Flinders University

# Optimising Laser Tattoo Removal: A Comprehensive Analysis of Parameters, Techniques, and Safety Protocols

By

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*Thesis*
*Submitted to Flinders University*
*for the degree of*

**Doctor of Philosophy**

College of Science and Engineering

November 2025

# CONTENTS

# ABSTRACT

Tattoos have become increasingly popular as a means of cultural identity and self-expression, leading to a corresponding rise in tattoo removal procedures. Despite this growing trend, concerns about the chemical composition of tattoo inks and the safety of laser removal treatments remain insufficiently addressed. This thesis investigates the chemical and toxicological implications of laser tattoo removal, with particular emphasis on the transformation of ink constituents under laser exposure.

Chapter One provides a comprehensive review of the history of tattooing, ink composition, and laser-based tattoo removal techniques. It also discusses related health concerns, as well as the chemical and cytotoxic profiles of tattoo inks and their laser degradation by-products.

Chapter two evaluated the components of commercially available tattoo inks (LY, GY, GR, BO and reference pigments PY14, PY65, PO13, PB15). Through analysis and using range of analytical techniques, including IR, NMR, XRD, Raman, EDX-SEM and ICP-OES, the components were identified. It was discovered that several of the tattoo inks studied were mislabelled, containing undeclared pigments and additional elements not listed on their bottle labels or safety data sheets. These new findings highlight significant inconsistencies in labelling practices, raising concerns about consumer safety, regulatory oversight, and the transparency of tattoo ink manufacturers. The presence of unlabelled components in inks suggests the potential for unknown health risks and underscores the need for stricter regulations and monitoring of the tattoo ink industry.

Certain tattoo inks are resistant to removal using laser methods because of their composition. This includes the removal of yellow pigments and tattoo inks containing titanium dioxide ($TiO_2$). Research outlined in chapter three describes a novel study focused on advancing the understanding of tattoo pigment photodegradation by investigating how $TiO_2$, a common additive in tattoo inks, influences the degradation of yellow pigments under 532 nm laser light. This study investigated several yellow pigments and tattoo inks before and after exposure to 532 nm QS Nd:YAG laser irradiation. A variety of analytical techniques were employed, including EDX-SEM, DLS, XRD, and GC-MS, to characterise the pigments and their degradation products. Results indicated that $TiO_2$ alters the degradation pathway, forming large particle agglomerates with ink components. This interaction reduced the amount of evolved of volatile fragments during laser irradiation, which could have implications for the effectiveness of tattoo removal and the safety of the degradation by-products. The behaviour of $TiO_2$ in tattoo inks provides valuable insights into the challenges of laser tattoo removal and the complexity of pigment interactions during the process. In addition, some of the degradation products were identified to be potentially harmful to the human body.

Chapter four addressed a critical gap in the literature by exploring the effects of laser treatments on tattooed dark skin. Melanin, a natural pigment abundant in darker skin tones, was found to interfere with laser therapies, leading to suboptimal results. The study specifically investigated the degradation of yellow pigments in the presence of melanin under laser irradiation. Yellow tattoo inks, reference pigments, and pigment-melanin mixtures were treated with a 532 nm QS Nd:YAG laser. The resulting degradation products, as well as their morphology and particle size, were analysed using GC-MS, SEM, and DLS. Findings revealed that melanin behaves similarly to $TiO_2$, altering pigment degradation pathways and reducing the formation of volatile fragments. These results provide a better understanding of the interaction between tattoo pigments and melanin, offering insights into the challenges faced during laser tattoo removal on darker skin tones and emphasizing the need for tailored treatment protocols.

Chapter five of this project used GC-MS to identify the degradation products formed on the irradiation of yellow pigments and inks. The chapter reports the formation *o*-toluidine, 2-methoxyphenyl isocyanate, and *o*-toluene isocyanate, compounds that have not been reported previously for these pigments. These compounds and the inks were assessed for their cytotoxic effects on HaCaT skin cells as breakdown products of tattoo inks and is one of the first cytotoxicity assessments of this kind. Experimental results showed that unirradiated inks and pigments reduced cell viability to approximately 50%, indicating inherent toxicity even before laser treatment. However, irradiated ink samples exhibited significantly heightened toxicity, with higher concentrations causing severe cell death. These findings underscore the potential health risks associated with both the use of tattoo inks and their breakdown during laser removal, raising concerns about the long-term safety of these practices.

In conclusion, this study further advances the analytical understanding of tattoo inks and their laser-induced transformation. The findings emphasize the urgent need for improved regulatory standards for tattoo inks, greater awareness of the health implications of tattooing and laser removal, and the development of safer practices in the tattoo industry. Through the application of analytical techniques this research contributes valuable insight into pigment behaviour under laser irradiation particularly regarding challenges associated with darker skin tones and lays a scientific foundation for future investigations into the chemical safety and efficacy of tattoo removal technologies.

# DECLARATION

I certify that this thesis:

1. does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university

2. and the research within will not be submitted for any other future degree or diploma without the permission of Flinders University; and

3. to the best of my knowledge and belief, does not contain any material previously published or written by another person except where due reference is made in the text.

4. has been completed without the use of generative artificial intelligence tools

Signed : Batool Abdullah M Aljubran

November 2025

# ACKNOWLEDGEMENTS

Completing a PhD thesis is a monumental job, and I could not have accomplished it without the assistance and guidance of many people. I am grateful to everyone who has supported me along on my journey, both academically and emotionally.

I would like to begin by expressing sincere gratitude to Prof. Claire Lenehan, my principal supervisor. Her unrelenting dedication, vast expertise, and insightful input have been the foundations of this research. Their mentoring has influenced not only my academic work but also my whole attitude to research. I am deeply indebted to her for her support and encouragement.

I extend my sincere appreciation to my co-supervisor, Prof. Kirstin Ross, and Dr. Ula Alexander, whose knowledge and unflinching support were critical to the successful completion of this thesis. Their distinct viewpoint and direction gave a well-rounded approach to my study, considerably improving the quality and depth of my work.

I would also be grateful to my academic institution for its assistance and resources, without which this research would not have been feasible. I would like to express my sincere gratitude to King Faisal University in Saudi Arabia for generously sponsoring my scholarship and providing me with the opportunity to pursue my studies. I would like to acknowledge Flinders University for providing the facilities, resources, and academic environment that supported the completion of this research.

I am also deeply thankful to the academic staff at Flinders University, including Dr. Liu-Fei Tan, A/Prof. Martin Johnston, Prof. Justin Chalker, Dr. Jason Gascooke, Ms. Amjad Alotaibi, Mr. Benjamin Boyle, Dr. Tristan Fraser, Ms. Jolene Anthony, and Dr. Alex Sibley for their invaluable support, guidance, and assessment throughout the course of my research. Their expertise and encouragement have been instrumental to my academic journey.

I would also like to thank my family, especially my mum, my husband, my kids and my sisters. Their unending encouragement, support, and understanding assisted me through every stage of my academic path. Their unflinching confidence in me has been my motivator. Furthermore, I want to thank my friends and colleagues for their significant ideas and moral support during my research process.

This doctoral dissertation is the outcome of an extended and challenging journey, and it could not have been achievable without the collaborative efforts of individuals named above. Each of you has contributed in your own unique manner, and I am grateful for your participation in this historic achievement. Thank you for your unfailing encouragement and support.

# LIST OF PUBLICATIONS

1- **Aljubran, B. A.**, Ross. K, Alexander. U, Lenehan. C. E, : Decoding Tattoo Inks: A Multi-Technique Analysis Reveals Discrepancies in Ingredient Composition and Elemental Content When Compared to Label Claims. Journal of Environmental Health, 88(2).pp.8-18 DOI https://doi.org/10.70387/001c.143999.

2- **Aljubran, B. A.**, Ross, K.E., Alexander, U. and Lenehan, C.E., 2025. Challenges in laser tattoo removal: the impact of titanium dioxide on photodegradation of yellow inks. Archives of Toxicology, pp.1-15. DOI https://doi.org/10.1007/s00204-025-03989-2.

3- **Aljubran, B. A.**, Ross. K, Alexander. U, Lenehan. C. E, : Analytical Investigation of Melanin's Impact on the Laser Fragmentation and Morphology of Yellow Tattoo Pigments. (accepted by Photochemical & Photobiological Sciences).

4- **Aljubran, B. A.**, Tan. L, Ross. K, Alexander. U, Lenehan. C. E, : Chemical and Cytotoxic Profiling of Tattoo Ink Degradation Products Generated by Laser Irradiation. (Submitted to Journal of Exposure science & Environmental Epidemiology ).

**Publications not presented in this thesis**

1- Samuel J. Tonkin, Harshal D. Patel, Jasmine M. M. Pople, Le Nhan Pham, Daniel J. Lewis, **Batool A. Aljubran,** Jason R. Gascooke, Christopher T. Gibson, Martin R. Johnston, Witold M. Bloch, Alex C. Bissember, Zhongfan Jia, Michelle L. Coote and Justin M. Chalker: Thermal Imaging Using Sulfur Polymer Optics (accepted by Nature Communications)

# CONFERENCE AND PRESENTATION

1. Aljubran, B. A., Ross. K, Alexander. U, Lenehan. C. E, : *Challenges in Laser Tattoo Removal: The Impact of Titanium Dioxide on Photodegradation of Yellow Inks,* [Poster] presented at 28th annual RACI R&D Topic Analytical and Environmental Chemistry Students Conference (2022), Flinders University, Australia (4th - 7th December 2022).

2. Aljubran, B. A., Ross. K, Alexander. U, Lenehan. C. E, : *Challenges in Laser Tattoo Removal: The Impact of Titanium Dioxide on Photodegradation of Yellow Inks,* [Poster] presented at Molecular Science and Technology Higher Degree Research conference (2022), Flinders University, Australia (12th December 2022).

3. Aljubran, B. A., Ross. K, Alexander. U, Lenehan. C. E, : *Laser Tattoo Removal on Dark Skin: Overcoming Melanin-Induced Challenges,* [Presentation ] presented at Molecular Science and Technology Higher Degree Research conference (2023), Flinders University, Australia (24th October 2023).

4. Aljubran, B. A., Tan. L, Ross. K, Alexander. U, Lenehan. C. E, : *Assessing the Impact of Tattoo Ink Laser Degradation Products on Skin Cell Viability: A Toxicological Investigation,* [Poster] presented at 17th IFEH World Congress on Environmental Health. (2024), Perth, Australia (20th - 24th May 2024).

