of Indican, Natural Indigo and Indirubin.** Plant leaves were collected after transport to drying areas and crushed in ethanol to maintain state. Afterwards, they were dried and resuspended according to analysis protocol. Fresh *Indigofera tinctoria* L. plant leaves of all three crops from two different fields (A and B) and harvest in the morning are shown.

**Table 6.5: Samples 2: Transported Plant Leaves Composition Regarding Amounts of Indican, Natural Indigo and Indirubin.** Fresh *Indigofera tinctoria* L. plants of all three crops from two different fields (A and B) and harvest in the morning. MV and SD are displayed.

| Sample | Molecules (mg/g RM) | | |
|---|---|---|---|
| | Indican | Indigo molecule | Indirubin |
| 2.1A | 3.2±2.8 | 1.7±0.4 | 3.5±0.8 |
| 2.1B | 5.5±8.8 | 1.0±0.2 | 1.2±0.2 |
| 2.2A | 36.6±8.8 | 9.5±1.7 | 6.0±1.0 |
| 2.2B | 29.3±10.3 | 6.9±1.7 | 4.4±0.2 |
| 2.3A | 31.8±11.5 | 10.0±0.9 | 1.7±0.3 |
| 2.3B | 30.4±9.4 | 8.1±0.6 | 2.5±0.8 |

With 36.6 ± 8.8 mg/g RM indican, the crop 2 sample from field A harvested in the morning (2.2A) showed the largest total quantity of the dye precursor. The second highest amount of indican (31.8 ± 11.5 mg/g RM) was found in sample 2.3A from the same field of another crop. The comparatively low amount of indican measured in the samples 2 from crop 1 (MV crop 1: 4.4 mg/g RM) was striking. The detected amounts of indigo molecule and indirubin in samples 2 of crop 1 from both fields were lower than those of crop 2 and 3.

Overall, the use of 5% (w/v) of the dried samples 2 for application to the hair resulted in a coloration (cf. figure 6.39). The natural pH of these suspensions ranged between 5.8 to 6.2. Similar results to the staining with samples 1 were observed. Initially, every color result was strikingly green which was, in addition to visual assessment, also evident from the low a* values and higher b* values (a* = -9.3 to -12.0; b* = 1.7 to 10.9). Over the course of 14 days, the a* values increased in the more reddish direction (a* = -1.0 to -7.9) and b* values decreased into the blue direction (b* = -4.1 to 1.7). The color of strands 2.1A, 2.1B, 2.2A and 2.2B became brighter which was reflected in higher L* values of strands measured 14 days after color application ($\Delta$L* = 2.7, 4.1, 2.4 and 7.4). All the changes described within 14 days could also be expressed with $\Delta$E values between 4.3 to 15.0.

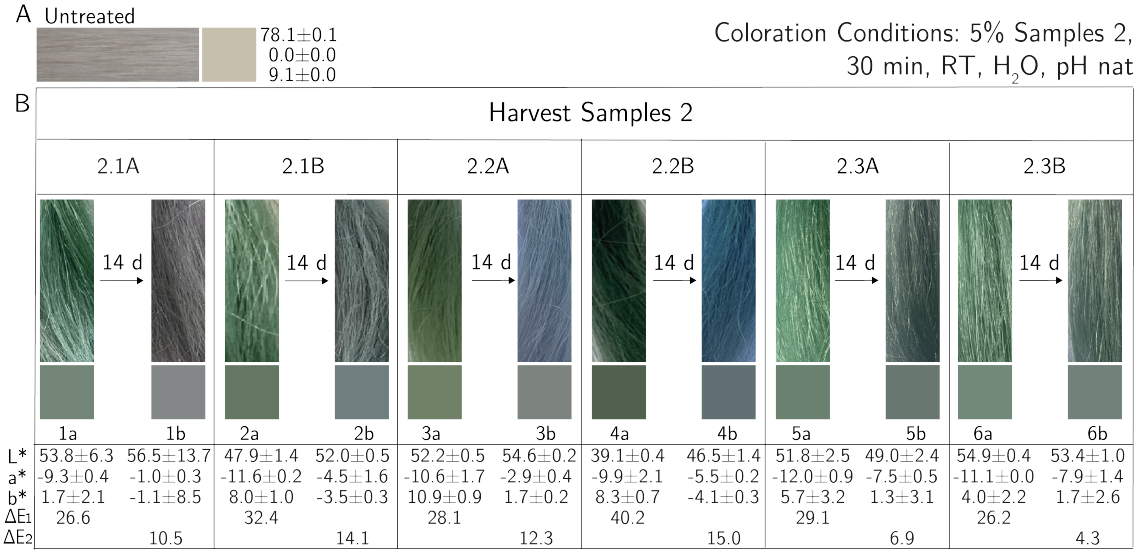



**Figure 6.39: Coloration of Transported *Indigofera tinctoria* L. Samples 2 on Hair.** A: Untreated strand. B: Colored strands. 5% (w/v) of dry sample were suspended in water, a strand was added for 30 min at RT at natural pH. $\Delta E_1$ represents the color difference between the freshly treated strand and the untreated reference strand (day 0). $\Delta E_2$ indicates the color change of the treated strand after 14 days, relative to its initial color on the respective day 0.

When dyeing the hair with samples 2, no parallelism could be established between the use of more indican and a more intense dyeing result. E.g. when comparing colorations on strands 2.1A & B with 2.2 & B, which samples showed clearly different amounts of indican contained, no such distinction could be found (cf. chapter 6.1.2.2). A color change was observed within 14 days on all strands, but it did not move in the usual purple direction, but rather toward gray/blue or stayed green. These results were in line with those of the coloration of samples 1 (cf. chapter 6.5.2.1).

The results showed that after transport of freshly harvested samples to drying areas on a tractor, indican remained the dominant compound in terms of quantity, with the highest concentrations found in the samples from the second harvest. The dyeing experiments again revealed a striking green initial coloration, which changed to gray-blue or stayed greenish within 14 days. Despite high indican contents, no direct correlation to color intensity could be established, indicating complex interactions between molecular structure, matrix effects, and processing influences. The observations confirm that even the transport of the plant material can have an influence on subsequent color development and should therefore be considered a critical factor in raw material processing.

### 6.5.2.3 Samples 3: *Indigofera tinctoria* L. Leaves after Drying, Threshing, Cleaning Process and Final Packaging

In the final processing step of the *Indigofera tinctoria* L. harvest, samples 3 were taken after drying, threshing, coarse cleaning using air flow, and final packaging. The aim was to analyze the molecular composition of the fully processed plant material and evaluate its dyeing potential (cf. figure 6.40).

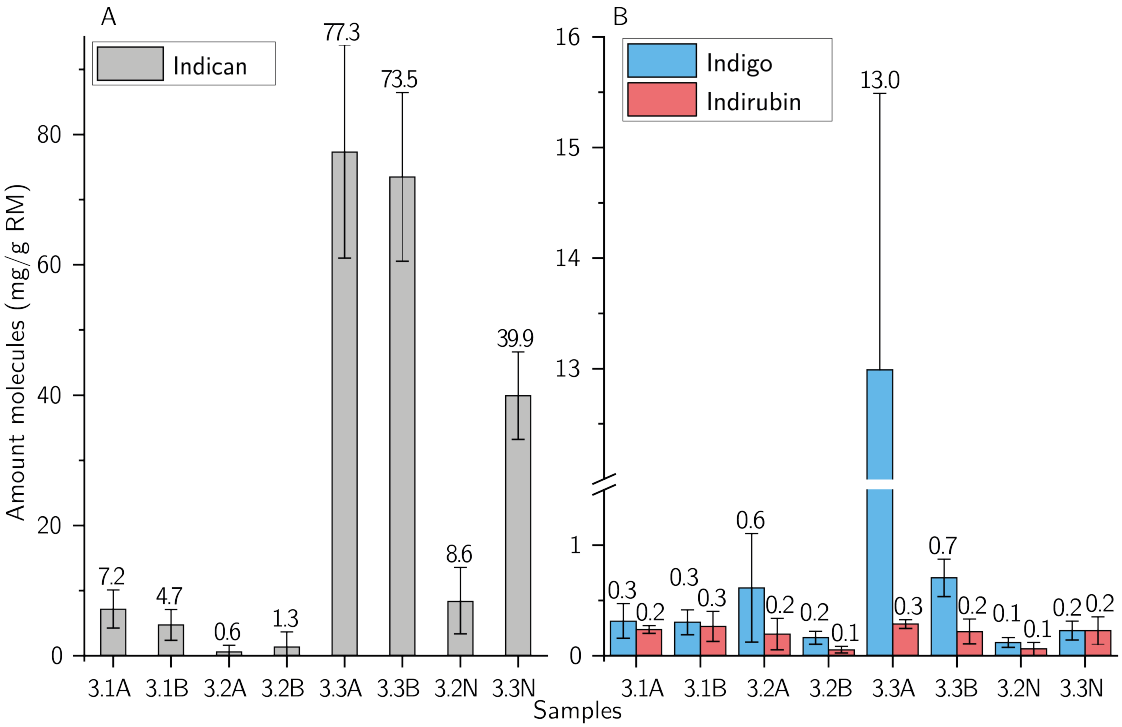



**Figure 6.40: Samples 3: Bar Charts of Dried, Threshed, Roughly Cleaned and Final Packaged Plant Leaves Composition Regarding Amounts of Indican, Natural Indigo and Indirubin.** Amounts of detected indican strongly outweighed indigo and indirubin. For clarity, molecule amounts are displayed in two separate diagrams. A: Amount of detected indican among all samples 3. B: Quantities of detected natural indigo and indirubin among all samples 3. Plant leaves were collected after drying, threshing, roughly cleaning and final packaged. *Indigofera tinctoria* L. plant's leaves of all three crops from two different fields (A and B) and harvest in the morning are shown. Evening harvest samples were only collected from field A for crop 2 and 3.

These samples 3 represent the condition of the material as it is typically intended for processing or sale. In all samples, the amount of detected indican strongly outweighed indigo and indirubin. For clarity, two diagrams were created. One shows the amount

of indican in the samples 3, the other shows the amounts of natural indigo and indirubin among the samples (cf. figure 6.40).

**Table 6.6: Final Packaged Samples 3: Plant Composition Regarding Amounts of Indican, Natural Indigo and Indirubin.** Dried, threshed, roughly cleaned and final packaged *Indigofera tinctoria* L. plant leaves of all three crops from two different fields (A and B) and harvest in the evening (N). MV and SD are displayed.

| Sample | Molecules (mg/g RM) | | |
|---|---|---|---|
| | Indican | Indigo molecule | Indirubin |
| 3.1A | 7.2±2.9 | 0.3±0.2 | 0.2±0.0 |
| 3.1B | 4.7±2.4 | 0.3±0.1 | 0.3±0.1 |
| 3.2A | 0.6±1.0 | 0.6±0.5 | 0.2±0.1 |
| 3.2B | 1.3±2.3 | 0.2±0.1 | 0.1±0.0 |
| 3.3A | 77.3±16.3 | 13.0±2.5 | 0.3±0.0 |
| 3.3B | 73.5±12.9 | 0.7±0.2 | 0.2±0.1 |
| 3.2N | 8.6±5.1 | 0.1±0.0 | 0.1±0.1 |
| 3.3N | 39.9±6.7 | 0.2±0.1 | 0.2±0.1 |

What was striking when examining sample 3 was the very high indican contents in crop 3 (3.3A: 77.3 ± 16.3 mg/g RM; 3.3B: 73.5 ± 12.9 mg/g RM; 3.3N: 39.9 ± 6.7 mg/g RM). The largest total amount of indican among all samples of the harvest studies was found in sample 3.3A. The most natural indigo was detected in 3.3A with 13.0 ± 2.5 mg/g RM and the largest amount of indirubin was found in 3.1B and 3.3A with 0.3 ± 0.1/0.0 mg/g RM. A specialty of sample 3.2A was that the quantity of indican and natural indigo was the same (indican and natural indigo: 0.6 ± 1.0 mg/g RM).

According to the usual protocol, samples 3 were added to water in a concentration of 5% (w/v) and strands were dyed (cf. figure 6.41). The natural pH of dyeing suspension ranged between 6.0 and 6.5.

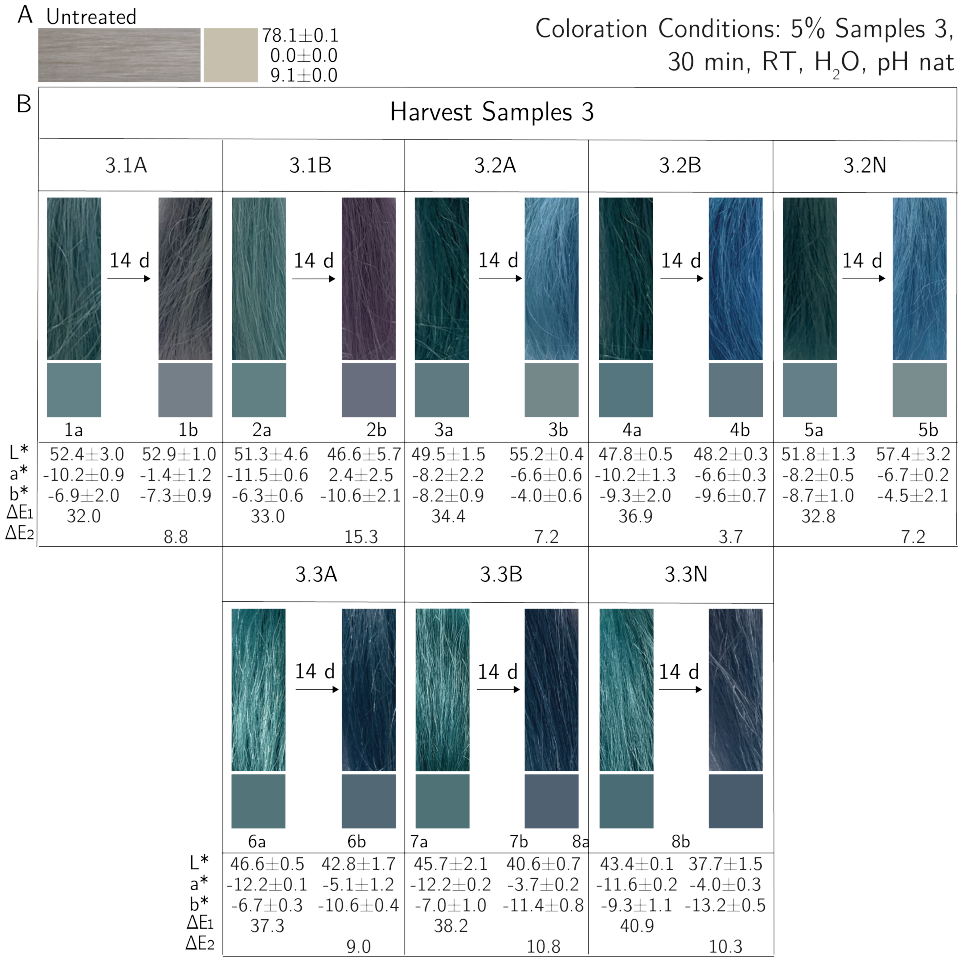



**Figure 6.41: Coloration of Dried, Threshed, Cleaned and Packaged *Indigofera tinctoria* L. Samples 3 on Hair.** A: Untreated strand. B: Colored strands. 5% (w/v) of dry sample were suspended in water, a strand was added for 30 min at RT at natural pH. $\Delta E_1$ represents the color difference between the freshly treated strand and the untreated reference strand (day 0). $\Delta E_2$ indicates the color change of the treated strand after 14 days, relative to its initial color on the respective day 0.