5. Aljubran, B. A., Tan. L, Ross. K, Alexander. U, Lenehan. C. E, : *Assessing the Impact of Tattoo Ink Laser Degradation Products on Skin Cell Viability: A Toxicological Investigation,* [Presentation] presented at 42nd State Conference Program Environmental Health Australia (2024), Australia (20th September 2024).

6. Aljubran, B. A., Tan. L, Ross. K, Alexander. U, Lenehan. C. E, : *Assessing the Impact of Tattoo Ink Laser Degradation Products on Skin Cell Viability: A Toxicological Investigation,* [Poster] presented at Molecular Science and Technology Higher Degree Research conference (2024), Flinders University, Australia (5th November 2024).

7. Aljubran, B. A., Tan. L, Ross. K, Alexander. U, Lenehan. C. E, : *Assessing the Impact of Tattoo Ink Laser Degradation Products on Skin Cell Viability: A Toxicological Investigation,*[Poster] presented at 30th annual RACI R&D Topic Analytical and Environmental Chemistry Students Conference (2024), University of Tasmania, Australia (1st - 4th December 2024).

8. Aljubran, B. A. [virtual participation] at 7th World Congress of Tattoo and Pigment Research (WCTP 2025), Rome, Italy (7th -9th May 2025).

9. Aljubran, B. A. [Attending] at 14th Annual Conference of the Flinders Institute for Nanoscale Science &Technology, Flinders University, Australia (18th June 2025).

# AWARD AND ACTIVITIES

1- Best Poster Presentation Award. Presented at Molecular Science and Technology Higher Degree Research conference (2022), Flinders University, Australia (12th December 2022).

2- Horizon Professional Development Award Certificate, Sliver& Gold: 2024, Flinders University, Australia.

3- Horizon Professional Development Award Certificate, Platinum: 2024, Flinders University, Australia.

4- Al-Bataliya Award for Creativity and Excellence – Distinguished University Student. Presented by the Al-Bataliya Community, Saudi Arabia (2024).

5- Highly Commend Poster Presentation award. Presented at 30th RACI R&D Topics Conference held in the University of Tasmania, Australia (1-4th December 2024).

# LIST OF FIGURES

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| **Pigment yellow 74** | PY 74 |
| **Pigment yellow 14** | PY 14 |
| **Pigment yellow 65** | PY 65 |
| **Pigment orang 13** | PO 13 |
| **Pigment blue 15** | PB 15 |
| **Lemon yellow ink** | LY INK |
| **Golden yellow ink** | GY INK |
| **Golden rod ink** | GR INK |
| **Bright Orange** | BO INK |
| **Titanium Dioxide** | $TiO_2$ |
| **Extracted Melanin** | EM |
| **Synthetic -Melanin** | SM |
| **Gas chromatography -Mass spectroscopy** | GC-MS |
| **Liquid chromatography -Mass spectroscopy** | LC-MS |
| **Gas chromatography -Flam ionisation detection** | GC-FID |
| **Nuclear Magnetic Resonance** | NMR |
| **Dynamic Light Scattering** | DLS |
| **Scanning Electron Microscope** | SEM |
| **Raman spectroscopy** | Raman |
| **X-ray Powder Diffraction** | XRD |
| **Infrared Spectroscopy** | IR |

| | |
|---|---|
| **Inductively coupled plasma-optical emission spectroscopy** | ICP-OES |
| **Crystal violet** | CV |
| **Dimethyl sulfoxide** | DMSO |
| **Ethylenediamine tetra acetic acid** | EDTA |
| **Foetal bovine serum** | FBS |
| **3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide** | MTT |
| **Phosphate-buffered saline** | PBS |

# 1 CHAPTER 1: INTRODUCTION

## 1.1 History of tattooing

Tattooing is a form of permanent body art, created by applying decorative ink to the skin, and has been practiced since ancient times [1]. Tattoos have a history dating back to 12,000 BC and have been discovered on early mummies from Peru, Egypt, and Russia, suggesting that tattooing was a global occurrence. "Ötzi", the oldest European iceman mummy, dates back to 3370–3100 BC and has 61 tattoos spread throughout his body [2, 3]. For thousands of years, tattooing was a laborious and agonising process that included manual piercing of the skin for each tattoo [4]. The introduction of the electric tattooing instrument in the late 1800s changed the art form [4].

People tattoo their skin for a variety of purposes, such as interpretations of feelings, representing cultural or spiritual beliefs, commemorating significant life events, or simply for aesthetic and personal identity reasons [1, 5-7]. In comparison to some cultures, such as Polynesian tribes, the purpose of tattoos in the Western world has become more ambiguous throughout the centuries [8-10]. In recent years, the global population of tattooed individuals has grown dramatically [7,11], driven by technological advancements in tattooing and reduced costs of these services [12,13]. Research indicates that over 25% of the world's population between the ages of 15 and 35 have tattoos [7,14], with the prevalence rates of tattooing ranging from 11.7% to 31.5% in industrialized countries [15]. In addition, most currently accessible surveys indicated that tattoos are becoming increasingly common in Western countries [16]. Approximately 20% of people in Germany, 25% of people in Australia, and 29% of people in the United States of America have tattoos [16-18].

## 1.2 Tattooing procedures and biological distribution

The word tattoo is derived from the Tahitian word "tatu", which means "to mark something" [19,20]. In the past, sharp devices, such as flint knives, were used to incise the skin, allowing the subsequent insertion of colorants into the resulting wounds for the purpose of pigmentation. Conversely, current tattoo artists utilise disposable needles that are inked and affixed to rotary machines [20]. This enables accurate pigment implantation in the human skin, decreasing the wounded skin area and allowing for more complicated tattoo patterns (Fig. 1.1) [4,20,21]. Tattoos are usually applied in private homes or in specific tattoo parlours. Tattoos can be either black or multicoloured. A survey done in Germany in 2010 found that over 60% of tattoos are either totally or partly black [10,22]. Tattoos can be found on almost every part of the human body, including the mucous membranes and the eyelids [23]. Tattoos are categorized into five types, as outlined in Table 1.1[4].

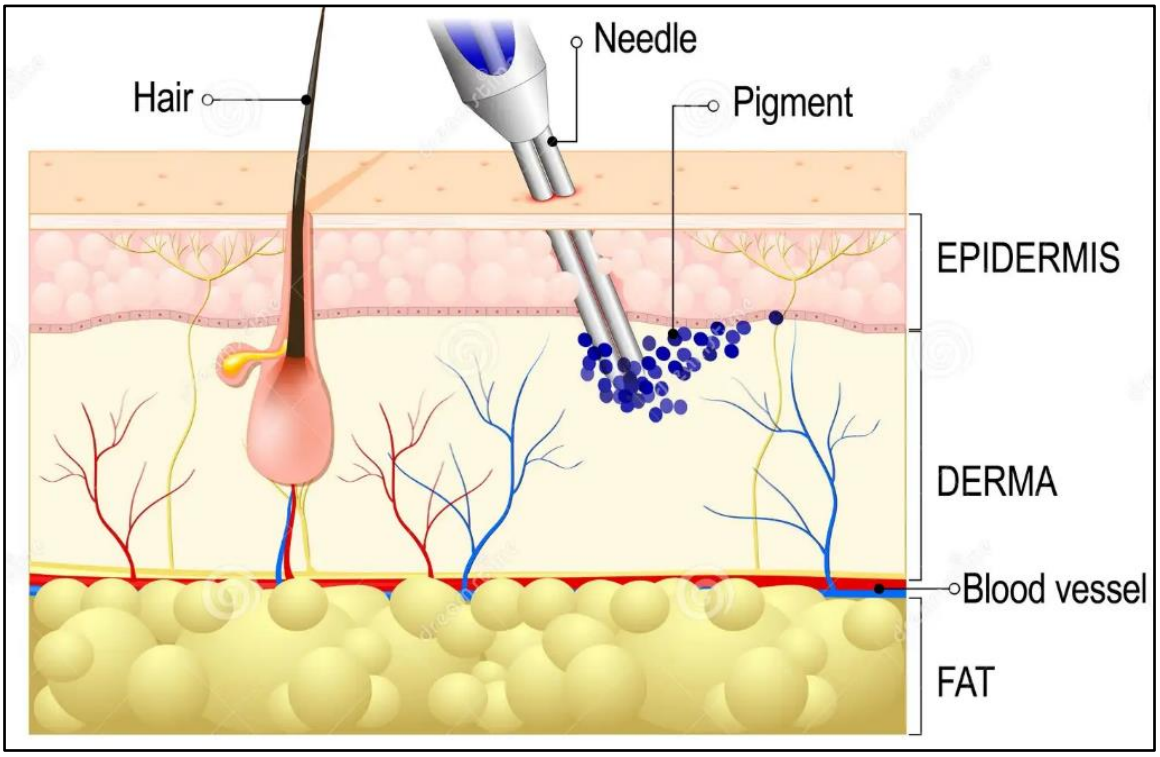



**Figure 1.1: Tattooing procedure refers to the injection of pigment (via tattoo ink) into the dermis of the skin.**

**Table 1.1: A summary of the tattoo types.**

| Types of tattoos | Mechanism | Description | Reference |
|---|---|---|---|
| **Professional tattoos** | Pigment insertion using a tattoo machine | Most common type of tattoo<br><br>Applied using a machine with a rapidly vibrating needle<br><br>Produces uniform and high-density ink deposition<br><br>Use professional-grade inks composed of organic, inorganic, and organometallic pigments [4,10,24] | [4,10,24] |
| **Amateur tattoos** | Manual or homemade device-based ink insertion | Created using readily available pigments such as charcoal, pen ink, or soot<br><br>Applied with hand-held needles or improvised tattoo machines<br><br>Typically, less intense and more variable in colour compared to professional tattoos [20] | [20,4,10,25] |

| | | | |
|---|---|---|---|
| | | Needles are not hollow, resulting in less ink penetration<br><br>Generally easier to remove due to superficial ink placement [4,10,25] | |
| **Cosmetic tattoos** | Micropigmentation using fine needles | Increasing in popularity for aesthetic purposes<br><br>Use colours such as brown, pink, and red to mimic makeup (e.g., eyebrows, eyeliner, lips) [24]<br><br>Common pigments include titanium dioxide ($TiO_2$) and ferric oxide<br><br>Removal is challenging due to pigment composition<br><br>Laser treatment may cause pigment oxidation, leading to paradoxical hyperpigmentation [10] | [10,24] |
| **Traumatic tattoos** | Pigment deposition via abrasion or explosive force | Occurs unintentionally through skin injuries (e.g., road rash or blast trauma)<br><br>Inks become trapped in the dermis following wound reepithelialisation [4,26]<br><br>Caused by exogenous materials embedded in the skin following mechanical trauma<br><br>Removal may be difficult if particles are located in the deep dermis or contain explosive residues [10] | [4,10,26] |
| **Medical tattoos** | Pigment insertion for clinical purposes | Used for medical indications such as marking radiation therapy sites, medical device access points, or corneal tattoos [26].<br><br>Typically, grey or blue-black in colour<br><br>Commonly made with Indian ink or carbon-based pigments | [26,24] |

| | |
|---|---|
| | Serve as permanent reference points in clinical procedures [24] |

A tattoo's colour is caused by pigment particles remaining in the dermis and absorbing light at a certain spectral wavelength. The concentration of tattoo pigment injected into the skin can exhibit significant variation, with experimentally recorded values spanning from 0.60 to 9.42 mg/cm$^2$ [27,28]. Following tattooing, a portion of the tattoo ink injected into the skin exits through the wounded surface. Another portion of the tattoo ink injected into the skin is either actively carried by migrating cells or removed passively by lymph or blood vessels [29]. This transport has been reported to be influenced by the size of the colourant particles [30]. Immune system cells identify tattoo pigments as foreign materials when the pigments are injected into the skin. These cells try to phagocytose the pigments or break them down so the pigments can be eliminated from the skin and transported into the lymphatic system [31]. However, tattoo pigment particles are often quite large, have a low chemical reactivity (fairly inert), and are difficult to phagocytose or break; the immune system eventually becomes compromised by encircling and isolating the pigment, which helps to keep it from moving around and ultimately fixes the pigment in place [31]. As time passes, immune cells may persist in their attempts to phagocytose and move the pigment particles, and some patients may have strong and effective immune systems that finally manage to break down or transport part or all of the pigments [31]. As a result, tattoo pigments can be seen in the local lymph nodes, but they can also move to other organs, including the liver, lungs, or kidney [29, 32]. Studies in mice have shown that Kupffer cells in the liver capture pigment particles from tattoo solutions, as shown by particle analysis using electron microscopy [33].

## 1.3 Composition of tattoo inks

In the past, tattooists used self-mixed black soot to create traditional tattoo ink, and certain inks are still produced in this manner today [34]. Commercial tattoo ink suspensions may comprise numerous distinct chemical compounds that include vehicles (water, glycerine, and other alcoholic derivatives), additives (surfactants, polycyclic aromatic hydrocarbons, nanoparticles, and polymers), and pigments [8, 35, 36]. Solvents, preservatives, and a variety of other ingredients are blended with the colouring pigment [8]. The composition of tattoo ink is described in Fig. 1.2.

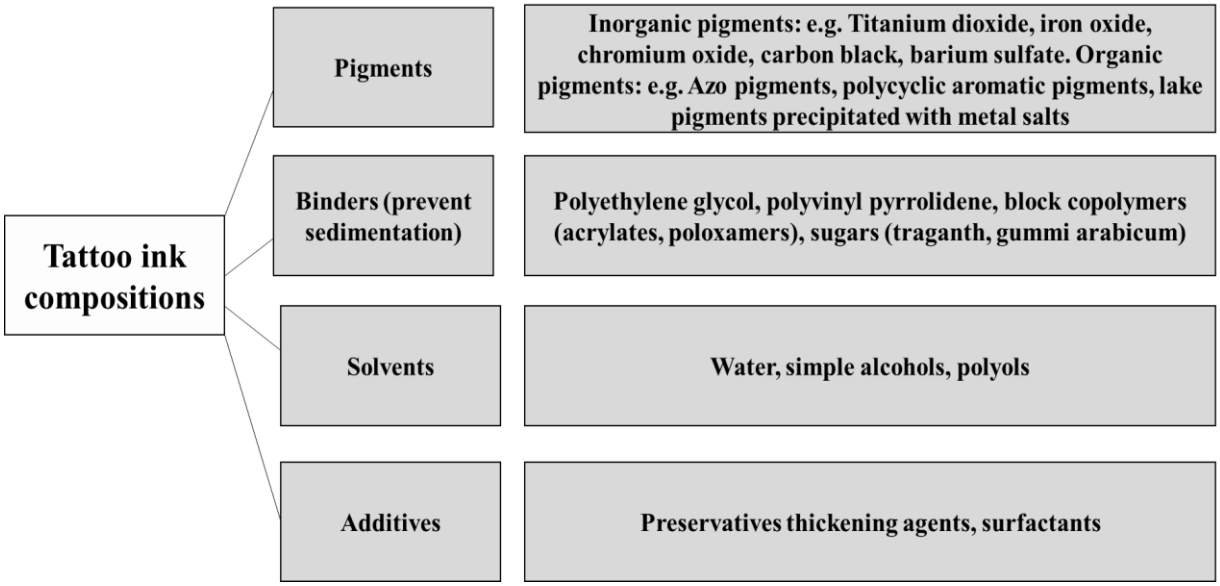



**Figure 1.2: The basic ingredients of tattoo inks [14].**

Historically, inorganic compounds such as mercury (II) oxide (red), cobalt (II) aluminate (blue), chromium (III) oxide (green), manganese violet, titanium dioxide (white), and iron oxides (brown) were commonly used as tattoo pigments [37]. Over time, these organometallic pigments have been gradually supplemented, and in many cases, replaced by organic pigments, which now make up the majority of modern tattoo inks [38]. To achieve specific tones or enhance brightness, metals such as aluminium, calcium, and cadmium are often incorporated into organic pigment formulations [35, 39]. Nevertheless, inorganic pigments remain prevalent in micropigmentation inks used for permanent cosmetics, including eyebrow makeup, eyeliner, and lip colour, due to their superior resistance to light and heat, improved setting characteristics, and larger particle sizes that make removal more challenging [35, 39].

### 1.3.1 Pigments

In chemical terms, colourants can be classified as either pigments or dyes. Although tattoo inks are frequently referred to as dyes, this terminology should be avoided because dyes are water-soluble and cannot be used to permanently decorate the skin [40]. Tattoo inks may contain various inorganic and/or organic pigments to achieve the desired colour [9,27]. The use of insoluble pigments ensures the stability of a tattoo in the skin. Pigment particles are synthesised in the chemical industry to form small solid-state particles, with dimensions ranging from a few micrometres to fewer than 100 nanometres [9].

**Inorganic pigments**

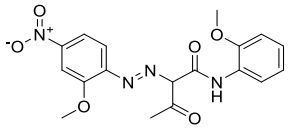
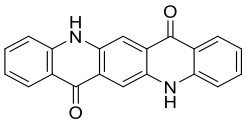
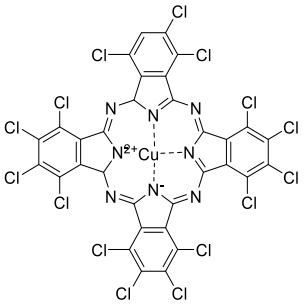
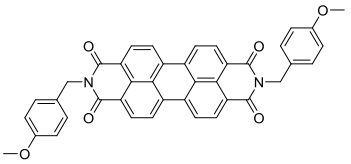
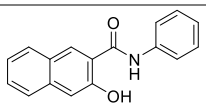
In tattooing, titanium dioxide (white) and other carbon derivatives (black) are the predominant inorganic pigments [8, 37] . Black pigments are created via flame soot methods. One class of coloured

inorganic pigments is based on iron oxides, which exist in yellow (Fe O[OH]), red ($Fe_2O_3$), and black ($Fe_3O_4$). Another colour category is based on heavy metals like cadmium sulphide (CdS, yellow), mercury sulphide (HgS, red), chromium oxide ($Cr_2O_3$, green), or cobalt spinel ($CoAl_2O_4$, blue) [8]. Due to the danger associated with such heavy metal compounds, their use has decreased. Two major inorganic pigments that continue to be used are $TiO_2$, mainly for lightening colours, and carbon black, primarily for black tattoos [8, 41].

**Organic pigments**

The preference for using organic pigments in tattoo inks stems from their exceptional colour-matching strength, lightfastness, resistance to enzymatic degradation, dispersion, and modest production costs [42]. Quinacridones, azo dyes, phthalocyanines, and black carbon dyes are the four classes into which the majority of pigments belong [19, 43]. Table 1.2 presents the chemical structure and colours of each type. Such pigments have a high light absorption, resulting in great colour intensity and, as a result, a dazzling colour in the skin, which may be the primary reason for their use in tattoos [9]. Pigments are characterised chemically as azo or polycyclic pigments. Azo pigments include mono-azo (greenish to medium yellow, reddish yellow to orange) and di-azo (greenish, reddish to orange red) compounds. Polycyclic pigments often have a condensed aromatic or heterocyclic ring structure and can include b-naphthol (orange to medium red), naphthol AS (medium red to violet) moieties [44]. Organometallic pigments with nickel, copper, or cobalt [45] can include phthalocyanines (green and blue) and quinacridone pigments (bluish red, red, violet) [44].

**Table 1.2: Chemical structure of different type of organic pigments [9, 44].**

| Group of pigment | Example | Chemical structure |
|---|---|---|
| **Azo** | Pigment Yellow 74 |  |
| **Quinacridones** | Pigment Violet 19 |  |
| **Phthalocyanines** | Phthalocyanine Green |  |
| **Black carbon** | Pigment Black 32 |  |
| **Polycyclic pigments** | Naphthol AS |  |

### 1.3.2 Solvents and additives

Powdered pigment must be combined with a fluid medium because tattooing with dried powder is almost impossible. They are also nearly insoluble in many of the solvents that may be applied to human skin [27]. To minimise particle sedimentation, emulsifiers, binding agents (such as polyvinylpyrrolidone and polythene glycol), and thixotropic additives are employed to incorporate pigment particles into aqueous solutions. This includes a mixture of heterogeneous components in which the solute particles do not dissolve but instead become suspended in most of the solvent and float around freely in the medium. Antifoam agents, such as polydimethylsiloxane, are utilised in the suspension to inhibit foam production during agitation [8].

### 1.3.3 Preservatives

Preservatives are commonly used into tattoo ink compositions at concentrations of up to 1.5% by weight to prevent microbial contamination post-opening [46]. Preservatives such as phenol, phenoxyethanol, benzoic acid, different isothiazolinones, and formaldehyde are found in tattoo inks. Even when the compounds are authorised to be used according to cosmetics standards, preservatives are seldom documented on the list of ingredients [8, 47].

## 1.4 Regulation of tattoo ink composition

Numerous tattoo pigments were originally designed for purposes other than tattooing and often lack established safety profiles for this specific application [48, 49]. Due to the diverse chemical compositions of tattoo pigments, which influence their properties and associated risks, developing comprehensive and effective regulatory frameworks for these substances presents a significant, but essential challenge [21].