The darkest color result of all sample 3 colorations on day 0 was achieved with 3.3N (L* = 43.4 ± 0.1). All strands initially had a* values in the green direction and b* values in the blue direction (a* = -8.2 to -12.2; b* = -6.2 to -9.3). Within 14 days, the biggest change of color was detected within coloration of sample 3.1B ($\Delta E = 15.3$). The change took place into a darker and more blueish direction, but the biggest difference was seen in the a* value – it increased by 13.9 to 2.4 ± 2.5. It

was noticeable that only the strands treated with sample 3.1B and all samples 3 of crop 3 (3.3A, 3.3B and 3.3N) became darker within 14 days indicated by the measured $\Delta L^*$ values between -3.8 and -5.7. Furthermore, only when staining with previously mentioned samples, the characteristic color shift toward purple was detected. In all other colorations, a color change was also observed, but either towards gray or blue. Samples 3.3A, 3.3B, and 3.3N showed the most comparable dyeing behavior to the plant material observed in all other coloration experiments apart from the harvest investigation underlying this work.

Analysis of the fully processed samples showed that indican was still the dominant compound in terms of quantity, but in significantly lower concentrations than in earlier processing stages in cases of crop 1 and 2, except for samples 3 of crop 3. The dyeing tests yielded predominantly greenish-bluish shades with variable color stability. Only individual samples, such as 3.2B and crop 3, showed the characteristic color shift towards purple, as known from previous InCL applications. These results illustrate that the final processing of the plant material has a significant influence on the composition and dyeing behavior. For consistent color performance, targeted control of the process parameters up to the final packaging is therefore essential.

#### 6.5.2.4 Comparison of Indican Content in Crop 1, 2 and 3

Since indican, as the central dye precursor in *Indigofera tinctoria* L., contributes significantly to dyeing performance (cf. chapter 6.1.2.2), its content was recorded in summary and compared across all harvest steps and times. The aim was to identify differences between the three harvests (crop 1–3) and between the respective processing stages (samples 1–3) in order to draw conclusions about the optimal harvesting and processing conditions for maximum indican yield (cf. figure 6.42).

The evaluation showed that the highest indican contents were detected in the third harvest (crop 3), especially in the samples from the third processing stage (samples 3) with 63.6 ± 12.0 mg/g RM. The highest amounts of indican in all samples together were also found in crop 3. When all crops are considered, samples 1 were overall the ones that showed the most indican content (crop 1: 24.5 ± 15.0 mg/g RM; crop 2: 29.6 ± 18.9 mg/g RM; crop 3: 27.6 ± 8.8 mg/g RM). The most striking was that samples 3 of crop 1 and 2 (crop 1: 4.8 ± 3.4 mg/g RM; crop 2: 5.9 ± 2.6 mg/g RM) contained significantly lower indican content than crop 3.

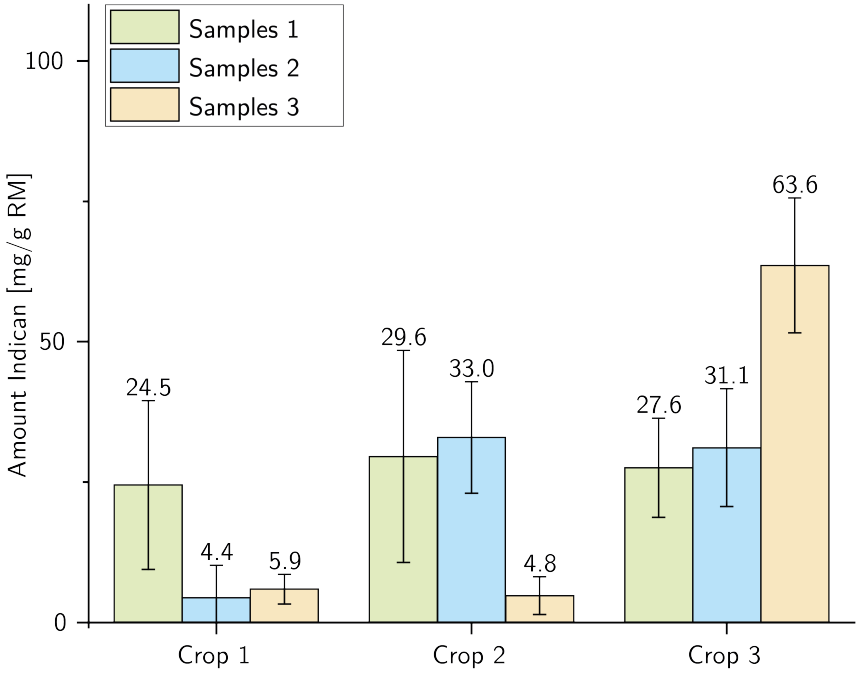



**Figure 6.42: Bar Chart for Comparison of Indican Content of all Samples 1-3.** MV and SD of indican content in all samples of every crop are displayed.

**Table 6.7: Amounts of Indican in Samples 1-3 Divided by Crop 1-3.** MV of indican content in all samples of a crop with SD.

| Crop | Sample | Amount of Indican [mg/g RM] |
|---|---|---|
| 1 | 1 | 14.5±15.0 |
| | 2 | 4.4±5.8 |
| | 3 | 5.9±2.6 |
| 2 | 1 | 29.6±18.9 |
| | 2 | 33.0±10.0 |
| | 3 | 4.8±3.4 |
| 3 | 1 | 27.6±8.8 |
| | 2 | 31.1±10.5 |
| | 3 | 63.6±12.0 |

The comprehensive analysis of samples from the three harvest stages revealed significant differences in the composition and dyeing performance of the plant material during processing. The early processing stages in particular showed higher levels of indican with exception of samples 3 of crop 3, while indigo and indirubin were found in greater quantities in the later stages of sampling. It was shown that both the time of harvest and the immediate processing of the plant material are crucial for preserving the indican content – a key factor in the development of high-performance natural dyes. These results underscored the importance of controlled process management and targeted sampling to ensure consistent quality of plant-based dyes. Furthermore, they provided a basis for optimizing raw material processing with a view to reproducible and efficient natural dyeing.

## 6.6 Dye Compound Analysis from Hair and *Indigofera tinctoria* L. Pathway Inductions

In the following section, the compounds which are responsible for the color outcome on hair are extracted and analyzed. From this, together with the previous results of the staining under various influences, a new *Indigofera tinctoria* L. biosynthesis pathway is presented that takes these findings into account in the context of hair coloration.

### 6.6.1 Chemical Analysis of Dye Compounds from Colored Hair Strands

Some of the results presented in this chapter have been previously published in the article by Klaas *et al.* (©2025 Wiley), and are reused here with permission. The article was developed as part of the research presented in this dissertation [126].

In order to better understand the chemical processes involved in dye formation on hair after the application of indigo-based dye, colored strands were analyzed. The aim was to quantitatively measure the dye molecules formed on the hair – in particular indican, indigo and indirubin – and to analyze their development over time. Important insights into the molecular basis of the dyeing results should be obtained.

Chemical extraction of dye compounds from the hair fiber after coloration with 5% (w/v) InCL in water (1), 5% (w/v) InCL with 1% (w/v) ascorbic acid and 1% (w/v) isatin (2), as well as with 1% (w/v) InHD p. and 50 µL $\beta$-D-glucosidase formulation (3) both freshly after dyeing procedure (a) and 14 days after application (b) were carried out. The differently treated strands were ground (cf. figure 6.43). The coloration approaches were performed at 37 °C and separately stirred in water for 60 min and blow dried directly before examination. This was done to prevent the extraction of color precursors that were not bonded to the hair fiber but that remained on the hair surface. The strands examined 14 days after color application were not treated with water to recreate the color development process on the hair [126].

Dyeing temperature of 37 °C was applied to create same reaction conditions for every strand.

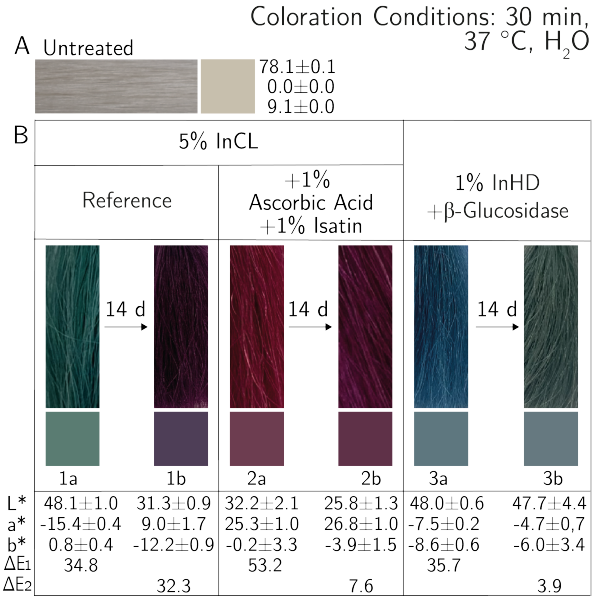



**Figure 6.43: Hair Colorations with 5% *Indigofera tinctoria* L., with the Addition of 1% Isatin and 1% Ascorbic Acid, as well as with 1% (w/v) InHD and 50 µL $\beta$-glucosidase Formulation.** A: Untreated strand. B: Colored strands. Each coloration was carried out for 30 min at 37 °C and measured both freshly dyed and 14 days after application. $\Delta E_1$ represents the color difference between the freshly treated strand and the untreated reference strand (day 0). $\Delta E_2$ indicates the color change of the treated strand after 14 days, relative to its initial color on the respective day 0. Adapted with permission from Klaas *et al.* (©2025 Wiley). [126]

The ground hair fibers were extracted both with DMSO and UPW/ACN at 80 °C for 30 min, centrifuged and the supernatant was used for HPLC quantification of natural indigo, indirubin and isatin (cf. figure 6.44 and 6.8).

The differences in color outcome in terms of L*a*b*-color values between strands 1a and 1b was $\Delta E_2 = 32.3$, between 2a and 2b it amounted in $\Delta E_2 = 7.6$ as well as $\Delta E_2 = 3.9$ between 3a and 3b. Parallel to this, the amount of indirubin on strands shifted from 1a to 1b from 0 µg/g strand to 271.4 µg/g strand. At the same time the amount of detectable indigo molecule decreased by 27.5% (189.5 to 137.5 µg/g strand). For strands 3a and 3b the amount of traced indigo molecules decreased by 43.7% (from 130.4 to 73.4 µg/g strand). The amount of indirubin increased from 61.1 to 75.8 µg/g strand by 19.4%. Largest quantity of indirubin in total was extracted from the red colored strands with 5% (w/v) InCL, 1% (w/v) ascorbic

acid and 1% (w/v) isatin at 37 °C both on day 0 (2a) and day 14 (2b). Initially 467.7 µg/g strand of indirubin could be detected on the hair fiber whereas hardly any indigo molecule was formed (10.5 µg/g strand).

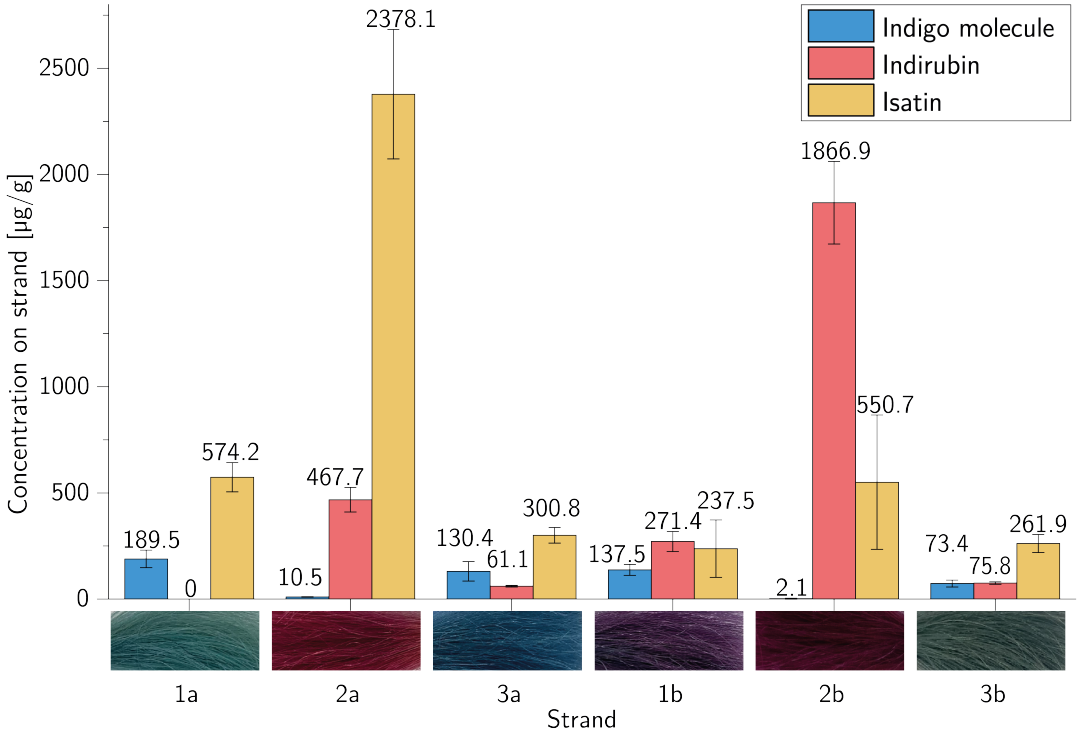



**Figure 6.44: Bar Chart of Extracted Molecule Concentrations from Colored Strands.** Strands examined were treated with 5% (w/v) InCL in water at 37 °C freshly after dyeing procedure (1a) and after 14 days (1b), strands colored with 5% InCL, 1% (w/v) ascorbic acid and 1% (w/v) isatin at 37 °C freshly after dyeing procedure (2a) and after 14 days (2b) and strands colored with 1% (w/v) InHD p. and 50 µL $\beta$-D-glucosidase formulation at 37 °C. Extracted and quantified molecules (MV $\pm$ SD) were natural indigo, indirubin and isatin. Image of the corresponding colored strands below. Adapted with permission from Klaas *et al.* (©2025 Wiley). [126]

Within 14 days after staining the amount of indirubin increased by 74.9% to 1866.9 µg/g strand and the quantity of indigo molecule decreased further by 80% to 2.1 µg/g strand. The L*a*b*-color measurement difference between strand 2a and 2b is reflected with $\Delta$E of 7.6.

A correlation could be established between the detected amounts of indigo, indirubin and isatin molecules on the hair after color formation on and the color measured

by spectrophotometer both on day 0 and day 14. The purple color formation in the 5% (w/v) dyed strands was related to the formation of the delayed indirubin molecule. An increase in formation of indirubin on the hair, compared to direct indigo formation, was observed in every strand examined between day 0 and day 14.

Chemical analysis of the dyed hair strands showed that the dye composition changed significantly after application. In all cases examined, the amount of indirubin on the hair increased within 14 days, while the indigo concentration decreased. This effect was particularly pronounced in red dyeing with InCL, isatin, and ascorbic acid, where indirubin was identified as the dominant dye. The measured color values correlated with the detected molecular quantities and confirmed the role of indirubin as the main contributor to the purple/red color development. These results prove that color formation on hair is influenced not only by the starting materials, but also by time-dependent reactions on the hair fiber as a key aspect for the development of stable, plant-based hair dyes.

**Table 6.8: Concentration of Indigo molecule, Indirubin and Isatin Extracted from Colored Strands.** Strands treated with 5% (w/v) InCL in water at 37 °C freshly after dyeing procedure (1a) and after 14 days (1b), strands colored with 5% InCL, 1% (w/v) ascorbic acid and 1% (w/v) isatin at 37 °C freshly after dyeing procedure (2a) and after 14 days (2b) and strands colored with 2% (w/v) InHD p. and 50 µL $\beta$-D-glucosidase formulation at 37 °C freshly after dyeing procedure (3a) and after 14 days (3b). MV and SD are displayed. [126]

| **Strand** | **Molecules (mg/g strand)** | | |
|---|---|---|---|
| | Indigo molecule | Indirubin | Isatin |
| 1a | 189.5±40 | 0.0±0.0 | 574.2±70 |
| 2a | 10.5±0.0 | 467.7±60 | 2378.1±30 |
| 3a | 130.4±46.0 | 61.1±3.9 | 300.8±36.9 |
| 1b | 137.5±30 | 271.4±50 | 237.5±140 |
| 2b | 2.1±0.0 | 1866.9±190 | 550.7±320 |
| 3b | 73.4±16.3 | 75.8±6.0 | 261.9±42.5 |

### 6.6.2 Revised Biosynthesis Pathway of Dye Molecules from *Indigofera tinctoria* L.

Some of the results presented in this chapter have been previously published in the paper of Klaas *et al.* (©2025 Wiley), and are reused here with permission. The article was developed as part of the research presented in this dissertation [126].

Based on the data presented above (cf. chapter 6.1 and 6.6.1), a new reaction pathway for the conversion of indican into dye solution and color formation on the hair was developed. Additionally, possible reaction sites of additives are proposed.

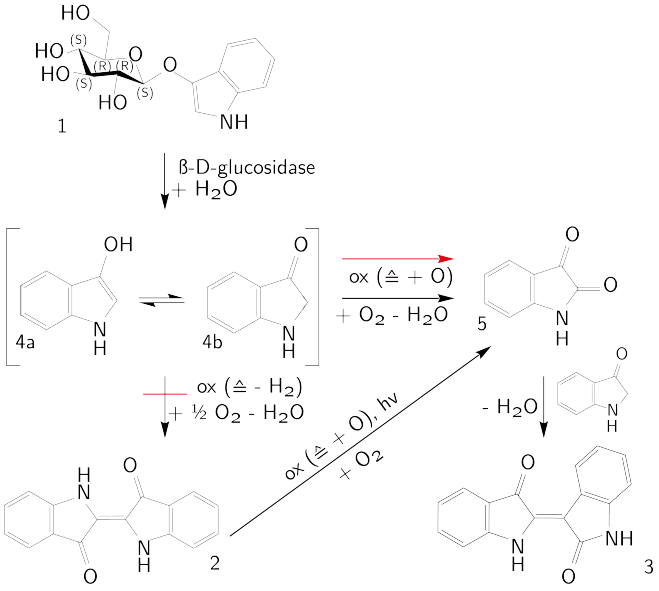



**Figure 6.45: Revised Biosynthesis Pathway from Indican to Either Natural Indigo or Indirubin with Indicated Reaction Sites of Additives.** Proposed indigo molecule/indirubin synthesis pathway from *Indigofera tinctoria* L. in accordance with Blackburn *et al.* [129]. Possible reaction sites of additives are marked in red. **1**: indican, **2**: indigo molecule, **3**: indirubin, **4a**, **4b**: indoxyl molecules, **5**: isatin. The pathway has not been investigated at the molecular level but can be deduced from the observations described. Adapted with permission from Klaas *et al.* (©2025 Wiley). [126]

This revised pathway built on previous investigations and descriptions of, among others, Maugard *et al.* (2002) and Sagwan-Barkdoll *et al.* (2025). [63,129–131]. Until now, indigo and indirubin molecule formation has not been studied on hair, but in coloration context for textile industry, as well as in microbial whole cell or enzyme system (cf. figure 3.2.2.1). It was aimed to describe the chemical reactions taking

place and involving additives such as isatin and reducing agents and explain their influence on color development.

The pathway started with the color precursor indican **1**, which is present as a glycolyzed molecule in the leaves of the indigo plant. By enzymatic hydrolysis and the $H_2O$ naturally contained in leaves, the glucose moiety is cleaved off. As a result, indoxyl, either in enol **4a** or keto **4b** tautomer, is formed. Through their functional groups, these tautomers are accessible to further reactions. Two different oxidations could take place from the color precursor. Indigo molecule **2** through oxidative coupling of two of the indoxyl molecules is formed. The connection is made at the C2 position of the indole ring and is formed by splitting off hydrogen and absorbing 1/2 equivalent $O_2$ through oxidation. The formation of the C=C double bond takes place there through proton transfer and as a consequence, the blue color pigment is formed. The other oxidation path is the formation of isatin **5** through oxidation by molecular oxygen. This step leads to the elimination of a water molecule. [7, 8, 61, 132–134] Isatin, together with an indoxyl molecule, takes part in indirubin **3** formation through a condensation reaction. A specialty of the proposed new indigo biosynthesis pathway is the photo-induced degradation of the indigo molecule. This photodynamic process oxidizes indigo to isatin under the influence of light and oxygen. The light energy activates oxygen to form reactive oxygen species, which subsequently interfer with the substrate and lead to the reaction taking place. However, although indirubin is a structural isomer of indigo molecule, it shows a high stability towards light [68].

Further, the pathway following indican hydrolysis can be influenced by the addition of $H_2$-donors, such as cysteine or ascorbic acid. They stop indigo molecule formation and cause an increase of concentration in indoxyl molecule. As a consequence, indoxyl is to a higher extent available for the synthesis of isatin and subsequently preferred requirements for indirubin formation are created [61] Further, indigo molecule can also be degraded through photooxidation into isatin [101, 129, 135, 136]. This would result in an even higher proportion of the precursors isatin being present on the hair.

The pathway has not been investigated at the molecular level but can be deduced from the observations described. The targeted control of these enzymatic and oxidative reactions enables a selective conversion of the indigo precursor with an extended color range.

# 7 Discussion

The demand for natural and environmentally friendly cosmetic products has increased significantly in recent years. Particularly in the field of hair dyes, there is growing interest in plant-based alternatives to synthetic, petrol-based and non-biodegradable dyes, which are associated with allergenic or potentially toxic substances [137–139]. In this context, indigo, derived from *Indigofera tinctoria* L., is one of the oldest known natural dyes and is increasingly being rediscovered as a sustainable option for hair coloration [140]. In contrast to synthetic oxidative hair dyes, which require aggressive developers such as hydrogen peroxide or synthetic indigo which requires strong reducing agents, dyeing with natural indigo takes place sustainably through enzymatic release of dye precursors. This reduces potential damage to the hair structure and minimizes the risk of influence on the human body and environment [48, 140].

The dyeing properties of *Indigofera tinctoria* L. investigated in this study show that both blue and reddish shades can be achieved on hair through targeted extraction and application techniques – without the use of synthetic additives. *Indigofera tinctoria* L. therefore offers not only an ecological, but also a functional, alternative in the field of hair coloring. Furthermore, conclusions could be drawn about the influence of the harvest on the raw material quality and its impact on coloration. In addition, a new *Indigofera tinctoria* L. synthesis pathway was induced from performed experiments. This incorporates the current state of research as well as new findings and targeted color formation.

## 7.1 Investigation of the *Indigofera tinctoria* L. Dyeing Properties

The different coloration approaches with *Indigofera tinctoria* L. on hair has provided insights into the importance of individual plant components, coloring substrates and physico-chemical conditions in color formation. There were also some insights into the extract production of the indigo plant for color application on hair. Furthermore, the immediate red coloration on the hair was achieved by applying InCL with ascorbic acid and isatin at an elevated application temperature, and insights into

the harvesting process and its effect on the quality of the plant material were given. Finally, the color molecules were again extracted from the hair fiber and quantified, providing information about the coloring with InCL.

Hair coloration with 5% (w/v) InCL led to a shift in color by $\Delta E = 33.0$ from initial blue to purple after 14 days without shielding from UV radiation and without any intermediate washing between day 0 and day 14. After that, the color changed slowly and slightly ($\Delta E = 6.1$ within a year), which is why the period of 14 days was chosen as the reference for further stability tests. Understanding color formation in indigo biosynthesis process suggests that, alongside indigo molecule, indirubin forms over time through oxygen-driven side reactions using oxygen as an oxidizing agent. The overlap of both molecules may result in the perception of a purple hue. [7,57,58,61]

A purple color shift was observed at an InCL concentration in suspension of 2% (w/v). However, a greater difference in the brightness of the strands fresh after application could be seen from a concentration of 3% (w/v) InCL. From this concentration onwards, the color shift within 14 days on a strand continued to increase with increased application concentration.

### 7.1.1 Coloration with Indigo Components on Hair

Comparing the InCL coloration approach with the equivalent amounts of synthetic indigo molecule in InCL coloration on hair, no equivalent analogous shift was observed. With a $\Delta E$ of 5.3 between strands treated with 13.125 mg synthetic indigo on day 0 and day 14, the color development of the strand within 14 days after application was significantly less pronounced than with the plant material at the corresponding concentration of 5% (w/v) InCL ($\Delta E = 33.0$) and could be largely caused by the uneven color distribution on strand resulting from bad solubility of synthetic indigo in EtOH. For this reason, it can be ruled out that the synthetic indigo molecule itself is responsible for the color shift on the hair fiber within 14 days. The reason for the described color change must be found elsewhere in the synthesis pathway of *Indigofera tinctoria* L.. A specialty here, however, was that from an equivalent concentration of indigo molecule of 2% (w/v) InCL the strands always appeared lighter (higher L* values) on day 14 compared to day 0. Experiments by Tello-Burgos *et al.* (2020) and Novotná *et al.* (2003) have shown that, due to

photooxidation, the indigo molecule decomposes over time in the solid state into isatin, among other degradation products [141–144]. Less indigo molecule on hair fiber is assumed to be in accordance with less color intensity and therefore lighter colored strands over time.

When looking at the pure indican dyed strands, it was noticeable that even small amounts were sufficient for a vibrant blue color result. The color shift initially observed within InCL treated strands, was also visible on the strands colored from a concentration of 0.12% (w/v) indican in water mixed with commercial $\beta$-D-glucosidase formulation which was equivalent to 60 mg/strand of indican. These results suggest that the color formation reactions can also take place only with the help of the moisture naturally present in the hair, which is dependent on relative environmental humidity and ranges between 7% to 12% in virgin human hair. [15, 16] The indigo precursor indoxyl might remain on or in the hair fiber in certain quantities from an InCL concentration of 2% (w/v). On the one hand this could lead to the development of indoxyl into isatin due to the ambient oxygen and, on the other hand, to the dimerization of indoxyl and isatin to indirubin under the influence of oxygen on hair. However, isatin resulting from photooxidation of indigo molecule could also be available for indirubin synthesis. This also fits with the fact that no red shift can be seen in synthetic indigo dyeing. Although isatin may be produced by photooxidation, there are no indoxyl molecules present that would be necessary for indirubin synthesis.

All in all, from the comparison of synthetic indigo to the coloration with indican, it can be ruled out that indigo molecule is transformed into indirubin causing the purple color shift in the course of 14 days, as this was not observed in the synthetic indigo dyeings. It can also be ruled out that the other plant components in *Indigofera tinctoria* L., besides indican, are initially responsible for the color change, since the shift also occurred when dyeing with indican alone.

Furthermore, it was observed that coloring with 5% (w/v) InCL (in 50 mL water – corresponding to 84 mg indican) showed a coloration the most comparable to 30 mg indican ($\Delta E = 2.8$). This suggests that when coloring with InCL, there are disruptive factors in the plant system that prevent the indican contained in the plant from developing its full color potential.