In Australia, tattoo inks are not classified as therapeutic goods and therefore fall outside the regulatory scope of the Therapeutic Goods Administration (TGA). Instead, regulation of the chemical components in tattoo inks is managed by the National Industrial Chemicals Notification and Assessment Scheme (NICNAS) [50, 51]. NICNAS generally does not regulate the importation of tattoo ink chemicals if they are already listed on the Australian Inventory of Chemical Substances (AICS) [50, 51].

Although limited data are available on the contamination or adulteration of tattoo inks, some evidence points to issues such as incorrect labelling [51]. A 2016 NICNAS report examining tattoo ink composition in Australia found that several products were non-compliant with regulatory standards, inaccurately labelled, or unsuitable for use [52]. This report has provided a foundation for risk assessment and the development of public health management strategies Additionally, a survey conducted among tattoo artists in central Brisbane and Melbourne revealed a general lack of awareness regarding the chemical composition of the inks they used, as well as the potential health risks associated with these substances [50].

In Europe, tattoo inks are regulated by the European Union's General Product Safety Directive [53]. According to this directive, a producer is required to place only safe items on the market, and a comprehensive list of contents must be included on the product label, accomplished through classification, labelling, and packaging[21, 34].

## 1.5 Tattoo regret

Tattoo art has been practiced in many cultures for thousands of years and is now popular among people of all ages, social classes, and jobs. The increased prevalence of tattoos has led to a corresponding increase in the rate of tattoo regret [13, 54]. A 2016 American study found that 23% of tattooed individuals regretted their tattoos, up from 14% in 2012 [55]. Research in Saudi Arabia, which included 181 tattooed participants, indicated that 58% regretted their tattoo, and 42.5% tried to remove it [13]. Although most people are satisfied with their tattoos, many are obtained impulsively, often before the age of 18, or under the influence of alcohol or recreational substances [56]. Therefore, these people may experience emotions of humiliation, low self-esteem, and stigma as a result of their tattoos, and many may want to have them removed [4, 57]. Common reasons for regret include getting the tattoo at a young age, changes in personality or lifestyle, pressure from family or spouse, poorly designed or meaningless tattoos, and medical issues such as allergic reactions or irritation, which may increase over time [13, 54, 58, 59]. Additionally, pruritus is the most frequently reported issue associated with tattoos in Saudi Arabia [13].

## 1.6 Tattoo removal

There is a growing demand for tattoo removal[10] and tattoo removal efforts can be dated back to ancient Egypt, with a variety of methods attempted over the past century [24]. Throughout history, many techniques, such as surgical excision, dermabrasion, and chemical destruction, have been employed to eliminate tattoos. Some of these old methods have caused harm to adjacent skin tissues, leading to scars and only partial removal of the tattoo itself [10, 24]. In the last 20 years, lasers have revolutionised the process of tattoo removal and have become the prevailing treatment paradigm. Advancements in laser techniques with a high-energy, short pulse have improved tattoo removal treatments, making them more effective with fewer adverse outcomes [10, 24].

### 1.6.1 Mechanical methods

For centuries, tattoo removal relied primarily on chemical and mechanical methods, much like those practiced in ancient Rome, until more advanced techniques emerged approximately 50 years ago [4]. Salabrasion appears to be the most effective method for removing amateur tattoos [60]. This technique was first documented by the Greek physician Aetius approximately 1479 years ago [61]. Salabrasion involves abrading the skin, followed by the application of salts or other chemicals to the wounded area, which is then covered with a surgical dressing [62]. To decrease scarring and hypopigmentation, this procedure is modified by removing the salt directly after salabrasion [60]. However, this procedure has fallen out of favour over the last decade and is currently rarely used due to the danger of scarring [60].

Another method is dermabrasion, which involves destroying the skin and the tattoo pigment within it with a wire brush or diamond fraise [63]. During this procedure, a rapidly spinning diamond fraise wheel or a wire brush abrades the skin, which is normally prepped with a skin refrigerant to form a hard surface [64]. Traumatic tattoos are typically superficial, requiring only one treatment. Professional and amateur tattoos appear to be deeper and may demand multiple treatments [65]. For complicated traumatic tattoos, dermabrasion has also been utilised in conjunction with surgical excision [65]. Dermabrasion removes the tattoo's superficial layer, exposing the deeper layer, which can then be medically erased [60].

The third method involves a simple surgical approach for tattoo removal. Ideally, this procedure results in a linear scar; however, tattoos are often located in areas with limited excess tissue, necessitating staged removal [4]. Surgical excision is a viable method for eliminating small tattoos in regions with sufficient skin laxity, as well as cosmetic or traumatic tattoos. In areas with greater skin laxity, surgery may produce an optimal scar [60]. One advantage of surgical excision is the potential for complete tattoo removal in a single procedure. However, for larger tattoos, particularly in areas with higher skin tension, successful removal may require multiple surgeries, each carrying an increased risk of complications [60].

Mechanical tattoo removal methods are less time-consuming and more affordable than thermal techniques. However, their primary drawback is the high risk of scarring. Hypertrophic scars often occur when tissue is extensively dissected to eliminate all tattoo ink [66]. Additionally, residual tattoo pigment is commonly left behind after treatment [66]. Other adverse effects of these invasive methods include postoperative pain, potential bleeding, and an increased risk of infections or other complications [60, 66, 67].

### 1.6.2 Thermal methods (Laser techniques)

Pigment particles are believed to remain within the dermis for two primary reasons. First, they are phagocytised by stationary macrophages within the dermis [8]. Second, their size often exceeds the capacity for phagocytosis or transport through lymphatic collectors, causing them to remain in place [8]. Consequently, any method that reduces particle size can decrease the amount of pigment retained in the skin. A key factor in particle size reduction is the light-induced degradation of pigment molecules, which occurs continuously when tattooed skin is exposed to light [8, 45].

A significantly safer and more effective method for tattoo removal emerged in 1966 when Goldman *et al.* (1965) documented the first use of a laser for tattoo removal [68]. Three years later, a case series using a quality-switched (QS) Ruby laser was published, marking a pivotal advancement in the field

[26]. Since then, laser treatment has become the gold standard for tattoo removal. Historically, QS lasers were the primary choice for this procedure. The first commercially available QS laser was the Ruby laser, followed by the QS neodymium-doped yttrium aluminium garnet (Nd:YAG) and QS Alexandrite lasers. These three lasers remain widely used by practitioners today. However, picosecond lasers are increasingly popular and are marketed as offering higher efficacy compared to QS lasers [26]. Key factors influencing the effectiveness of laser tattoo removal include skin pigmentation, pulse duration, spot size, and fluence [4]. Additionally, different laser wavelengths are more effective for targeting specific pigment colours (Table 1.3), a topic explored in greater detail in Section 1.7.3.

**Table 1.3: Efficacy of Q-switched lasers for specific tattoo colours [10,69].**

| Q-switched laser | Wavelength (nm) | Tattoo colours most effectively removed |
|---|---|---|
| **Nd: YAG** | 1064 | Black, blue |
| **Nd: YAG** | 532 | Red, orange, yellow, brown |
| **Alexandrite** | 755 | Black, blue, green |
| **Ruby** | 694 | Black, blue, green |

The principle underlying laser tattoo removal is the theory of selective photothermolysis. First introduced by Anderson and Parrish (1983), this concept forms the foundation for using lasers to target specific materials in the skin, such as melanin, pigments, water, and oxyhemoglobin, while minimizing damage to surrounding tissues [70]. These materials, known as chromophores, effectively absorb laser energy at specific wavelengths, with different chromophores exhibiting peak absorption at varying wavelengths [40].

When a chromophore absorbs a photon of a particular wavelength, a chemical reaction occurs, generating heat that disperses through the surrounding tissue. This heat leads to rapid thermal expansion, fragmenting the target material into smaller particles, often accompanied by shock waves (Fig. 1.3) [40, 71]. Additionally, this process may cause molecular decomposition, producing potentially harmful by-products such as hydrogen cyanide and aromatic amines [40, 71].

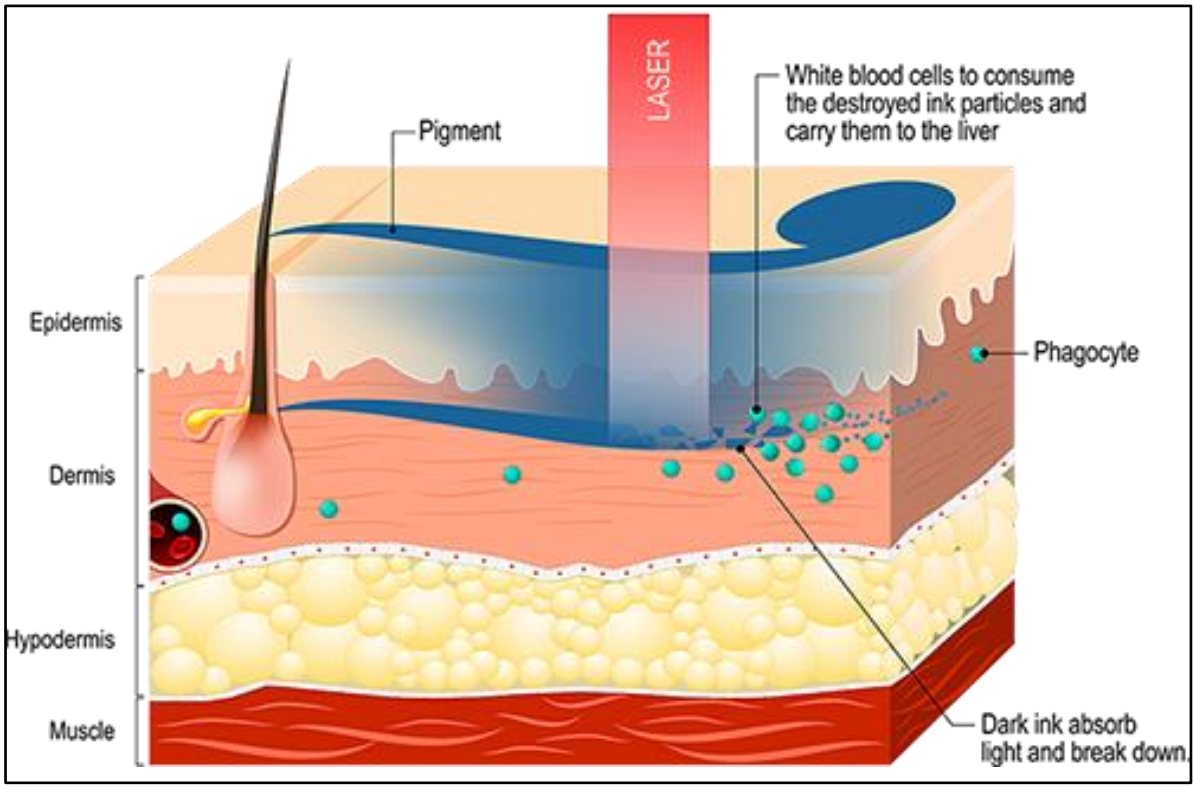



**Figure 1.3: General principle of laser tattoo removal [72].**

The thermal relaxation time (TRT) of a chromophore refers to the time required for it to dissipate 50% of the heat absorbed during laser treatment [10, 40]. According to the theory of selective photothermolysis, a chromophore can be effectively destroyed without harming surrounding tissues if it is heated for a duration shorter than its TRT [10, 40]. Notably, TRT is directly proportional to the size of the chromophore, the larger the chromophore, the longer its TRT, and vice versa. In practice, the TRT of a chromophore is calculated as the square of its diameter in millimetres. For example, a chromophore with a diameter of 0.1 micrometres ($10^{-4}$ mm) has a TRT of approximately 10 nanoseconds ($10^{-8}$ seconds) [4, 10, 26, 70].