### 7.1.2 Influence of Substrate on Dye Outcome of InCL

In addition to dyeing hair, other substrates were colored with 5% (w/v) InCL and examined for color development within 14 days after application using a color spectrophotometer. The color change of the hair ($\Delta E = 33.0$) in particular and the preservation of the color on the other tested substrates cotton ($\Delta E = 4.6$), viscose ($\Delta E = 6.6$), silk ($\Delta E = 6.5$), hemp ($\Delta E = 5.5$), and linen ($\Delta E = 4.9$) showed that the hair played a decisive role in the purple color development. A major difference between the selected substrates and the hair fiber is that only the latter contains the animal protein keratin consisting of large amounts of cysteine. The other substrates mainly contain cellulose or, in the case of silk, the proteins fibroin and sericin. [23, 80, 84]

In the context of influencing the product selectivity from indigo precursors, it is known that cysteine can play a role in shifting production towards indirubin [64, 72]. The exact cysteine content in yak is not yet known, but it has a very similar molecular structure to human hair, which contains cysteine in quantities of 12% in epicuticle and 35% in exocuticle and even higher amounts in the cortex [35–37, 145]. Further, Plowman *et al.* (2003), Parry *et al.* (2006) and Deb-Choudhury (2018) found that some cysteine in wool as well as in hair is accessible for external treatments [28]. This could therefore, among other factors explained in the following, have an influence on the color development with InCL over the course of 14 days as stated before. [28–30]. It is assumed that there is an interaction between the cysteine bound in the yak belly hair and the color precursors of the indigo applied which influences the color outcome. This emphasizes the special role that hair plays in InCL coloration and explains why hair, as the substrate for dyeing, needs to be examined more closely.

### 7.1.3 Influence of Physical-Chemical Parameters on Coloration with InCL on Hair

This chapter systematically examines the influence of several parameters such as post-treatment, wash fastness, temperature, pH value, and molecular properties on dyeing performance with *Indigofera tinctoria* L.. The aim is to better understand the underlying mechanisms and to derive recommendations for the optimized application of plant-based hair dyes.

#### 7.1.3.1 Aqueous Post-Treatment and Color Stability

To investigate color stability, an aqueous post-treatment was performed in which freshly InCL-dyed hair strands were stirred in 50 mL of deionized water for 90 min. Over a period of 14 days, the treated strands showed only a color change of $\Delta$E of 1.7, indicating increased color stability. In particular, the change to a more reddish color within 14 days of application in strands that had not been post-treated, could no longer be observed. The water post-treatment must have therefore change the dyed hair fiber in such a way that no color other than the initially visible one was formed. At the same time, the color itself remained stable as no lightening of the strands was visible. To place this phenomenon in the context previous findings, one explanation for these observations could be that after the usual rinsing suggested in the dyeing protocol, color precursors remained on or in the hair and influence the color outcome after time. In contrast, the post-treatment ensured that either the remaining precursors were removed from the hair fiber after the color application, or that they no longer reacted due to the changed environment, e.g. oxygen concentration, after coloration. [146] In context of product development and applicability for consumers of hair dyeing products, the performed water post-treatment is not ideally feasible. In addition to practical reasons, it would increase application time and costs [147, 148]. Nevertheless, these results provide information about possible color formation processes that take place on the hair after the completion of coloration application and give a good base for further research for alternative post-treatments.

#### 7.1.3.2 Wash Resistance and Hair Penetration of *Indigofera tinctoria* L. Dye

Color stability on hair directly after completed color application was tested by wash stability tests of five cycles with six washes each. The color of 5% (w/v) InCL treated strands changed by a maximum $\Delta$E of 11.5 over the course a total of 30 washes. 1% (w/v) InHD treated strands showed a maximum $\Delta$E of 9.6 and InCL red dyed strands of $\Delta$E = 13.5. The overall good performance of the indigo dyed hair in the wash stability tests was also recognizable by the small increase in L* value, where higher values reflect brighter results. The maximum L* increase was observed in the InHD strand after 24 washes with +9.4. However, this value has decreased in most

wash cycles for all strands. These results indicated a stable anchoring between the hair fiber and the applied color molecules.

The penetration and performance of the dyes into hair depends heavily on the molecule size and water solubility. Water causes the salt bridges in the hair to dissolve, which leads to swelling and easier penetration of substances than in dry state [149, 150]. Furthermore, Morel *et al.* (2008) introduced a revised descriptor for molecule sizes. It measures the longest dimension of a molecule in its optimal envelope and serves as a measure of how well a dye can penetrate hair. Beyond that, the penetration also depends on whether the charge of a molecule. It was stated that virgin hair can be penetrated by non-ionic molecules up to a size of $L_D$ = 9.5 Å [33, 151]. As a consequence, the water-soluble dye precursor indoxyl, with a molecular diameter of $L_D$ = 5.29 Å, is able to penetrate into the hair fiber through the cell-membrane-complex (CMC) [43]. Same applies to water-soluble isatin ($L_D$ = 4.94 Å) and cysteine ($L_D$ = 5.20 Å) as well as ascorbic acid ($L_D$ = 6.45 Å; $S_7$ = 7.75 Å; $S_8$ = 5.25 Å; $S_9$ = 3.74 Å). In addition, despite showing higher $L_D$ values, dye molecules indigo ($L_D$ = 6.67 Å) and indirubin ($L_D$ = 8.00 Å) are still able to diffuse into the hair. These molecules are not water-soluble, which is why they practically cannot penetrate into the hair or be removed again. Especially the InCL and the InCL-red dyed strands showed a greater change in the a* and b* values than in the L* value throughout the washing cycles ($|a^*|_{max}$ = 10.2; $|b^*|_{max}$ = 11.0). This might result from the color development inside the hair which is promoted by the aqueous environment during wash fastness tests. After hair washing, some water remains inside the hair fiber, amounting to approx. 20% moisture content [31]. The reactions of the molecules that were not washed out of the hair could therefore have taken place more quickly.

When considering the stability of the color on and in the hair fiber dyed with InCL and InCL with ascorbic acid and isatin, in particular the L* value, which decreased during washing cycles, indicated good wash fastness. The InHD p. treated strand showed slightly increasing L* values among the washing cycles carried out. However, after 30 washes the $\Delta L^*$ value was 7.6, which could be perceived by human eye when observed accurately. All in all, analogous to evaluation of synthetic dyes, the dyed strands that have undergone the wash fastness test, could be classified as permanent because they offered long-term color change for more than 24 washes. [10, 39].

The microscopic examinations of dyed hair cross section provided further insight into the staining mechanism on the hair. All of the strands examined showed that the dye had penetrated the cortex after dye application. The dyes, whose penetration depth in the microtome cuts was measured under a light microscope, were always deposited under the cuticle in the cortex. The penetration depth was temperature-dependent: At room temperature, distribution was limited and sharply defined to the outer area of the cortex, while at 37 °C, deeper and more even distribution was observed. This can be explained by increased molecular movement and improved permeability of the hair structure when heated. At the same time, the cuticle appears to exert a stronger barrier effect at low temperatures, which is partially overcome at 37 °C. [152–158]

Interestingly, the amount of dye that penetrates did not increase proportionally to the penetration depth. This could be due to the temperature-induced aggregation of the dye, which could lead to altered distribution within the hair fiber. At the same time, the concentration of dye on hair surface could be higher at RT, resulting in stronger local staining, while at 37 °C, distribution was more even through the hair. The comparison between the blue and red dye components was particularly striking. While the blue components were clearly visible at both dyeing temperatures and tended to accumulate in the outer cortex region, the red components showed a more diffuse spreading that is barely visible at RT. This indicates differences in the formation of the dyes inside the fiber. The indigo color precursor molecules first penetrate the hair and dimerize in the cortex to form larger, water-insoluble indigo molecules. The concept is already partly known from the synthetic, oxidative dyes, which form large color molecules inside the hair fiber trough reaction of two smaller dye precursors [39, 42]. At the same time, the side reactions from indoxyl to isatin and consequently to indirubin could also take place over time in the hair fiber. This was observed in the microtome cuts from InCL-dyed strands and confirmed through ball mill experiments (cf. chapter 6.6.1). However, this process is stopped by aqueous post-treatment, as any remaining water-soluble volatiles are washed down again – the initial color remains stable. Moreover, these findings are in line with the synthetic indigo molecule not having the same dyeing properties on hair as the plant material because it cannot penetrate the hair so easily as the dye precursors and form a stable color there.

What was also noticeable in the microtome sections were the grainy color spots in the cortex. This could indicate that the indigo molecules accumulate in the hair to form

agglomerates, which then become even more firmly anchored and therefore cannot be washed out of the hair, as their sizes exceed the penetration paths diameters of CMC. Agglomeration is a known phenomenon of pigments in general and of indigo molecule in particular [56, 144, 159, 160].

#### 7.1.3.3 Influence of pH Value on the Color Result

Different pH of dyeing suspension during application on hair had an influence on color outcome. The results at pH 5, 6.8 (nat) and 7 were comparable (day 0: $\Delta$E between 2.3 to 6.9; day 14: $\Delta$E between 1.9 to 9.2). A change in the color after 14 days was observed at both pH 3 and pH 9, whereby at pH 9 both the fresh and the later color result were more intense than at pH 3 (day 0: $\Delta E = 4.3$; day 14: $\Delta E = 10.6$). Only at pH 11 was no comparable coloration of the hair after application identified – it remained almost uncolored. The influence of different pH on color outcome affects not only the hair and the enzyme contained in InCL, which is responsible for color release, but also the color precursors. These three components are considered individually below.

Regarding the **hair**, the pH value of the surrounding water primarily affects the cuticle [19, 32]. At a lower pH, it lays flat, whereas the individual scales swell at a higher pH, making the hair more susceptible to the penetration of external substances. When in contact with water with a neutral pH, such as when rinsing, the cuticle closes again. [43] In context with the results shown this phenomenon, which would be reflected in a more intense color on the hair, cannot be recognized. Therefore, the influence of pH on the two other components mentioned are considered.

A deviation of pH value from an **enzyme**'s optimum leads to a significant decrease in its activity. The pH value influences the charge and the spatial structure, especially the active center of the enzyme and consequently it can no longer bind or convert the substrate properly. [161] Although the optimum for the $\beta$-glucosidase from *Indigofera tinctoria* L. is not known exactly, according to literature, the pH tolerance of $\beta$-glucosidases in general is high in the range of 3.5 to 9 [161]. This is only partially consistent with the presented results. Even if the enzyme pH tolerance of the range was exceeded at pH 11, it was still present at pH 3. However, the similar trend in the colorations at pH 3 and pH 9 suggests that both ends of the activity range have been reached. Furthermore, it is assumed that, due to higher enzyme activity, a

higher amount of color precursor could be cleaved and thus a higher concentration of indoxyl was present, which in turn leads to a higher color shift, in line with previous results.

The **color precursors** that are present in the pH-adjusted suspension after they have been released by the remaining enzyme activity at the specific pH value, could also partly be effected. In an acidic environment, indoxyl is predominantly present in its neutral, protonated form, as the hydroxyl group (-OH) is not deprotonated. As the pH rises, the hydroxyl group is successively deprotonated, resulting in an indoxylate anion (indoxyl-O-). This process can be described by the following acid-base equilibrium (cf. eq. 7.1.3.3).

$$\text{Indoxyl–OH} \rightleftharpoons \text{Indoxyl–O}^- + \text{H}^+$$

The resulting phenolate-like anion is a better substrate for oxidative processes (e.g. atmospheric oxygen) than the neutral form due to its increased electron density on oxygen. This results in an increased reactivity towards autoxidative dimerization to indigo in the basic range. [162] However, this influence cannot be clearly observed either.

Taken all the observations together, the most decisive factor in the influence of pH is probably the glucose cleaving enzyme, which, although not exactly as expected, could react strongly to the changed dyeing conditions. The natural InCL pH value is recommended for future dyeing purposes.

The investigations show that hair coloring with *Indigofera tinctoria* L. is influenced by several physical and chemical parameters. Particularly relevant are post-treatment, wash fastness, temperature, pH value, and the molecular structure of the dyes. Recommendations for the most effective coloring conditions in the context of application to hair were provided. Nevertheless, further research was conducted to determine whether extracts from plant material can enhance the desired coloring properties.

### 7.1.4 Comparison of Coloration with Indigo Extracts on Hair

Indican as the stable dye precursor of indigo was identified through TLC, CC and HPLC [9]. Two approaches for extract production from InCL were tested and evaluated concerning their feasibility for use in cosmetic formulations for hair coloration as well as their concentrations of indican contained. Firstly, a heat-denatured indigo extract in liquid (InHD liq.) and powdered form (InHD p.) were created using hot water. Secondly, an ethanolic indigo extract (InEE) and its residue (InEER) was produced using ethanolic Soxhlet extraction.

#### 7.1.4.1 Evaluation of Coloration with InHD p. on Hair

The aim of the production of heat-denatured indigo extracts (InHD liq. and InHD p.) was to prevent the plant's own enzyme activity and, as a result, to protect the color precursor in an aqueous environment from enzymatic sugar cleavage. This should stabilize the indican. Treatment of hair with both InHD extracts did not result in any coloration. Only after addition of $\beta$-D-glucosidase formulation to the dye preparation could a color outcome be achieved again. The color result was darker than the coloration with 5% (w/v) InCL by using only 1% (w/v) of powdered InHD p. extract. The decisive color precursor of the plant material was present in InHD p. containing 103.1 mg indican/1 g InHD p. whereas 1 g InCL comprised 33.6 mg indican. However, the indican amount of 1% (w/v) InHD p. (51.55 mg) used for coloration in 50 mL for one strand did not exceed that in 5% (w/v) InCL (84 mg). This suggests that, in addition to increasing the indican concentration, the extract development also improves the raw material properties for hair coloring purposes.

Another important aspect of coloring with InHD was the significantly reduced color change within 14 days after the application (InHD liq.: $\Delta E = 1.6$; InHD p.: $\Delta E = 4.9$) which was only perceptible by close observation for InHD liq. or at a glance in case of InHD p. [109]. These results show that the indican-cleaving enzyme $\beta$-D-glucosidase, naturally present in *Indigofera tinctoria* L. was deactivated through hot water stirring in InHD production, as its denaturation temperature was exceeded and its active structure destroyed [98]. Consequently, the three components crucial for coloration activation – dye precursor indican, $\beta$-D-glucosidase for sugar cleavage and water for the hydrolysis – were separated and prepared separately. [6] This

provided an outlook for the storage opportunities of color components. As soon as all three components were added together again, e.g. in form of addition of active $\beta$-D-glucosidase formulation or InEER to an aqueous InHD solution, the enzymatic cleavage of indican could take place again and coloration was induced. This concept of the controlled combination of dye precursor and enzyme is known from patent EP 0 843 993 B1, although the precursor originated from a synthetic source. [163] In addition, the isolation of phenolic glycosides from *Lawsonia inermis* L. plant leaves by various extraction steps is known for evaluation of antioxidant capacities and structural profile determination from Hsouna *et al.* (2011). [164]

In plants, the dye precursor is stored in an aqueous environment but separated from the enzyme by cell compartimentation [6]. This leads to the assumption that InHD liq./p. could be incorporated into an stable aqueous cosmetic formulation. Furthermore, through controlled addition of a second aqueous enzyme formulation shortly before application, the indoxyl release can be initiated again and color formation can take place on hair. However, stability tests, especially concerning ingredients in cosmetic formulation other than the three mentioned, have to be carried out to confirm this hypothesis. The absence of a significant color shift during the hair dyeing process indicates that coloration with InHD liq. and InHD p. requires a smaller amount of indican compared to coloration with InCL, while still achieving a more intense color result.