Beyond the thermal effects, photon absorption by chromophores also generates pressure forces, which form the basis of inertial confinement time (ICT). Similar to TRT, ICT represents the time threshold within which mechanical stress is applied to destroy a particle [26]. When a chromophore is struck with sufficient energy in a period shorter than its ICT, the resulting mechanical disruption is known as the photoacoustic effect [26]. As a result, shorter pulse durations than the TRT of the targeted region are significant in cosmetic laser pigmentation treatment protocols [40]. However, a laser with a pulse duration range still requires several treatments to fully destroy the tattoo ink into smaller particle sizes. Many tattoo pigments are estimated to have a TRT in the picosecond range, as *in vivo* studies have shown that their particle sizes typically range from 40 to 300 nm [11]. According to the TRT

formula, the TRTs of carbon particles with diameters of 40, 100, 200, and 300 nm are 19.12, 119.5, 478, and 1,060 ps, respectively [25].

Picosecond laser pulses are considered more effective for targeting tattoo particles, as they deliver thermal energy more precisely and produce stronger photothermal and photomechanical effects compared to conventional nanosecond-domain lasers[11]. Tattoo fragmentation is caused by photoacoustic impacts rather than photothermal effects with shorter picosecond pulse durations [73]. In terms of side effects after laser irradiation, such as tattoo darkening, applying a picosecond laser creates less heating, allowing additional darkening to be prevented and other heat-related adverse effects to be minimised.

The picosecond laser may also provide better mechanical breakdown of pigment particles (breaking up particles into smaller fragments that are more easily phagocytosed) [74]. Mi Soo Choi *et al.* (2018) [11] used Hartley guinea pigs to study the impacts of picosecond laser on multicoloured tattoo treatment [11]. Their study clearly showed that the 532-nm wavelength laser with a picosecond pulse duration was the most useful in erasing orange and yellow tattoos (Fig. 1.4). Picosecond lasers showed less epidermal injury and faster healing than 532 nm nanosecond lasers [4, 11].

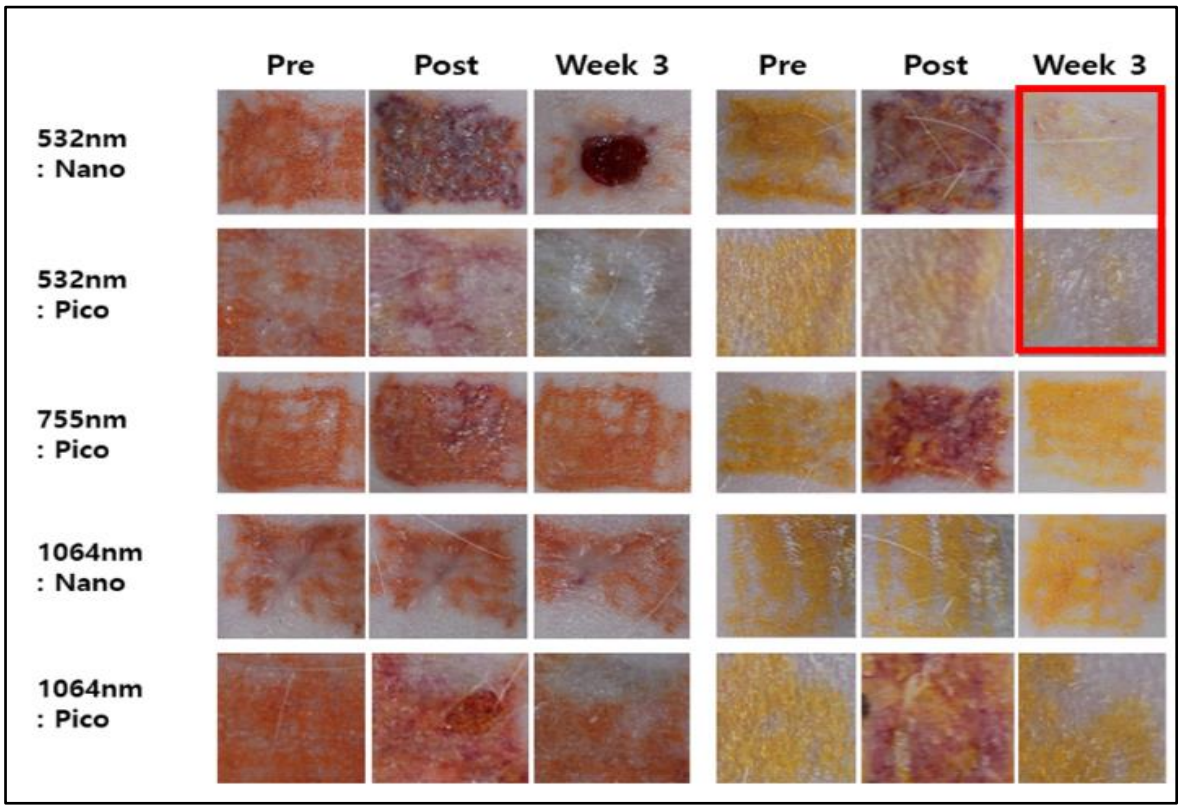



**Figure 1.4: Clinical photos of alteration in orange and yellow pigments over time [11].**

## 1.7 Factors that affect tattoo pigment degradation by laser

Understanding the variables involved in tattoo removal is essential to evaluate the laser response rate. Three key factors should be considered: the type of laser used, the type of skin, and tattoo-specific parameters such as the style, depth, and size of the tattoo (Table 1.4) [75].

**Table 1.4: Summary of the primary variables and factors that influence pigment degradation [75].**

| Main variable | Characteristics |
|---|---|
| **Tattoo** | Type of tattoo, aged, depth, volume surface area, colour of tattoo, size, and morphology of particles. |
| **Laser** | Type of QS laser, wavelength, spot size, fluence, pulse duration. |
| **Host Factors** | Age and type of skin. |

### 1.7.1 Particle size

Tattoo ink particle size is a critical factor in laser tattoo removal, because it influences the absorption of laser energy and the subsequent breakdown of pigments. According to the principle of selective photothermolysis, lasers with pulse durations in the nanosecond range or shorter are required for effective removal of tattoo particles, which typically range from 30 to 300 nm in size [36, 76]. Short TRT of around 10 ns are characteristic of such particle sizes [36, 76].

Research using transmission electron microscopy (TEM) has provided a broader perspective on pigment sizes [44]. One study analysed both *in vitro* and *in vivo* human tattoo biopsies, revealing that tattoo pigments can range from as small as 10 nm to as large as 5000 nm [77]. Specifically, black pigments were identified within intracellular lysosomes *in vivo*, with sizes ≤ 100 nm [76, 77].

Building on this, Høgsberg *et al.* (2011) classified tattoo pigments based on their colour and size [36]. Their analysis of single-pigment inks revealed that black pigments were the smallest, while white pigments, typically composed of $TiO_2$, were the largest (Fig. 1.5). Coloured pigments such as blue (PB15), red (PR170), and yellow (PY74) fell in between, with average sizes of 81 nm, 119–142 nm, and 154 nm, respectively. In contrast, white pigments had mean diameters ranging from 317 to 738 nm [36].

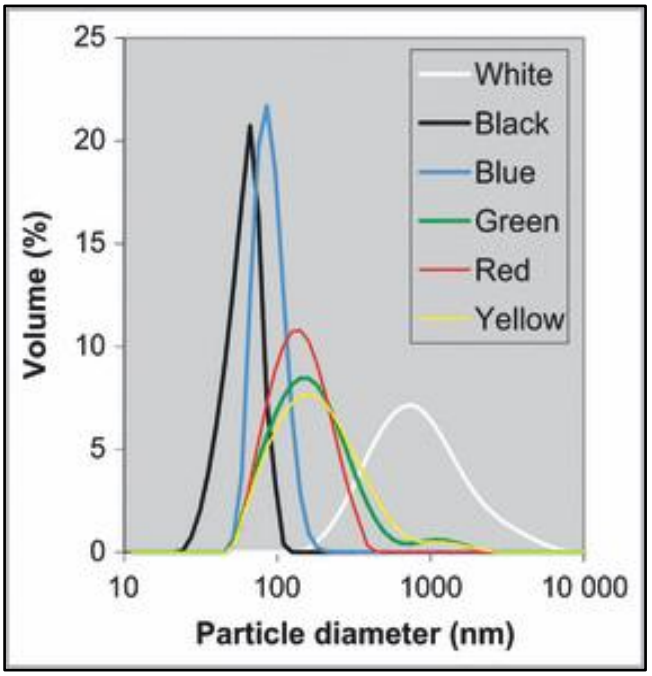



**Figure 1.5: Laser diffraction particle size characteristics of six tattoo inks [36].**

The study further explored how pigment aggregation and $TiO_2$ content influence particle size and distribution [36]. As shown in Fig. 1.6, $TiO_2$ not only increased particle dimensions, but also caused bulk aggregation. This was especially evident in inks combining multiple pigments. For example, a yellow ink containing PY65, PG7, and $TiO_2$ exhibited large particles and aggregates in its unfiltered form. When filtered through syringe filters with a 200 nm pore size, these aggregates were significantly reduced. Inks with single pigments were typically analysed without filtration, while combination inks, particularly those with $TiO_2$, were evaluated both before and after filtering [36].