For instance, the coloration result of 1% (w/v) InHD p. was best comparable to the coloration of 4.4 mg pure indican on one strand ($\Delta E = 5.5$). The quantity did not lead to a pronounced color shift within 14 days with pure coloring ($\Delta E = 6.3$). This observation supports the assumption that plant – derived components present in InCL and to a certain extent in InHD p. – in addition to the color precursors – may interfere with the dyeing process, necessitating a higher amount of raw material to achieve visible coloration. Consequently, this results in a higher concentration of color precursors on the hair, which enhances the visible color change.

The *Indigofera tinctoria* L. plant represents a complex biochemical system, in which its constituents and their influence on coloration, are not yet fully understood. One such component is chlorophyll, which is abundant in the leaves and contributes to the plant's coloration [165]. During the preparation of InHD extracts, a filtration step was included. Since chlorophyll is bound within cell components in organelles or compartments, which results in larger cell components, it is likely that it was partially

removed by filters with a pore size of 2–3 µm. This would reduce its competition with the actual dye precursors during hair application [166, 167].

Higher concentrations of InHD p. than 1% (w/v) led to less intense color results on hair. At concentrations of 2% and 3% (w/v), a blue hue was still visible, while from a concentration of 5% (w/v) onwards, the strand remained almost colorless. At the same time, the quantities of 50 µL commercial $\beta$-D-glucosidase formulation added remained the same. One possible explanation for the lack of color formation at the higher concentrations of InHD p. is that the added enzyme is confronted with increasing amounts of plant components. This can happen due to low enzyme specificity towards the color precursor or due to inhibition by other plant components [168]. Although, this was not the case with higher amounts of InCL, indicating an coordinated plant system and higher enzyme specificity of the plant's own enzyme. And at the same time parallel increased quantities of the precursor and enzyme in the system. In addition, the principle of a special case of uncompetitive enzyme inhibition, substrate excess inhibition, could come into play. Increased substrate concentrations as with InHD p. can lead to the formation of a catalytically inactive enzyme-substrate complex, which consequently no longer initiates color precursor cleavage. [90, 169] As a result, no color formation takes place.

Another explanation for observed coloration behavior could be that in InHD p. there are colorless plant components which compete with indoxyl related to the bonding on the hair. Higher concentrated InHD p. goes along with higher amounts of polyphenols on hair surface which are able to attach [102, 170]. As a consequence, this could influence the permeability of the cuticle, observed by Won *et al.*, who discovered that polyphenols can interact with the hair to provide an improved protective layer on the hair shaft. Hydrogen bonds and $\pi$-$\pi$ stacking bonds form a protective polyphenol layer on the hair shaft, which can influence the diffusion of other substances into the hair. [19, 170, 171] However, this was not observed when dyeing with InCL. Yet, in conclusion, it cannot be clarified whether these results can be transferred to the observations made when dyeing with InHD p..

A further observation regarding the colorations with InHD p. with reactivation through a commercial $\beta$-D-glucosidase formulation was that at higher pH (9 and 11) than the adjusted pH during extract production (pH 7, nat), color formation on hair did not take place. At lower pH (3 and 5) less intense coloration and a pronounced color shift within 14 days ($\Delta$E = 31.4 and 28.3) took place. From literature it is

known that besides the pH tolerance range between 3.5 and 9, the pH optimum of $\beta$-glucosidases is between 5 to 6.5 [172]. With reference to the discussion of the results of the pH influence on the coloration with InCL, the pH tolerance of the enzyme added could have been exceeded by pH adjustment to 9 [161]. However, the color result at pH 3 shows strong similarities to that at pH 5, which comes closest to the optimum. What also speaks for a high enzyme activity at the lower pH values is the recurring color shift. It is assumed that due to higher enzyme activity, a higher amount of color precursor could be cleaved in shorter time and thus a higher concentration of indoxyl was present, which in turn leads to a higher color shift, in line with previous results. At pH 7, however, less enzyme activity might have led to a suitable amount of color precursors available for an intense coloration but not enough to cause a color change towards purple within 14 days.

Based on the observations, in terms of using the lowest possible raw material concentration to save resources, lower InHD p. concentrations at pH between 3 and 5 should be tested in combination with commercial $\beta$-D-glucosidase. When using 1% (w/v), a pH of 7 should still be used for most stable and intense results. Furthermore, the possibility of producing this extract on a large scale, as carried out by Indfrag Biosciences Pvt Ltd at the production plant, shows a perspective on product development. An important foundation for extract development and consequently convenient cosmetic dye formulations have been laid.

#### 7.1.4.2 Evaluation of Coloration with InEE on Hair

The ethanolic Soxhlet extraction of InCL aimed for separation of indican in the ethanolic extract (InEE) and $\beta$-D-glucosidase (the still active indigo own enzyme) in the extraction residue (InEER). The extracted amount of indican in every Soxhlet cycle of the 5 g InCL used was calculated via HPLC. 92.5% (120.3 mg) of the total extractable indican was identified in InEE within eleven cycles which corresponded to 1,327 mL. Regarding an efficient extraction method for product development, it is advisable to extract only until cycle eleven, because the last 7.5% of indican extraction stretched 14 extra cycles. This ensures that the largest part of the color precursor has been extracted from InCL with the minimum possible use of solvent contact, corresponding energy and costs overall. Furthermore, the premature, uncontrolled reaction of indican and $\beta$-D-glucosidase which takes places as soon as they meet in

an aqueous environment was significantly reduced. [173] However, the content of indican in 1 g InEE was found to be 124.0 mg. Therefore, Soxhlet extraction was the most productive method for indican extraction from InCL so far.

At the same time, the enzyme-containing InEER was separated from most of the EtOH-soluble components of the InCL. Through mass spectrometry of ethanolic InCL extract, 26 phytocompounds were identified by Mishra *et al.* (2020). Among others, alkaloids, flavonoids, tannins and phenols, saponins, steroids, glycosides, terpenoids, chlorophyll and anthraquinones were found. [174, 175] It can therefore be assumed that the majority of these components were no longer in the InEER, but in the InEE. For staining with the introduced extracts, this means that the color precursor indican was separated from the enzyme, which could also be demonstrated practically. Both components on their own did not show the characteristic blue indigo color on strand. Although, a slight blueish color was visible on strand treated with 3% (w/v) InEER. This could be caused by the small amount of indican remaining in InEER after the Soxhlet extraction (0.6 mg/g InEER, 3% (w/v) $\triangleq$ 0.9 mg indican/strand), which led to a light color when water is added together with the enzyme contained.

However, mixing and stirring 1% (w/v) InEE and 3% (w/v) InEER in water for 5 min showed bad solubility, but a characteristic blue coloration on strand. The color was best comparable with the dyeing result of 1% or 2% (w/v) InCL. The strands treated with InEE and InEER differed from both of these strands by $\Delta E = 5.7$, whereas it deviated by $\Delta E$ of 10.0 from 5% (w/v) InCL treated strand. The color development in the course of 14 days was lower with a $\Delta E$ of 5.2 than of the comparison strand with InCL ($\Delta E_{1\%} = 5.8$; $\Delta E_{2\%} = 11.3$). 14 days after both coloration treatments, the differently treated strands varied from each other by $\Delta E$ of 27.4. The reactivation of InHD p. with InEER led to a more intense color result. InEE and InHD p. both reactivated with InEER showed an initial color difference of $\Delta E = 9.4$.

For the strands treated with InEE, which were reactivated with the commercial $\beta$-D-glucosidase formulation, the color outcome on day 0 was best comparable with the 3% InCL treatment ($\Delta E = 6.6$) but within 14 days the color result changed in the purple direction, so that these strands differed again by $\Delta E$ of 18.1. Although this was less than the change with 5% (w/v) InCL, it was too intensive for the application requirements towards permanent hair dyes [42]. The most intensive overall color result with at the same time a relatively low color shift was achieved with 1% (w/v) InHD p. in combination with 3% InEER. The color result was most

similar to the one with 3% InCL ($\Delta E = 6.6$). Further, the color development within 14 days was relatively stable with $\Delta E$ of 6.7.

All strands that were dyed using InEE were left with a greasy film on the hair that could not be washed off with water. This is undesirable for future treatments on the head.

#### 7.1.4.3 Application Potential of Ethanolic and Aqueous Indigo Extracts

According to the EU Cosmetics Regulation, water is the preferred solvent in cosmetic formulations as it offers the highest safety, best compatibility and uncomplicated regulatory handling. EtOH, on the other hand, should be used selectively and with caution, especially when special technical or preservative properties are required [176]. The manageability of the indican extracts InHD p. and InEE showed advantages of the aqueous extract (InHD p.), although its indican content was lower than that of InEE (103.1 mg vs. 124.0 mg indican). It could be distributed more evenly in the water base used for coloration, removed from the hair without leaving residues and offered better possibilities for product development. Furthermore, the color shift turned out less pronounced. Dyeing feasibility of InEE in terms of prevention of oily residues on the hair after application could not be improved by shaking experiments with PE. Even though TLC was able to prove that the indican from the InEE resolubilized in water remains in the water phase and the non-polar chlorophyll, as well as dye molecules natural indigo and indirubin, pass into the non-polar PE, the feasibility of the extract was not improved. The oiliness of an ethanolic plant extract is usually due to the co-extraction of lipophilic natural substances such as fatty acids, waxes, terpenoids or chlorophyll. As EtOH has amphiphilic properties, it also dissolves non-polar components at high concentrations, which can appear in the extract as an oily fraction. The sticky film on hair surface after application of water phase remained. Nevertheless, ethanolic indigo extract has advantageous properties for product development. The stronger color development within 14 days after application of InEE compared to InHD p., both with $\beta$-D-glucosidase formulation, indicates, in the context of previous results, that the color precursor is more concentrated in the ethanolic extract. Further work would have to be done on a possible extract development and its stability in cosmetic formulation.

Nonetheless, recently it was observed that consumer are increasingly interested in sustainable, biobased and environmental friendly cosmetic products which should be considered regarding all ingredients of the natural hair color formulations. [177–180]

### 7.1.5 Red Coloration with InCL and Formation of Indirubin on Hair

An immediate stable red color result was achieved for the first time using InCL or InHD. The addition of isatin and cysteine to InCL suspension and application of the same at 37 °C on hair, led to the shade, which is a novelty for hair coloration with InCL [126]. Furthermore, the color remained more stable between day 0 and day 14 ($\Delta E = 7.6$) than strands colored merely with InCL. Instead of cysteine, ascorbic acid in combination with isatin, improves the coloration results further in terms of intensity and stability.

It is known in the context of Kim *et al.*'s (2019) and Han *et al.*'s (2011) observations in microbial biosynthesis of indigo precursors, that cysteine influences product selectivity towards red indirubin synthesis. What contrasts with findings from Kim *et al.* (2019) in relation to hair dyeing with InCL, no specificity in the reducing agent concerning cysteine could be recognized. From the observed coloration it can be concluded that in case no cysteine but ascorbic acid is added, it is assumed that no intermediate product 2-cysteinylindoleninone is formed. This suggests that the reduction of the intermediate product indoxyl is sufficient to prevent the major formation of indigo molecule on hair. [64,72,181] At the same time, through adaption of dyeing conditions primarily indirubin is produced from dye precursors – noticeable due to the red color on the hair.

Furthermore, increased temperature had several advantages which are relevant in this context. First, diffusion rates were accelerated, which allows the penetration and distribution of dye molecules into hair or cotton fibers. As a consequence, color outcome after dyeing procedure was intensified. [155, 157] This was also noticeable in microtome cuts as the strands treated at 37 °C had the highest relative penetration depths (34.5-59.2%) compared to the ones at RT (5.4-24.8%). Second, higher reaction temperatures sped up the reaction kinetics of enzymatic cleavage of the sugar moiety from indican and therefore indoxyl release was advanced. [152, 154].

However, exceeding the optimal temperature, protein denaturation can occur, as observed with InHD production [182–184]. Third, increased temperatures enhance kinetics of the reaction between indoxyl and isatin. This results in indirubin being formed more quickly and the red color result being directly visible on the hair. [185] Fourth, aggregated dyestuff contained in the dye bath might be broken down into monomers by raising temperatures. These may then enter the interior of fibers more easily which results in intensified and more uniform dyeing [153, 155, 156]. At the same time, with a view to product development, the required temperature must still be acceptable for use on the human head which is guaranteed at human body temperature of 37 °C [33, 186].

With regard to the use of found additives for the formulation of cosmetics for natural red hair coloration, apart from InCL, only the use of isatin is explicitly regulated in the Regulation of the European Parliament and of the Council on Cosmetic Products (EU CPR (Reg. 1223/2009)). It can be safely used with a maximum concentration of 1.6% and with an indication on the packaging. Both cysteine and ascorbic acid are not explicitly mentioned and are therefore not prohibited under the EU CPR (Reg. 1223/2009). [176] The incorporation of these additives into a cosmetic formulation must be tested. However, an important basis has been laid for the use of indigo to achieve two different, stable shades on the hair.

The red color result is also achievable on the other tested substrates cotton, viscose, silk, hemp and linen. The results fit in with the fact that the reducing agent was added externally and the substrate therefore no longer plays a major role in color formation as it did with InCL. This innovation in dyeing with InCL provides a significantly expanded color spectrum. In addition to the well-known indigo blue textile dyeing, this shows the possibility of natural red textile dyeing [2].

### 7.1.6 Influence of Harvest and Processing on Raw Material Quality for Hair Coloration and Composition of *Indigofera tinctoria* L.

The quality of raw material *Indigofera tinctoria* L. depends on several aspects during the cultivation and harvest of the plant [1]. The aim was to clarify to what extent plant cultivation influences the quality of the plant material with regard to hair color-

ing applications. Therefore, the plant's processing was considered. The source of plant material this work was based on was supplied by Indfrag Biosciences Pvt Ltd (Bangalore, India). It was cultivated in Tindivanam, Tamil Nadu, India on a farm run by KMA Exports which was specialized in cultivation of indigo for coloring. The harvest and raw material processing steps were subdivided into the following sampling steps:

1. Fresh plant leaves from field
2. Fresh plant leaves after transport to drying areas before drying
3. Plant leaves dried, threshed, cleaned roughly and final packaged.

These steps will be examined in the following concerning the influence of raw material processing the molecular composition and on hair coloration. For organizational reasons, the plant material analyzed was not the same as that used for all other colorations in this work.

#### 7.1.6.1 Molecular Composition and Indican as a Dye Precursor

The molecular composition of the *Indigofera tinctoria* L. samples across three harvests and several processing steps was quantified via HPLC. The structured comparison of the results revealed both patterns and contradictory developments. For instance, samples 1 showed comparable indican values across all three crops (24.5 to 29.6 mg/g RM), suggesting similar cultivation conditions. However, this trend was not consistently across all fields and processing stages. Field B appeared to yield higher indican contents than Field A in early harvest, potentially indicating better suitability. Yet, this trend was not maintained throughout all harvests and processing steps, making a clear conclusion difficult. Particularly striking are the differences between the three harvests and the partly opposite developments along the processing chain. Crops 2 and 3 stood out clearly in sampling step 2 and in sampling step 3, in particular crop 3 showed high indican contents. These ranged between 77.3 to 3.2 mg/g RM. The standard deviation of the measured molecule quantities in the samples was large – it fluctuated from ±0.1 to ±19.5 mg/g RM. In particular, when mean values of the amounts of molecules in raw material were above 5 mg/g RM, the standard deviation increased. This limited the statistical reliability of the observed trends. Statements

about increases or decreases in molecules must therefore be interpreted with caution. The measured indigo and indirubin quantities varied between samples. No trend could be identified. The highest indigo content was found in samples 1.3A with 13.4 mg/g RM. The heterogeneity underscores the sensitivity of plant dye precursors to environmental and process conditions and highlights the need for a differentiated approach. At the same time, there are several limitations to the significance of the results, which are listed systematically below.

An important factor concerning indican content in *Indigofera tinctoria* L. leaves was found to be access to direct sunlight during plant cultivation and growth. At low light intensity, the production of secondary metabolites, including indican, is stimulated. This is because light, especially in high amounts, favors photosynthesis, but at the same time can limit the synthesis of certain secondary metabolites, such as indican. [187] However, light intensity is particularly high in the Tamil Nadu region during the growing months of March to June [188, 189] – a factor that is difficult to control in outdoor cultivation. However, samples 1 showed comparable values of indican content among all three crops (24.5 to 29.6 mg/g RM) indicating similar cultivation conditions of the analyzed plants throughout all three harvests.

Transported samples 2 showed divergent trends across crops. Samples 2 of crop 1 exhibited an 82.2% decrease of average indican amounts compared to samples 1. This was different for crop 2 as the measured indican quantities increased by 10.3%, but remained within the standard deviation. This suggests that during transport of fresh plants of crop 2 to the drying areas, much less indican was degraded by plant's own enzymatic activity than in crop 1. Samples 2 of crop 3 showed 12.7% more indican than samples 1 of the same crop. The concentrations of natural indigo and indirubin are comparatively high in the samples 1 and 2 of crop 2 and 3. The simultaneous increase in indican and its degradation products indigo and indirubin in certain samples contradicts the expected conversion logic. This indicates complex, possibly not fully understood enzymatic or chemical processes that limit the significance of individual concentration comparisons. Applied sample processing by crushing the fresh plant leaves in EtOH, which naturally contain water, ensures that the water concentration fell below the required proportion for the enzymatic release of indican. This allows the original concentration of this color precursor to be maintained. However, based on the evaluation protocol, it could be ruled out that any by-products or the water content of the samples increased due

to environmental conditions. As a consequence, this would have led to decreasing concentrations of molecules of interest in corresponding analyses and might be an explanation for partially unexpected results. The transportation step in the processing chain is particularly sensitive, as loading the indigo branches onto the tractor can destroy the natural compartmentalization of the dye precursors and the enzyme through mechanical destruction or thermal influence of the sunlight, which can lead to premature degradation. [190–194] Furthermore, natural variability e.g. genetic differences between plants, microclimatic conditions e.g. light intensity, soil quality and temperature, as well as the degree of ripeness of the plant at the time of harvest significantly influence the biosynthesis of secondary metabolites such as indican. [128, 195] This variability is particularly relevant because the samples did not originate from identical plants across all process steps.

After drying, threshing, rough cleaning through air suction, and final packaging, all samples 3 had higher indican concentrations than indigo or indirubin as well. The largest total amount of indican within samples 3, and in total, was found in crop 3 of the morning harvest from field A (3.3A). However, also the samples 3 of crop 3 from field B (3.3B) and those harvested in the evening (3.3N) exhibited high indican concentrations (3.3B: 73.5±12.9; 3.3N: 39.9±6.7). While the amounts of indican measured in samples 3 for crops 1 and 2 were 5.9±2.6 and 4.8±3.4 on average, which falls below the indican concentration found in prior used InCL (33.6 mg/g RM). Even though the measured amount of indican in samples 3 of crop 1 exceeded samples 2 of crop 1 by 1.5 mg/g RM, it was still within its SD. Therefore, the decrease in indican concentration within the harvesting process of crops 1 and 2 was in line with the processing steps in which the color precursor may degrade for the reasons mentioned above. According to literature, drying is the decisive step in the preservation of plant material after harvesting, as it minimizes microbial and enzymatic degradation and thus extends the shelf life [128, 195]. Various drying processes were tested for the extraction of dye precursors from *Isatis tinctoria* L. plant leaves. It was found that drying at RT or at 40 °C resulted in the highest concentrations of indican. The time of harvest also plays a role: plants harvested early or at an early stage of maturity contain lower amounts of color precursors [190].

In addition to drying, the storage of the dried material is an important factor influencing the plant material quality. A low moisture content and protection from contact with air, microorganisms and insects are essential [128, 195] – which was

the case with the plant material examined. In India, there are particular challenges when processing plants. They are often dried in the sun, which can lead to mold growth and insect infestation due to high humidity and temperature. In addition, strong sunlight can have a negative impact on sensitive plant components. [128] As mentioned above, in addition to the drying process, several factors during cultivation also have a major influence on the molecular composition of plants. A special feature of crop 3 was that it was watered exclusively by rainwater. The leaves appeared greener and denser with more mature stems and foliage. This could be one approach to explain why relatively high amounts of indican were found in samples 3 of crop 3. Furthermore, the samples were not obtained from the same plant material across all processing steps. This means that it is not possible to directly trace molecular changes within a single plant. Differences could therefore be due to both process conditions and natural variability in the starting material. The plant material rich in indican may have been subject to particularly favorable cultivation and harvesting conditions.

In the underlying studies, heterogeneous observations were made depending on the crop underlining the complexity of harvest process. Indican content in crop 1 indicated a loss of the dye precursor during the transport of plant material to drying areas. The content then remained stable and no further indican was lost during drying. However, crop 2 showed a high stability during transportation but a significant decrease in indican content between transportation and final product, indicating sub-optimal drying. Crop 3 deviated because the indican content in the final product increased, which could not be explained by the harvesting process or sample preparation protocol. It is expected that high fluctuations in measured molecule quantities was due to the natural plant material that was examined, which was subject to several influencing factors, both during cultivation and harvesting process (light, temperature, humidity), that could not be fully controlled. These factors significantly influence the biosynthesis of secondary metabolites such as indican and make it difficult to clearly identify the causes of the observed differences in concentration. The method used for sample preparation and plant analysis was innovative but no systematic errors were identified. Despite potential susceptibility to inaccuracies, no consistent deviations were observed, supporting the reliability of the results.

In general, it can be stated that short transportation distances from the field to drying remain essential, as large amounts of plant material are both cultivated and required for extract production. Recommended drying methods such as hot air, vacuum, or freeze drying are not practical for economic and environmental reasons. The drying process currently used therefore represents a reasonable compromise between quality, sustainability, and cost [196, 197]. However, *Indigofera tinctoria* L. was found to be prone to fluctuations regarding molecule composition. The results provide valuable information on critical process steps and potential optimization approaches for the extraction of plant dyes. At the same time, they show that a reliable assessment of dye quality is only possible if the aforementioned limitations are taken into account. In the following, the color outcome of the samples taken during different harvest steps as well as from different crops is discussed.

#### 7.1.6.2 Influence of Raw Material Processing on Hair Coloration

The plant material analyzed was not the same as that used for all other colorations in this work. Therefore, both fully processed raw materials – the one used in coloration and extraction experiments of this work (InCL) and the one of the final packaged raw material (samples 3 of all crops) from the harvest studies – are compared regarding their coloration behavior. Initially, color outcome on strands treated with samples 3 of crop 1 and this of the prior used InCL differed by $\Delta$E of 8.6. Samples 3 of crop 2 showed a difference compared to the prior used InCL of $\Delta E = 11.0$ and final crop 3 differed by $\Delta E = 8.4$. When comparing the development of different dyeings on hair, the shift of previously used InCL material was higher than those of the samples of harvest analyses ($\Delta E_{InCL}$: 33.0; $\Delta E_{S3-C1}$: 11.8, $\Delta E_{S3-C2}$: 5.1, $\Delta E_{S3-C3}$: 10.0). On day 14, the colorations of the samples 3 to the usually used InCL differed by $\Delta E = 15.1$ to 26.8.

However, it was also noticeable that in samples 3 of all crops, the pH values of the color suspensions were between 6.0 and 6.5. The previously applied 5% (w/v) InCL suspension showed a higher natural pH of 6.8. This indicated that the raw materials differed significantly. Greatly varying coloring results, depending on the *Indigofera tinctoria* L. raw material used, were obtained. For this reason, no precise conclusions can be drawn about the InCL used for all dyeing experiments, but an overall impression can be gained based on the processing steps and harvest conditions.

Influencing factors on the plant's pH are both internal physiological processes and the environment, e.g. soil pH and properties, nutrients uptake, water availability, temperature, humidity and light conditions. Furthermore, drying causes chemical changes in the compounds contained in the leaf, such as polyphenols and flavonoids, as well as their concentration, which affect the pH value. [198, 199] The pH of plant material subsequently led to differences in color outcome. In chapter 6.2.4, the significance of pH on color intensity and color change over time was demonstrated.

Furthermore, the process of leaf drying influenced the plant material by increasing the pH value and changing the chemical composition of the plant compared to the dry, freshly harvested plant material. This could explain why samples 1 and 2 did not show a comparable coloration on hair to that of the dried leaves. Even though the leaves crushed in EtOH were dried afterwards, the sampling protocol significantly interfered with the drying process of the leaves. This makes direct comparison of dyeing with samples 1 and 2 to samples 3 difficult. [198, 199] Drying is therefore not a purely physical process, but a chemically active state that changes and preserves the plant material. This affects not only visible components such as chlorophyll, but presumably also less obvious ones such as polyphenols or flavonoids, which could influence coloring through physical adsorption or chemical interactions with dye precursors or hair proteins. [76, 200, 201] Samples 1 and 2 showed a greener color on hair, presumably due to EtOH treatment immediately after harvest. EtOH efficiently extracts lipophilic green plant pigment chlorophyll from the chloroplasts than water and inhibits its degradation by chlorophyllase. As a result, chlorophyll is more prominently displayed on the hair fiber, where it could initially attach physically to the hair via weak Van-der-Waals or hydrophobic interactions. It is degraded within 14 days by UV light and oxygen, particularly on its ring molecule. This creates reactive oxygen molecules that attack and destroy the double bonds in the tetrapyrrole ring. As a result, chlorophyll loses its green color and function. [76, 200–202] This phenomenon could be observed on the hair through the increasing blue coloration.

In contrast, the samples from stage 3 showed significantly more differentiated color characteristics depending on the harvest time and origin. The color has also changed over 14 days, but in different directions. Either the color impression was more grayish (3.1A), more reddish (3.1B), or came closer to the typical blue indigo color (3.2A, 3.2B, 3.2N, 3.3A, 3.3B and 3.3N). With regard to samples 3 of crop 2, no purple color shift could be observed within 14 days. On average, with a 5% (w/v) dyeing

of samples 3.2A, 3.2B and 3.2N in 50 mL water, 8.8 mg indican was applied to the hair fiber, which is most consistent with the dyeing with 7.4 mg pure indican. Here, too, a relatively small color shift was visible on the hair. The color shift of samples 3 of crop 3 (3.3A, 3.3B and 3.3N) was more prominent ranging between $\Delta$E 9.0 and 10.8. During these colorations, on average 158.9 mg indican was applied to the hair during coloring (5% (w/v) dyeing in 50 mL water). However, color outcome was also most consistent with colorations of 7.4 mg pure indican ($\Delta E = 2.4$), indicating once again the huge impact of cultivation influence and sample processing on dyeing characteristics of the plant material. Furthermore, the results suggests that chlorophyll is largely broken down during the drying of the leaves, unlike in samples 1 and 2. The highest indican contents in samples correlated with the most intense color results (lowest L* values), especially in crop 3. At the same time, the color shift towards purple/red was most pronounced in strands treated with indican-rich samples 3 of crop 3. This suggests that an increased indican concentration promotes red indirubin synthesis.

The results clearly show that drying plant material is not merely a physical process in which water and other volatile components are removed. Rather, profound chemical changes take place during drying that have a lasting effect on the composition of the material. In particular, sensitive compounds such as vitamins, antioxidants, and aromatic components may be lost. [203–205] Subsequently, it is not the original, fresh plant material that is preserved, but a chemically altered state with increased concentrations of the components due to the removal of water. Although these components were not analytically determined in the present study, they could influence relevant metabolic processes through physical adsorption on the hair surface or through chemical interactions with dye precursors or hair proteins, thereby contributing to the color result. [76, 198, 199, 202] In summary, drying affects the composition of plants through the loss of volatile and heat-sensitive components and through molecular changes, with the choice of drying method playing a decisive role in preserving the original composition. Therefore, drying is not only as a technical step in preservation, but a crucial chemical process that significantly determines the dyeing properties of the plant material.