Moreover, the presence of $TiO_2$ may alter how pigments interact with laser light. Although prior studies [36, 77], like those by Høgsberg *et al.* (2011), examined pigment aggregation across 58 tattoo inks, they did not fully assess how different forms, sizes, and concentrations of $TiO_2$ influence the degradation process under laser exposure. Given that $TiO_2$ affects photothermal interactions, its role deserves further investigation.

The effectiveness of laser tattoo removal is also highly dependent on both pigment size and colour. A study hypothesised that black ink responds more effectively to Q-switched lasers due to its high absorption and smaller particle size [78]. Conversely, white ink, with its larger particles and lower absorption capacity, shows poor responsiveness. These findings underscore the importance of understanding pigment characteristics for optimising laser-based removal techniques [78].

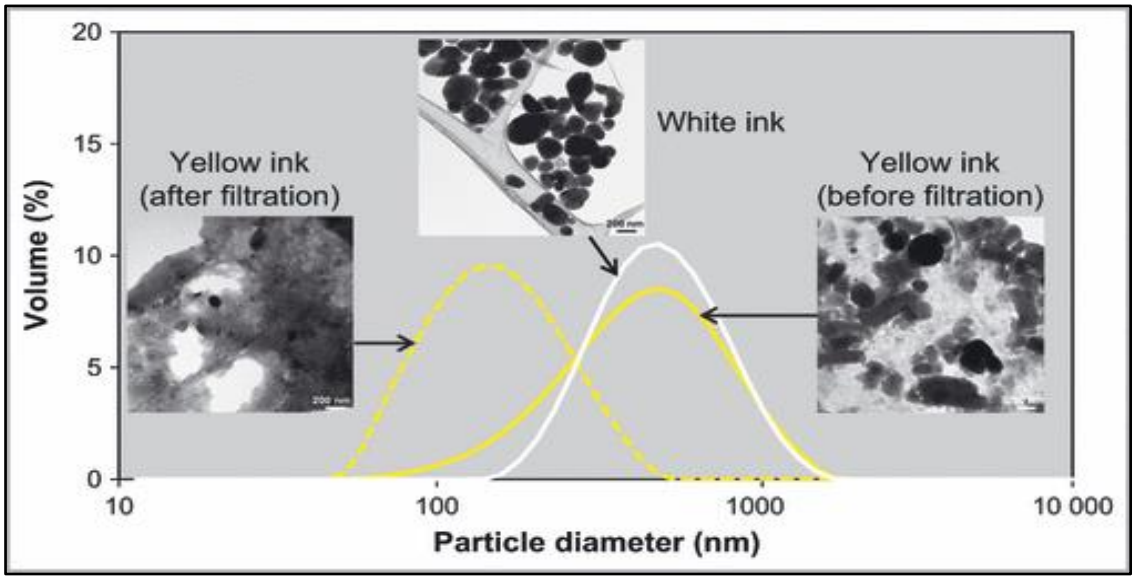



**Figure 1.6: Filtering a yellow ink to remove aggregates from $TiO_2$ in a sample of lemon-yellow ink [36].**

### 1.7.2 Morphology of particles

Carbon black, commonly used in black tattoo inks, is an inorganic pigment known for its diverse and complex morphologies [40]. Depending on the manufacturing process, carbon black particles can exhibit four main shapes: linear, spheroidal, ellipsoidal, and branching structures [40]. In contrast, coloured tattoo inks typically contain organic pigments, such as azo and polycyclic compounds, which are generally more amorphous than ideal crystalline structures. However, these organic pigments often undergo various post-synthesis treatments to enhance or modify their crystallinity [79, 80].

Despite these treatments, it is nearly impossible to determine the exact crystallinity state of pigment particles at the time of tattoo application. Once deposited into the dermis, the pigments may undergo further changes [40]. For example, different phagocytic processes in the skin can lead to aggregation of the initially dispersed particles. Consequently, both black and coloured tattoo pigments in the skin can display a wide range of particle sizes and shapes [40].

These morphological variations have significant implications for laser-tissue interactions. Differences in particle structure and aggregation can influence how pigments absorb and respond to laser energy, potentially leading to varied fragmentation mechanisms and treatment efficacy. This variability underscores a critical gap in current knowledge and highlights the need for further investigation into the influence of pigment crystallinity and morphology on laser tattoo removal outcomes.

### 1.7.3 Wavelength range

Selecting the appropriate laser wavelength is crucial, as different tattoo pigment chromophores absorb light at specific wavelengths[10]. For effective treatment, the chosen wavelength must match the absorption peak of the pigment [69]. For example, black and dark blue pigments strongly absorb 694 nm light wavelength, making the Q-switched ruby laser particularly effective for treating these colours. However, other pigment colours absorb this wavelength to a lesser extent [4].

In addition to pigment absorption, the penetration of light into the skin is influenced by wavelength (Fig. 1.7) [81]. When optical radiation enters turbid media such as skin, scattering occurs, which complicates light transmission [82]. However, as the wavelength increases, photons are scattered less, resulting in greater penetration depth [82]. For example, ultraviolet (UVB) radiation (~300 nm) penetrates only a few tenths of a millimetre, while infrared light (e.g., Nd:YAG at 1064 nm) can reach several millimetres [75]. However, this trend reverses beyond approximately 1100 nm, where increased absorption by water in the skin reduces penetration depth [40, 81]. The balance between absorption and penetration depth must be considered when selecting a laser for different tattoo colours and depths.

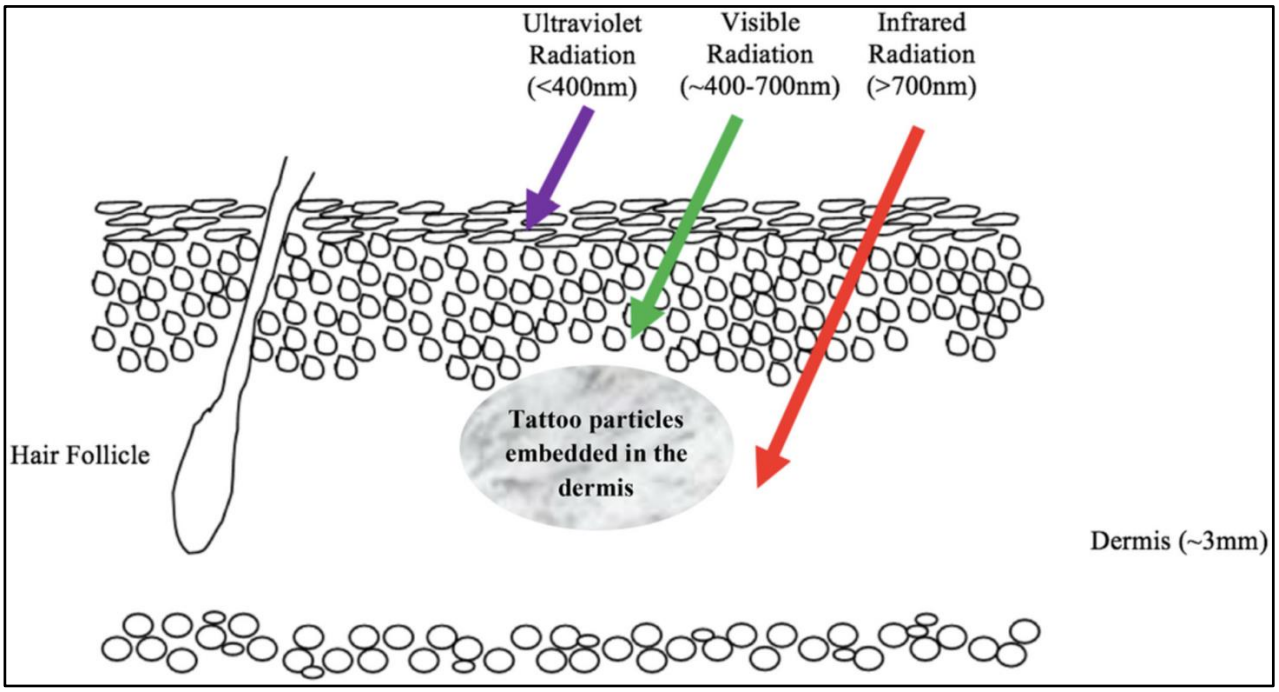



**Figure 1.7: Skin cross-section showing the relative penetration levels of various wavelength areas of electromagnetic radiation. Reproduced with permission from [81].**

The QS Nd:YAG laser is versatile, emitting both 1064 nm infrared and 532 nm green light using a potassium titanyl phosphate (KTP) crystal. This dual-wavelength capability allows it to treat a wide range of ink colours: the 1064 nm wavelength is well-suited for black and blue inks, while the 532 nm wavelength effectively removes red, orange, and yellow pigments (Fig. 1.8) [44] . Meanwhile, the QS 755 nm alexandrite laser is particularly effective for green inks, and the QS 694 nm ruby laser remains the best option for purple pigments [10].