All in all, the aim when growing *Indigofera tinctoria* L. should be to retain as much indican as possible in the plant material. This leads to higher quantities of colorants that can be subsequently produced, and therefore to higher efficiency,

which leads to reduced costs, as less plant material has to be used, for example, for extract development. In addition, the amount of waste products generated during production can be reduced. In short, maximizing the indican content in the plant is crucial to ensure efficient, cost-effective and high-quality indigo production. [190] The results show that the timing of cultivation, transport, drying, and storage contribute significantly to quality. The observed variations can largely be explained by natural variability and process-related influences.

### 7.1.7 Interpretation of Analysis of Indigo-Colored Hair

To gain a better understanding of the color-producing molecules on the hair, indigo-colored hair was ground using a ball mill and subsequently extracted with different solvents for detection of isatin, natural indigo and indirubin with the help of HPLC. This should lead to references of color formation mechanisms taking place on hair. Strands treated with 5% (w/v) InCL (1), with the addition of 1% (w/v) ascorbic acid and 1% (w/v) isatin (2) as well as 1% (w/v) InHD p. and 50 µL $\beta$-D-glucosidase formulation (3) at 37 °C for 30 min each on day 0 (a) and day 14 (b) were examined. The measured L*a*b*-color values combined with corresponding differences in amounts of molecules extracted from hair fiber through HPLC were considered. The influences of temperature on the coloration already described in chapter 7.1.5 also applies here. The difference in color of strands 1a to 1b was detected in both, the L*a*b* values and extracted molecules from hair. The quantity of detected indirubin increased and natural indigo decreased. Due to the known absorption spectra of indigo molecule and indirubin, these findings prove the hypothesis that the overall purple color impression on hair is caused by the superposition of both molecules [126]. It can also be stated that the color change does not decrease significantly within 14 days after staining with InCL. This suggests that despite application at 37 °C and the resulting increased reaction kinetics, not all color precursors have reacted and remain in the hair, which in turn leads to the formation of indirubin over a period of two days.

The strands 2a and 2b showed the highest total extracted amount of molecules from strands. Initially, 467.7 µg/g strand of indirubin can be detected on the hair fiber whereas hardly any indigo molecule was found. Within 14 days the amount of indirubin increased by 74.9% and the quantity of indigo molecule decreased

further by 80%. The difference between color of strand 2a and 2b was reflected with $\Delta$E of 7.6. The role of isatin as an educt in formation of indirubin was indicated by the tendencies of remaining amounts on strands after different coloration approaches and their color outcomes 14 days after application. For all colored strands, it could be observed that a larger amount of isatin is present on the freshly colored strands than on the strands on day 14. At the same time, the content of indigo molecules decreased and that of indirubin increased. Overall, it must be considered that this isatin was added for the color formation of the strands 2. Thus, not the absolute values of isatin between the differently treated strands can be compared, but the trends in color development on each colored strand separately over the course of 14 days. In addition, the time-consuming formation of indirubin on InCL-treated strands (1a and 1b) could be explained by the lack of isatin in the system [8, 61, 126]. This could be accelerated by the addition of isatin and the application of heat to the dyeing procedure. The reaction that takes place could therefore be influenced by the specific choice of additives and reaction conditions.

For strands 3a and 3b the amount of traced natural indigo decreased by 43.7%. At the same time, the amount of indirubin increased by 19.4%. The greater difference in the amount of indirubin than in the amount of indigo measured on InCL dyed hair (1a and 1b) compared to hair treated with InHD p. and 50 µL $\beta$-D-glucosidase formulation (3a and 3b) indicated the significant influence of indirubin on the color difference $\Delta$E (strands 1: 32.3; strands 3: 3.9).

The results also allow the assumption that the same phenomenon which Tello-Burgos *et al.* (2021) and Novotná *et al.* (2003) have observed applies here. Due to photooxidation, indigo molecule decomposes over time in the solid state, among other degradation products, into isatin [101, 129, 135, 141, 142, 206]. The resulting isatin could be available for subsequent indirubin synthesis on hair. This would also clarify the decrease in the indigo molecule concentration on the hair and the delayed formation of indirubin within 14 days. These reactions can also take place in dry state or only with the help of the moisture naturally present in the hair, which is dependent on relative environmental humidity (RH) and ranges between 7% to 12% in virgin human hair. [15, 16] Overall, it can be concluded that the visible color results on the indigo colored hair are related to the mixture of indigo and indirubin molecules and their proportions detected rather than the total amounts of dye molecules.

## 7.2 Proposed Color Formation Pathway of InCL on Hair

Based on the findings, a new *Indigofera tintoria* L. pathway, including the possibilities of influencing it, has been developed in the context of hair coloring.

During hair coloration with InCL it was observed that indirubin synthesis takes longer than the synthesis of the indigo molecule. The speed-determining step in this pathway is the conversion of indoxyl into isatin, due to the different speeds of different oxidation steps [74]. Therefore, indirubin synthesis can be sped up with the addition of external isatin into the system, especially at elevated temperatures.

It was found that the use of the reducing agent for the immediate red coloration from InCL in hair is not limited to cysteine. Even improved results were achieved with replacement by ascorbic acid. This indicates that, contrary to the assumption of Kim *et al.* (2019) in the context of microbial synthesis, in case where no cysteine is added, no intermediate product 2-cysteinylindoleninone is formed. It can be concluded that there is no such narrow specificity in the reducing agent for promoted indirubin synthesis and the reduction of the intermediate products in color synthesis is sufficient to prevent the major formation of indigo molecule in hair [64, 72, 181].

The findings provided the substantiation of controlling the shade of hair coloration with indican from InCL by theoretical explanation. The demand of consumers can thus be adressed. However, the revised pathway resulted from deductions of observations described and was not investigated at the molecular level.

## 7.3 Cross-Experiment Comparisons and Integration

A series of experimental studies on hair dyeing with InCL were performed, each shedding light on different aspects of the dyeing process – from chemical composition and color dynamics to physico-chemical influencing factors, extract development and substrate dependence were comprised. In order to bring the knowledge gained into an overarching context, this chapter is dedicated to a comparative analysis and integrative evaluation of the experiments carried out. Central patterns and

correlations that go beyond the individual findings to enable a more comprehensive understanding of color formation with natural indigo are identified in the following. Molecular mechanisms, as well as application-related and ecological aspects, are taken into account. The integration of the results not only serve the scientific classification, but also the derivation of recommendations for the development of sustainable, functional plant-based hair dyes.

### 7.3.1 Influence of Substrate and Ambient Conditions

The usage of different substrates for coloration and creation of various dyeing conditions showed that the hair structure has a decisive influence on the color formation with InCL. While no significant color change was observed on keratin-free substrates such as cotton, silk or linen, a characteristic shift from blue to violet occurred on human hair. This substrate-specific reaction can presumably be attributed to the presence of cysteine in keratin, which could play a role as a reaction partner or modulator in the formation of indirubin. [23, 28–30, 35–37, 64, 72, 80, 84]

In addition, physico-chemical parameters, such as the pH value of the dye solution or water post-treatment, as well as wash fastness tests, influence both the color intensity and the development of the color over time. It was shown that water post-treatment prevented the subsequent color shift, presumably by removing or inactivating remaining dye precursors on the hair surface. It should be emphasized that the natural pH value of the InCL dye suspension (approx. pH 6.8) provided the best results in terms of color intensity and stability. Both acidic and alkaline conditions led to undesirable effects like lower performance or increased color shift. For future applications, it is therefore recommended to maintain the natural pH value in order to ensure optimum color yield and high stability at the same time. For InHD p. at a concentration of 1% (w/v), the color outcome showed most preferable characteristics at pH 7 which was adjusted during InHD production. However, pH 3 and 5 might have a beneficial influence on coloration which, in the case of 1% (w/v) InHD p., led to a color shift that might indicate more color precursors on hair. Even if the color change is not intentional, this may demonstrate an opportunity to further reduce the concentration of InHD used and thus save resources thanks to the reaction conditions. [161, 172]

### 7.3.2 Color Formation on Hair and Molecular Mechanisms

The results obtained indicate that the color formation of InCL on hair is largely determined by the penetration of water-soluble dye precursors, mainly indoxyl, into the hair fiber. Water-soluble molecules involved in dye formation, such as indoxyl, isatin and cystein, whose lateral diameters are below the diffusion limits of non-ionic molecules of the CMC, can penetrate the hair and react intrafibrillarly to form water-insoluble indigo and indirubin. This in-situ color formation within the hair structure explains the observed high washing stability of the dye. [33, 149, 151]

Taken this together with the results discussed above, it can be assumed that the cleavage of glucose from indican takes place in aqueous environment of dyeing suspension. As soon as indoxyl is released, it penetrates the hair fiber and forms dyeing molecules either with another indoxyl or an isatin molecule. This is aided by the water content of the hair, the cysteine from keratin in the hair or color additives such as isatin, cysteine or ascorbic acid. The color is then firmly anchored inside. [33, 149–151] This also explains the delayed red (indirubin) formation, which then leads to a purple impression on the hair in the course of 14 days. The kinetically favored reaction of two indoxyl molecules to natural indigo takes place very quickly. The remaining indoxyl then reacts over time to form isatin on the one hand and, together with it, indirubin in the hair on the other.

Particularly interesting is the targeted development of red InCL hair dye which can be attributed to the formation of indirubin. This color change represents a novel extension of the natural color spectrum of plant-based hair dyes and was achieved without the use of synthetic additives. Furthermore, a new synthesis pathway, also considering the use additives, was experimentally derived.

### 7.3.3 Comparison of Plant-Based and Synthetic Systems

Synthetic indigo is widely used in textile industry but concerns towards its environmental impact arise. For coloration with plant-based dyes derived from *Indigofera tintoria* L. these concerns can be circumvented, although it entails a completely new field of application that needs to be explored. [4, 7, 180]

A major advantage of plant-based systems is the ability to specifically control color formation. This has been demonstrated by the development of extract-based approaches such as InHD and InEE. By separating the dye precursor indican from the cleaving enzyme, the reaction for color formation can be triggered at the desired time, e.g. directly before application, which ensures best possible performance on hair as well as a application method used with oxidative hair dyes. This controlled activation not only offers advantages in terms of color stability, but also with regard to the shelf life and formulation of cosmetic products.

Its worth noting that the water-based heat-denatured indigo extract InHD p. has proven to have regulatory and practical benefits, can be easily integrated into cosmetic formulations, and washed out without leaving residues meeting the requirements of the EU Cosmetics Regulation. In contrast, ethanolic extracts showed a higher dye concentration, but also cosmetically disadvantageous properties such as oily residues on the hair. Water-based systems are therefore a preferred basis for product development.

## 7.4 Methodological Considerations and Limitations

Various experimental approaches were systematically correlated to each other in order to investigate the influences of different parameters such as material properties, test conditions and extract production. The aim was to gain a comprehensive understanding of the mechanisms behind color formation. The experiments were carried out under standardized and reproducible conditions with multiple determinations to ensure a high degree of comparability of the results. This included uniform application quantities, constant exposure times and controlled environmental conditions. Both untreated plant material (InCL) and specifically prepared extracts of the same starting material (InHD, InEE) were examined in order to differentiate between various aspects of color formation.

### 7.4.1 Substrate for Indigo Coloration

Identical strands of yak belly hair were used as a substrate in order to minimize structural differences that are typically seen within hair of different origins. From the

results obtained from the experiments with yak belly hair, conclusions can be drawn about human hair. However, when doing so, some considerations must be taken into account. Yak belly hair has a higher porosity and less cuticula layers which might lead to a higher dye uptake in hair. This makes the dyeing process more efficient but comparability to human hair less reliable. Yak hair is the common material used to replace expensive human hair. However, human hair would first need to be bleached for experimental color applications, introducing further uncertainty factors. [35–37] In addition to hair fibers, textile materials such as cotton, silk, linen, viscose and hemp were also used to analyze substrate-specific effects. This selection enabled a targeted differentiation between keratin-based and cellulose-based substrates and provided important information on the role of the hair structure – in particular cysteine – in red dye formation over time.

### 7.4.2 Color Measurement of Substrates

The color results were documented using color measurements in the CIELAB color space. Each single color measurement was carried out four times. The color change was quantified using the $\Delta$E value, whereby measurements were carried out immediately after staining (day 0) and after 14 days. This period was used to observe possible subsequent color dynamics and to evaluate color stability. However, because strands of hair consist of many individual fibers, creating an irregular surface and light reflections, it was difficult to measure color without any influence. As recommended by Liberini *et al.* (2020), the color evaluations on which this work is based were supported by visual assessment. [110]

### 7.4.3 Coloration From Plant Material

The plant material is a complex system with various components that could influence coloration. Not all the ingredients were elucidated in detail, but the coloration with the color molecule indigo of synthetic origin, which is similar to the natural one, was compared with the color formation of the glycolyzed indigo precursor. This method proved to be helpful as it provided initial information on the factors influencing the color change within 14 days of application. To investigate physico-chemical influencing factors of the plant-based coloration, staining was carried out under

varying pH values (3-11) for both, InCL and InHD p. colorations. The activity of plant-derived and externally-cultivated $\beta$-D-glucosidase was evaluated indirectly via the color yield and the change in color over time. In addition, stirring in water as a post-treatment and wash stability tests were used to analyze the reactivity of remaining dye precursors originated from plant material and to determine their influence on color stability. Furthermore, the InCL extracts were evaluated in terms of their dyeing performance, color stability and cosmetic properties. Particular attention was paid to practical applicability, residue formation and regulatory suitability for cosmetic formulations.

### 7.4.4 Hair Microtome Cuts for Color Penetration Depth Evaluation

The microtome cuts of dyed hair strand were performed in a cryostat and evaluated using a light microscope to determine color quantitatively. It was not possible to quantify or identify the color molecules penetrating the hair fiber but only measure visually if and how far they have entered. However, this could not always be recognized as a sharp edge, but sometimes somewhat blurred. In some cases, it was not possible to clearly distinguish between hair pores and possible color particles in the hair. Since the penetration depth measurements were always carried out by the same person, no major fluctuations in the results are to be expected that would need to be taken into account. Nevertheless, in some cases the color molecules responsible for the exact shade of the strand could not be identified.

One irregularity was the uneven shape of the hair fibers, which complicated the calculation of the penetration depth of dye into hair. Since yak belly hair is a natural product, this factor could not be ruled out. Comparability was achieved through the calculation of relative penetration depth, as this took into account the individual hair radius.

Overall, the hypothesis concerning color penetration depth and the associated color stability was supported. A good overview of the penetration depth was created. The main layers of hair could be identified in cross-section, allowing a statement to be made about the location of dye.

### 7.4.5 Chromatography for Analysis of Plant Material

TLC was used for identification of target substances in plant material as well as extracts by comparing their running behaviors to a standard substance – the indigo molecule. Thanks to a specific enzyme-staining method and the unique color of individual molecules, it was possible to clearly identify target substances. In addition, the $R_f$ values of the substances made it possible to identify the higher polarity of indican compared to indigo, which was a further indicator of a suitable detection method [207, 208].

Using preparative column chromatography with silica has proven to be a suitable method for separating indican from *Indigofera tinctoria* L.. A central advantage was the use of InHD p. as a starting material and EtOH as a solvent for initial extraction, which prevented premature hydolysis of indican through denaturation of the plant's own enzyme and the lack of water in the system. Furthermore, the multiple extractions of InHD p. with EtOH make sure that the target substance is enriched in the extract. Subsequent separation of different components from plant material by EtOAc on silica was successfully carried out. Early elution of indigo and indirubin was assumed by color, but could not be confirmed by other analytical methods because the concentrations were too low. This matches the expectations as indican concentration in *Indigofera tinctoria* L. is higher than this of the dye molecules. The yield of 1.62% indican isolated can be rated as moderate but satisfactory in the context of plant-based natural product extraction. Confirmation of purity by $^1$H-NMR in acetone and comparison with literature data supported the qualitative analysis. [124]

Overall, the methodology proved to be suitable for the isolation and identification of indican. The combination of extraction, TLC analysis, enzymatic detection reaction and preparative chromatography was conclusively structured and led to reproducible results. For future work, optimization of the extraction conditions or the use of alternative solvents to increase the yield could be considered. Due to the time-consuming preparation, commercially available dye precursor was used for pure substance coloration.

HPLC was used to quantify individual components from complex plant extracts and the hair fiber. Due to the high chemical diversity in the samples, HPLC was chosen as

the analytical method because it offered high selectivity and separation performance. Sample preparation and solvent choice were important factors to dissolve and extract target substances from complex systems. A C18 reversed-phase column was used because it is well suited for medium-polar to non-polar compounds. The mobile phase was same for both sample preparations with ACN/$H_2O$ (45:55) suitable for indican and isatin and DMSO used for natural indigo and indirubin. It consisted of a gradient of ACN and $H_2O$ (with 0.1% (v/v) FA) whereby the proportion of ACN was increased constantly. The pH was kept acidic through the addition of formic acid to improve the separation of compounds. The gradient allowed separation of polar and less polar compounds by a run of 40 min at 0.6 mL/min. A DAD (diode array detector) was used to enable simultaneous detection at wavelengths 240 nm for indican, isatin and indirubin and 300 nm for indigo molecule detection. Furthermore, the LOD and LOQ were in the low µg range which is sufficient for natural product analysis [65, 114].

The advantage of the described conditions used was that several compounds could be detected without the necessity to change the method. The drawback was that the runtime was high because the flow rate of 0.6 mL/min was low. This enabled improved chromatographic resolution, particularly for structurally similar substances like indigo molecule and indirubin. This is particularly important in the case of complex samples such as plant material. Furthermore, every sample had to be dissolved in two different solvent systems to be able to quantify each of the molecules of interest. Excluding column conditioning and sample application, the runtime amounted to 80 min for one sample material. However, each sample needed to be tested at least 3 times, resulting in a measurement time of over 240 min. The HPLC method used showed a good separation efficiency of the target substances, high linear correlation for the standard substances ($R^2 > 0.98$) in a range from 0.5 to 20 mg/L, depending on the substrate. For faster analyses, the usage of a Ultra-High Performance Liquid Chromatography (UHPLC) could be considered. [209] The combination with a mass spectrometry could help to identify even unknown substances e.g. for extract evaluation. [210] Preliminary liquid-liquid or solid phase extraction of the samples could promote a more targeted examination as they would eliminate interfering plant components before HPLC analysis, possibly shortening analysis times. [211] All in all, the HPLC method developed is suitable for the reliable and reproducible quantification of the ingredients of interest from plant extracts. It provides a solid basis for phytochemical quality controls or bio-analytical questions.

### 7.4.6 Evaluation of Harvest Influence on Plant Quality

In summary, there were large fluctuations between the multiple determinations of the samples along the harvesting process of the *Indigofera tinctoria* L. plants. Uncertainties in the comparability between different harvests of the plant and the sampling steps are that, for organizational reasons, different people have taken the samples. This can lead to variations in the analyses despite the protocol. In addition, the same plant was not always used for each sampling from crop 1 to 3. Since small amounts of plants were used for the HPLC analyses, this can also lead to unwanted irregularities despite multiple determinations. However, greater fluctuations are to be expected with natural products, which are highly dependent on environmental factors. Finally, it can also be stated that although the different amounts of indican in the samples could be measured, they had no systematic influence on the dye outcome at the end. In general, small amounts of indican are sufficient for intensive color results on the hair. Factors in the plant, which could not be finally clarified at this time, play a greater role in color formation.

Nevertheless, it can be stated that a certain range of differences must be tolerated in the context of these studies, as the natural product is subject to a number of influencing factors that cannot be eliminated. A reliable insight into influencing factors and adjusting screws in the harvest of *Indigofera tinctoria* L. were provided.

### 7.4.7 Molecule Extraction from Hair and *Indigofera tinctoria* L. Pathway Inductions

The staining for the extraction experiments from hair fiber were carried out under controlled conditions at 37 °C to ensure a high degree of comparability of the results. This standardized temperature selection made it possible to specifically take into account the temperature-sensitive reaction mechanisms already described in chapter 7.1.5. It was also pointed out that the elevated temperature led to intensified results in every coloring approach. The systematic variation of the dyeing preparations – untreated plant extract (InCL), InCL with the additives ascorbic acid and isatin, as well as enzymatically activated InHD p. – allowed a differentiated consideration of the factors influencing color formation and development in the hair. The parallel color measurement in connection with the quantitative analysis of the color molecules extracted from the dyed hair fibers confirmed the previous hypotheses. The extraction

technique for the analysis of *Indigofera tinctoria* L. in wool by HPLC introduced by Mantzouris *et al.* (2014) was transferable to hair applications. Same extraction conditions in terms of temperature and solvent but longer extraction time (30 min vs. 5 min figured out by Mantzouris *et al.* 2014) were found to be the most suitable for mentioned research purpose. [117] This could be explained by the different nature of hair and wool already discussed, particularly with regard to the thicker and less porous hair cuticle [36, 37] resulting in the molecules being more firmly anchored in the hair and therefore having longer extraction times.

The inferred pathway that represents the color formation on the hair, provides a theoretical basis for the targeted control of hair color by varying reaction conditions and additives. However, it should be emphasized that the proposed reaction cascade is based on observed phenomena and has not been verified at the molecular level. Further analytical methods, such as liquid chromatography-mass spectrometry studies, would be required for complete validation.

All in all, the methods and experiments presented in this research made it possible to identify and evaluate important parameters in hair coloration with *Indigofera tintoria* L. in a reliable way. As a result, user-friendly hair coloring with the plant-based raw material was advanced.

# 8 Summary of Results

This dissertation has provided insights into the color mechanism that takes place after applying *Indigofera tinctoria* L. to hair. Important influencing factors, such as the biosynthesis pathway from dye precursor indican leading to the color molecules, and hair as a substrate to be dyed, were examined. It was found that, compared to synthetically produced indigo, plant-based material was not only more environmentally friendly and harmless to health, but also offers decisive advantages for dye application to hair. This was demonstrated both from dye outcome observation and from the derived color formation pathway starting with indican. As soon as the glucose moiety from the dye precursor was enzymatically cleaved in the aqueous environment of dye suspension, the resulting indoxyl molecules penetrated the hair fiber and then either dimerized to natural indigo or formed isatin which then reacted with another indoxyl molecule to form indirubin inside the cortex. Both color molecules are water-insoluble and larger than their precursors. Therefore, remaining permanently anchored in the hair fiber.

Hair as a dye substrate had a significant influence on color development after traditional application of crushed leaves of *Indigofera tinctoria* L., especially in the first two weeks after dyeing. The cysteine content in keratin, which is particularly high in the hair cortex, favored formation of indirubin over natural indigo. Extracting the color molecules from the hair fiber confirmed that a mixture of indigo and indirubin formed in the cortex, resulting in a violet color impression. These color developments could be influenced specifically – either by an aqueous indigo extract or by post-treatment with water.

Overall, this resulted in a high-performance hair dye with high color stability over many hair washes. The performance was comparable to permanent synthetic hair dyes. It was particularly noteworthy that the addition of natural additives to the dye bath and a suitable temperature for application to the head resulted in an immediate red coloration. The application of this mixture to human hair complies with EU regulations. The color produced in this way from indigo plant material is not only a novelty in plant-based hair dyeing, but opens up an unexpected innovative areas.

Three components were identified as essential for dyeing with *Indigofera tinctoria* L. or corresponding extracts: The dye precursor indican, the splitting enzyme $\beta$-D-

glucosidase, and water for hydrolysis. This knowledge can be used for stabilization and controlled color release for hair dyeing products – e.g. through a two-component system keeping indican and enzyme separated in an aqueous environment and only combining them shortly before application. A water-based extract from *Indigofera tinctoria* L. was preferred because it offered numerous advantages for product development. The enzyme activity can either originate from the indigo plant's own system or be obtained from other systems through fermentation.

In addition, important factors for harvesting the plant in India were highlighted in terms of color performance and composition. After sowing in February, the harvest takes place three times between April/May and June/July. It is crucial that the freshly harvested plant material is dried quickly. It must be considered that, in addition to its drying effect, the sun can also have a negative impact on sensitive plant components. Drying the plants at night is therefore particularly advantageous. In addition, the time of harvest and storage conditions influence the quality of the material. In general, it is of utmost importance to preserve as much of the dye precursor indican in the plants as possible in order to enable cost-efficient dyeing. Shelf life can be maximized through dry storage and protection from microorganisms and insects. At the same time, adjustments to the harvesting protocol with regard to the composition of the raw material must also be considered from the point of view of feasibility. The plant processing implemented to date therefore represents the best compromise between investment costs, process costs, sustainability, and raw material quality.

The results of this work demonstrate that sustainability and functionality are not mutually exclusive in hair coloration with *Indigofera tinctoria* L., but rather complementary. The plant-based indigo source enabled effective and stable hair colorations without the use of synthetic developers or potentially harmful additives. At the same time, it offers ecological advantages such as biodegradability, a lower environmental impact and the possibility of resource-saving processing. The targeted control of color formation - for example via extract-based systems or additives in dye bath - allows plant-based dyeing systems to be developed that meet the requirements of both product performance and environmental compatibility. At the same time, this approach ensures that initial color instability on the hair, caused by its specific protein structure, is overcome. This opens up promising prospects for the development of marketable, versatile, bio-based hair dyes that address the growing consumer interest in natural and sustainable cosmetic products.

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# Appendix

### Safety Instructions of Chemicals

None of the chemicals used were carcinogenic, mutagenic or reprotoxic (CMR) substances. The GHS list is provided below.

**Table 8.1: Chemicals Used, Information on Symbolism According to GHS and H- and P-Statements.**

| Chemical Name | CAS Number | GHS Pictogram | H-Statements | P-Statements |
|---|---|---|---|---|
| Acetone-$d_6$ | 666-52-4 |  | H225, H319, H336 | P210, P233, P240, P241, P242, P305 + P351 + P338 |
| Acetonitrile | 75-05-8 |  | H225, H302 + H312 + H332, H319 | P210, P240, P302 + P352, P305 + P351 + P338, P403 + P233 |
| Ammonium Thioglycolate | 5421-46-5 |  | H290, H301, H317, | P234, P261, P264, P280, P301 + P310, P302 + P352 |
| Ascorbic Acid | 50-81-7 | - | - | - |
| Cooling Liquid (Water-Glycol) | - |  | H302, H373 | P260, P264, P270, P280, P312, P501 |
| Collodial Silica | 7631-86-9 | - | - | - |
| L-Cysteine | 52-90-4 | - | - | - |
| Dimethyl Sulfoxide | 67-68-5 | - | - | - |

To be continued on the next page

| Chemical Name | CAS Number | GHS Pictogram | H-Statements | P-Statements |
|---|---|---|---|---|
| Ethanol | 64-17-5 |  | H225, H319 | P210, P264, P280, P303 + P361 + P353, P305 + P351 + P338, P337 + P313 |
| Ethyl Acetate | 141-78.6 |  | H225, H319, H336 | P210, P233, P240, P305 + P351 + P338, P403 + P235 |
| Formic Acid | 64-18-6 |  | H226, H290, H302, H314, H331 | P210, P260, P280, P303 + P361 + P353, P305 + P351 + P338, P370 + P378, P403 + P233, P403 + P235 |
| Formulation with $\beta$-D-glucosidase activity | non-commercial |  | H334 | P261, P304 + P340 |
| Indican | 487-60-5 | - | - | - |
| Indirubin | 479-41-4 |  | H315, H319, H335 | P261, P264, P271, P280, P302 + P352, P304 + P340, P305 + P351 + P338, P312 P321, P362 + P364, P332 + P313, P337 + P313, P403 + P233, P405, P501 |
| Isatin | 91-56-5 | - | - | - |

To be continued on the next page

| Chemical Name | CAS Number | GHS Pictogram | H-Statements | P-Statements |
|---|---|---|---|---|
| Hydrochloric Acid | 7647-01-0 |  | H314, H335, H290 | P234, P260, P264, P271, P280, P301 + P330 + P331, P304+P340, P305 + P351 + P338, P310, P363, P390, P403+P233, P405, P406, P501 |
| Petroleum Ether (50-70) | 64742-49-0 |  | H225, H304, H315, H336, H361f, H373, H411 | P210, P243, P273, P310 + P330 + P331, P373 + 378, P403 |
| Sea Sand | 14808-60-7 | - | - | - |
| Sodium Chloride | 7647-14-5 | - | - | - |

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# Affidavit

### Affidavit

I hereby declare and affirm that this doctoral dissertation is my own work and that I have not used any aids and sources other than those indicated. If electronic resources based on generative artificial intelligence (gAI) were used in the course of writing this dissertation, I confirm that my own work was the main and value-adding contribution and that complete documentation of all resources used is available in accordance with good scientific practice. I am responsible for any erroneous or distorted content, incorrect references, violations of data protection and copyright law or plagiarism that may have been generated by the gAI.

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