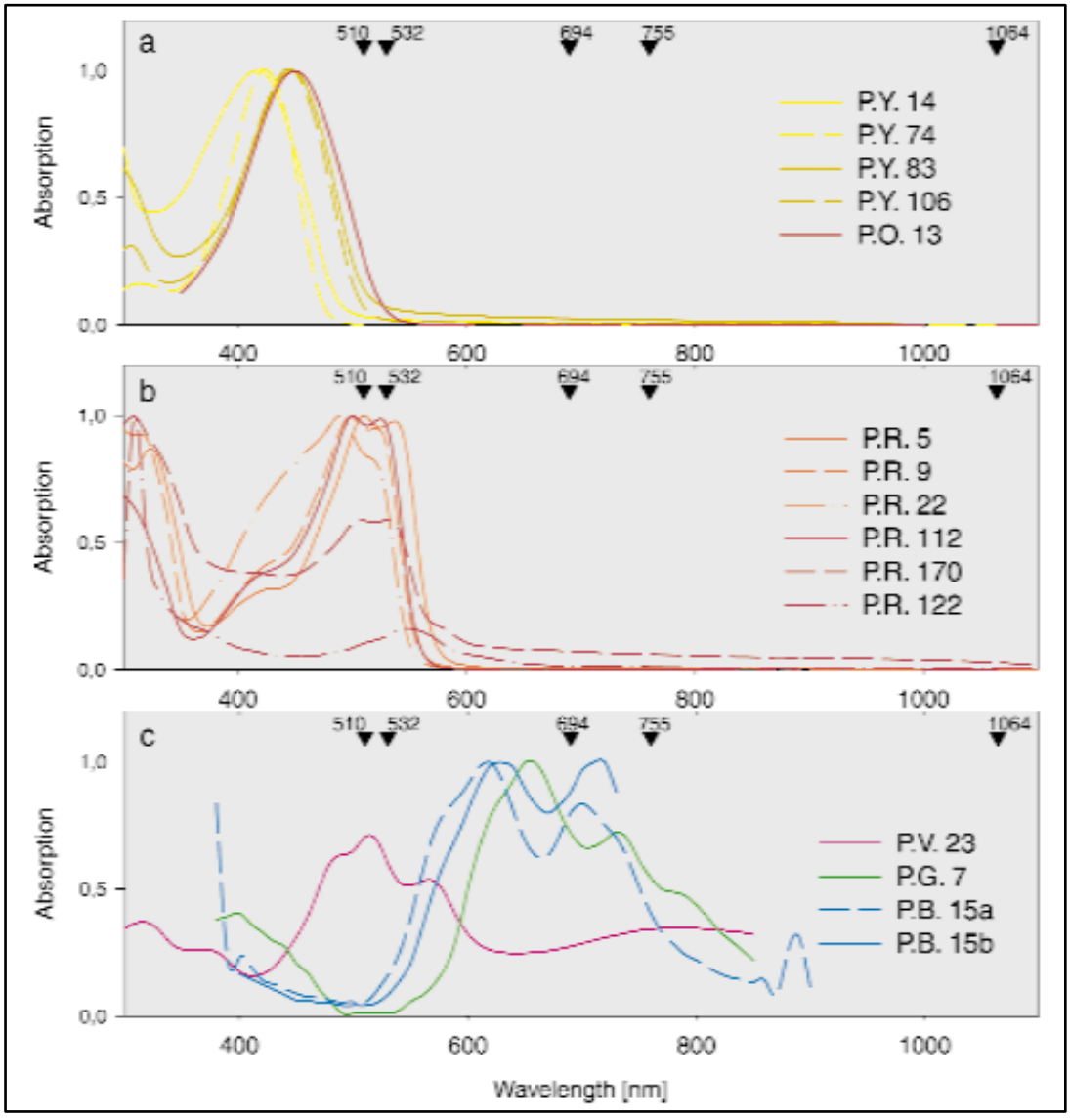



**Figure 1.8: The wavelength-dependent absorption of different pigments. The symbols inside the picture indicate the wavelength of Q-switched lasers used for tattoo removal (Figure is adapted from [44]).**

Comparative studies of the 694 nm ruby, 755 nm alexandrite, and 1064 nm Nd:YAG lasers for treating blue-black tattoos have shown that while the ruby laser clears pigment effectively, it is more frequently associated with long-term pigmentary changes, such as hypopigmentation or hyperpigmentation, especially in darker skin types [83].

Importantly, light with longer wavelengths also interacts less with epidermal melanin. This reduces the risk of unwanted pigmentary side effects and enhances laser safety in individuals with darker skin [4]. As summarised in Table 1.5, the clarity and safety of tattoo removal largely depend on choosing a laser wavelength that both matches the pigment's absorption properties and considers skin interactions.

**Table 1.5: Summary of the condition used to remove tattoo ink by laser with different colours.**

| Chemical class | Pigment colour | Optimal wavelength (nm) | Q-Switched Laser | Number of treatments | Percentage of clearance | Reference |
|---|---|---|---|---|---|---|
| **Carbon black** | Black | 1064 | Nd: YAG | 3-4 | 75-95% | [84] |
| **Phthalocyanine** | Green | 694, 755, 532 | Ruby and Alexandrite | 4-8 | 90% | [75] |
| **phthalocyanine** | Blue | 694, 755, 1064 | Nd: YAG | 3-6 | 75-100% | [85] |
| **Azo** | Red | 532 | Nd: YAG | 6 | >90% | [86, 87] |
| **Azo** | Yellow | 532 | Nd: YAG | 2-4 | >75% | [88] |

It should be noted that additional laser parameters can affect tattoo removal efficiency in addition to wavelength. Table 1.6 below indicates how other laser parameters might affect tattoo removal efficiency [26, 89, 90].

**Table 1.6: Summary of the parameters of laser device that should be taken into consideration for tattoo removal methods [26, 89, 90].**

| Parameter | Definition | High application | Low application |
|---|---|---|---|
| **Pulse width** | The length of time that each pulse of the laser is employed directly to the skin. This factor distinguishes Q-switched from picosecond lasers. | A pulse width higher than TRT is not practicable to achieve tattoo removal. | In accordance with the TRT idea and the size of the tattoo particle, a maximum of a nanosecond pulse is required to achieve the best results. |
| **Fluence** | Referred to as the "local dose" of a laser device, it is the energy per unit area ($J/cm^2$). | High fluence is used when the target chromophore amount is low. | Low fluence is applied to tattoos that have layered designs or strong colour. |
| **Spot size** | The laser beam diameter, measured in millimetres, is known as spot size. | Greater photon preservation due to increased spot size, increases skin penetration and reduces epidermal damage. | When a small spot size is used, photons are more likely to be scattered, resulting in less laser penetration and an increased danger of epidermal damage (as the action of the energy will tend to be more superficial) |
| **Repetition rate** | The repetition rate (RR) is the rate at which pulses are fired in one second; 1 Hz corresponds one pulse fired every second. | In broad and flat lesions, a high RR is frequently employed to decrease the course of treatment. | Low RR is employed when high precision is required, such as when the lesions are discrete, tiny, or placed on an uneven surface. |

### 1.7.4 Age of tattoo

Following tattooing, pigment particles are randomly dispersed within the dermis at depths ranging from 0.2 to 3 mm [40]. The exact position of these particles within the skin depends largely on the age of the tattoo. In relatively new tattoos excess pigment may still be present in the epidermis within the first one to two weeks. However, as keratinisation and epidermal regeneration occur, this superficial pigment is typically eliminated through sloughing at the damaged epidermal dermal junction [91].

Following this initial phase, the remaining pigment becomes embedded in various layers of the dermis. The body's immune response is triggered, and macrophages begin to phagocytose the pigment particles, transporting some to nearby lymph nodes [42]. Over time, this ongoing immune activity contributes to gradual pigment fading [42].

The age of the tattoo is a critical factor in determining the number of laser treatments required for removal. Older tattoos tend to have already undergone years of immune system processing, which reduces the amount of residual ink. The natural degradation of pigment molecular structure over time may also make older inks easier to fragment with laser treatment. In contrast, newer tattoos contain a higher concentration of intact pigment, making removal more difficult and requiring more sessions. Additionally, a tattoo must fully heal through all skin layers, typically over three to six months, before laser treatment is advisable [4, 75]. Initiating treatment too soon increases the risk of poor outcomes and side effects such as scarring or pigment changes [4, 75].

Environmental exposure, particularly to UV light, also plays a role in tattoo fading. Tattoos located on sun-exposed areas of the body may undergo pigment degradation over time, even without laser treatment [40]. UV radiation, with wavelengths between 280 and 400 nm, is efficiently absorbed by most tattoo pigments, leading to slow, long-term colour fading following repeated sun exposure [40]

### 1.7.5 Skin colour

Laser tattoo removal depends heavily on the interaction between laser light and skin chromophores, primarily melanin and oxyhemoglobin [7]. Among these, melanin is the dominant chromophore in the epidermis and plays a critical role in determining treatment effectiveness, especially across different skin tones [7].

Skin tone significantly influences laser-tattoo removal outcomes. Individuals with lighter skin contain less melanin, allowing the laser to focus more directly on tattoo pigments. This typically results in faster and more effective removal with fewer treatment sessions. In contrast, those with darker skin have higher melanin content, higher, which competes with tattoo ink for laser absorption. As a result, lasers may inadvertently target melanin, increasing the risk of pigmentary complications

such as hypopigmentation or hyperpigmentation [75] . To minimise these risks, lower laser fluence and adjusted parameters are used, which usually necessitate more sessions to achieve full ink clearance[4, 75]. Despite these challenges, tattoo removal can still be successfully achieved in darker skin types with careful laser selection and precise technique to avoid damage to surrounding tissue and reduce the risk of dyspigmentation [4, 75].

This relationship between melanin and laser treatment is further influenced by melanin's broad absorption spectrum, which spans wavelengths from 250 to 1200 nm [5]. This wide range enables most visible-light and near-infrared lasers to effectively target pigment [92, 93]. However, in darker skin tones, the high concentration of melanin in the basal layer increases the risk of non-specific thermal injury, leading to adverse effects such as focal atrophy, permanent dyspigmentation, textural changes, and scarring [94]. Competitive absorption by epidermal melanin also reduces the amount of laser energy reaching deeper dermal layers where tattoo pigments reside. This decreases treatment efficacy and increases the potential for complications if laser settings are not properly adjusted [92, 95].

Although darker skin absorbs more laser energy due to higher melanin content, melanin's absorption coefficient decreases with increasing laser wavelength [5, 96]. For example, the epidermis absorbs approximately four times more energy from a 694 nm ruby laser than from a 1064 nm Nd:YAG laser under the same exposure settings [5, 92], making longer wavelengths safer for darker skin types. The alexandrite laser (755 nm) and QS Nd:YAG laser (1064 nm) are considered safer alternatives for these individuals, as melanin absorbs these wavelengths less efficiently [73].

In addition to skin tone, pigment colour plays a crucial role in wavelength selection. The QS 694 nm ruby laser is highly effective for removing black and blue tattoo pigments, while the 532 nm green light is best suited for targeting red, orange, and yellow pigments [84]. However, both wavelengths are strongly absorbed by epidermal melanin, making them less suitable for darker skin types due to the increased risk of long-term dyspigmentation and other adverse effects [73]. By comparison, the longer wavelengths of 755 nm and 1064 nm not only provide better safety in melanized skin, but also maintain sufficient energy for pigment targeting [73].

Even when targeting tattoo pigments, melanin might absorb part of the laser energy, reducing the light intensity that reaches the ink particles. This interaction must be considered carefully, especially in darker skin types, to ensure effective pigment fragmentation while minimising damage to surrounding skin structures.

## 1.8 The difficulty and hazards of removing particular colours of tattoo ink

### 1.8.1 Titanium dioxide

$TiO_2$ is referred to as pigment white 6, has a C.I. of 77891, and is found in most (~75%) tattoo inks [97]. It is commonly used in the range from 38 % to 95 % to make blue inks and to change the brightness of other pigments [98]. In nature, the minerals anatase, rutile, and brookite are all examples of titanium dioxide, and the crystalline structures for $TiO_2$ are tetragonal, brookite orthorhombic, respectively, with rutile being the most stable phase and the others being metastable [99]. $TiO_2$ works well as an opacifier in paints, plastics, coatings, papers, inks, pharmaceuticals, sunscreens, toothpastes, and cosmetics. The opacity is controlled by optimising the $TiO_2$'s particle size, which can range from a few nanometres to several micrometres [8].

Under UV irradiation, the anatase form of titanium dioxide particularly displays photocatalytic activity [100], creating radical species. $TiO_2$'s chemical characteristics allow it to absorb UV radiation between 280 and 400 nm. $TiO_2$ allows light transmission, with negligible absorption at wavelengths greater than 400 nm. However, due to the high intensity of the nanosecond pulses commonly used in laser therapy, even a small relative absorption of other wavelengths is sufficient to notice a darkening response based on a $TiO_2$ reduction in the compound. It is unknown whether the quantity of $TiO_2$ influences the degree of darkening [98]. Tattoos with a high $TiO_2$ content, such as (8007) white (98.55 % $TiO_2$), might darken faster than those with a lower $TiO_2$ content, although this can only be verified through experimentation [98]. A better understanding of the identity, shape, and particle size of the darkening material produced after being exposed to laser light is required.

Titanium dioxide was classed as 2B by the International Agency for Research on Cancer in 2010, indicating that it may be harmful to humans [101]. $TiO_2$ nanoparticles have also been studied in a number of genotoxicity investigations involving DNA that has been oxidatively damaged in cell cultures and animal models, as well as comet test endpoints [102]. $TiO_2$ has the potential to produce DNA strand breaks in a concentration-dependent manner unaffected by exposure time. A standard comet test was shown to be capable of distinguishing between the genotoxicity of various forms of $TiO_2$, with anatase form having the highest genotoxic potential [103]. After being exposed to titanium dioxide, cell culture experiments show elevated amounts of oxidatively damaged DNA[103].

While many nanoparticles interact with cells without causing acute toxic responses, metal oxide nanoparticles such as $TiO_2$ nanoparticles have been shown to be associated with autophagy dysfunction with toxic consequences [104]. In addition, Viviana et al. (2016) investigated the impacts of $TiO_2$ nanoparticles (18 nm) on autophagy in human keratinocytes (HaCaT) cells at non-cytotoxic levels [105]. They found that $TiO_2$ nanoparticles caused a 15–25% loss of cell viability, above the ISO

non-cytotoxic threshold of 70 %. Under non-cytotoxic conditions, $TiO_2$ nanoparticles uptake resulted in a dose-dependent increase in autophagic effect [105]. The mitochondria were also revealed to be the primary target of tattoo ink toxicity in HaCaT cells [105], and this may be due to the existence of $TiO_2$ nanoparticles, as previously observed with HaCaT cells [35].

In a study conducted by Hering (2022), the interaction of UV light and laser light with tattoo pigments and the consequent effects on human skin cells were investigated in order to gain a better understanding of the photo-induced side effects reported in tattoos [21]. Electron microscopy demonstrated dermal fibroblast absorption of the tattoo pigments employed, including $TiO_2$ anatase and rutile, pigment orange 13, and carbon black. $TiO_2$ anatase dramatically lowered cell survival and elevated interleukin-8 release in 2D monolayer cultured fibroblasts. This study also raised issues about $TiO_2$ anatase and azo pigments like PO13 and their application in tattoo inks [21].

$TiO_2$ has been found to make the tattoo removal more difficult [98, 106]. In most clinics, laser removal of pigments based on $TiO_2$ is not achievable[98, 106]. $TiO_2$ acts strangely, changing colour after the treatment from white to filthy green, dark grey, blue, and even pale purple, or does not respond to laser light at all [7]. Repeated laser removal treatment of titanium-containing tattoo ink generally results in skin scarring or the tattoo becoming weaker [7]. In a study conducted by Kim et al. (2006), several tattoo ink colours were analysed using quantitative energy dispersive spectrometry (EDS) to determine how they reacted to the Nd-YAG laser in animal studies [106]. According to this study, titanium is a key ingredient in blue tattoo ink that causes resistance to Nd:YAG laser treatment[106].

The mechanisms behind how tattoos darken after laser therapy remain unclear [107]. One possible explanation is the laser-induced reduction of metallic compounds such as $TiO_2$ found in some tattoo inks [98].When tattooed skin is exposed to laser light, it experiences reduction and changes from white to black. This transformation is frequently unattractive from an aesthetic standpoint [108]. When a Q-switched ruby laser was used to remove tattoos with $TiO_2$ pigment, a black colour was observed [107]. The reduction of white and other titanium-containing inks is suggested to be the mechanism. High-intensity laser irradiation has been found to cause Ti (IV)to be reduced to Ti (III), causing the blue colour. By exposing a titanium-enriched sunscreen to radiation, this study also showed how the colour changed [107].

Currently, there is no research confirming how $TiO_2$ in laser tattoo removal behaves in real-life environments, including both living organisms (*in vivo*) and controlled test settings (*in vitro*). Existing studies are limited to laboratory experiments and do not reflect environmental or biological

conditions. Therefore, further research is required to understand the role of $TiO_2$ in the poor response to laser treatment and to determine whether this metal is the underlying cause.

Little is known about how the density of ink nanoparticles affects laser tattoo removal. One research paper reported ink re-aggregation and ejection at high speed; however, no analytical data were presented to support these claims [109]. Reaggregation would make it potentially difficult to remove tattoos, and this may have ramifications for the treated individual. Understanding the removal process requires collecting data on morphological changes in treated inks, as they likely play a key role.

Thus far, there has been a lack of research on the degradation products created by tattoo laser treatment. Due to the large number of patients treated with these laser systems, it is necessary and important to investigate the degradation products formed from the tattoo pigments based on $TiO_2$ treated with high laser intensities.

### 1.8.2 Yellow pigments

Organic yellow pigments can be classified as monoazo and diazo pigments [110]. A number of studies have assessed commonly used different pigments in tattoo inks, with yellow pigments found in dermatome shave samples taken from 104 individuals who had adverse responses to tattoos [111]. PY 74 had the highest frequency (5%) among yellow pigments that were identified in all biopsies N = 104 [111]. In addition, Hauri (2011) reported that the frequency of PY74 in the samples was 7%, which was the highest proportion among yellow pigments[112]. However, the frequencies of PY 65 and PY 14 were 2.1 and 0.5 percent, respectively [112].

Unregulated organic pigments include those that contain carcinogenic aromatic amines as a structural component (PY 74, PY 65, PY 14, PO13). These pigments frequently fail when examined under the authorized azo-dyes standard EN 14362 due to their inability to dissolve. This is a concern, because carcinogenic amines are sometimes produced when tattoo inks are exposed to UV light or laser treatment [112]. There is considerable cause to be concerned that these pigments may emit toxic compounds when exposed to light or when tattoos are erased using laser radiation [112].

Fading of a tattoo's colour is the most important indicator of success of laser tattoo removal. The development of the laser techniques such as alexandrite picosecond has resulted in the more efficient removal of green and blue pigments. On the other hand, yellow pigments have no proven and final therapy, as they do not absorb existing laser wavelengths effectively and are therefore more difficult to remove [88]. Yellow pigments, which are commonly composed of cadmium sulphide, ochre, PY74, chrome yellow, or curcuma yellow, are now treated using a QS frequency doubled Nd:YAG laser with a nanosecond pulse duration, although clearing remains challenging and uneven [88]. In addition,

*in vitro* investigations have shown that the wavelength of the yellow ink's absorption peak does not match the current wavelengths available in QS laser systems (440 and 470–485 nm) [88, 113]. Varma (2002) also reported that yellow tattoo ink has been found to darken in two out of every 31 tattoos [114]. However, this hue change did not occur following the initial therapy [114, 115].

Yellow ink has traditionally been hard to remove using QS laser instrument; thus, using picosecond lasers for effective therapy of yellow ink is of particular interest[10, 88]. For example, six patients with yellow tattoo inks had >75% removal after one to four laser treatments using picosecond frequency doubled 532 nm Nd:YAG [88]. Scientists suspected that picosecond technology's photomechanical effects were more important for ink breakdown than the direct photothermal impact on the targeted yellow chromophore [88].

### 1.8.2.1 Photodegradation and metabolism of yellow pigments

A previous study investigated the effect of UV light on pigment yellow 74[116]. The authors used a 6.5 kW xenon arc lamp with the emission filtered through WG320 glass filters (0.6 mm; Schott Glass Technologies, Puryea, PA) to achieve a spectrum with UV light content consistent with sunlight (UV-A. UV- B, -2l: l). This study found that the three main photodecomposition products were: *N*(2-methoxyphenyl)-3-oxobutanamide (*o*-acetoacetanisidide), 2-(hydroxyimine)-*N*-(2-methoxyphenyl)-3-oxobutanamide, *N,N*″-bis(2-methoxyphenyl)urea[116]. In addition, Cui et al. (2005) investigated PY7 metabolism and discovered that PY74 was metabolised by the cytochrome P450 (CYP450) enzymes CYP1A1 and CYP1A2 [117]. They also observed that the enzymes CYP1B1 and CYP3A4 metabolised PY74, although not as much as the other two, showing a lesser efficiency. PY 74 was also discovered to be metabolised in two stages. PY74 is hydroxylated first, and then o-demethylated, as shown in (Fig. 1.9) [117].

In another study, pigments (PY16, PY3, PY12, and PY100) were exposed to artificial light and analysed using pyrolysis GC-MS to investigate their photochemical degradation [118]. Artificial ageing was performed using a Ci4000 Xenon weather-ometer (Atlas, USA) equipped with a 6500 W water-cooled Xenon lamp, under conditions of 0.35 W/m² irradiance at 340 nm with 100% light exposure, reaching a total radiant exposure of 510 kJ/(m²·nm) [118]. Colorimetric analysis showed significant changes in the yellow pigments (PY3, PY12, and PY100), including a reduction in the yellowness/blueness value, indicating a decrease in the yellow component, and darkening, evidenced by a decline in the lightness/darkness value. Pyrograms of both unaged and aged samples revealed peaks for benzene, isocyanato, and aniline, with the aniline peak increasing after ageing due to greater availability of this pigment component for pyrolysis. This increase was attributed to the breaking of the amide bond during photoaging, leading to structural changes and associated colour alterations [118].

Investigating laser irradiation, one study reported the photodecomposition products of irradiated pigment yellow [119]. In this investigation, post-mortem tattooed pig skin and the breakdown products of the pigment yellow138 in aqueous solutions were both examined. Xylene, benzene, and chlorinated benzenes like hexachlorobenzene were the main products of laser irradiation of PY138 in post-mortem tattooed pig skin [119].

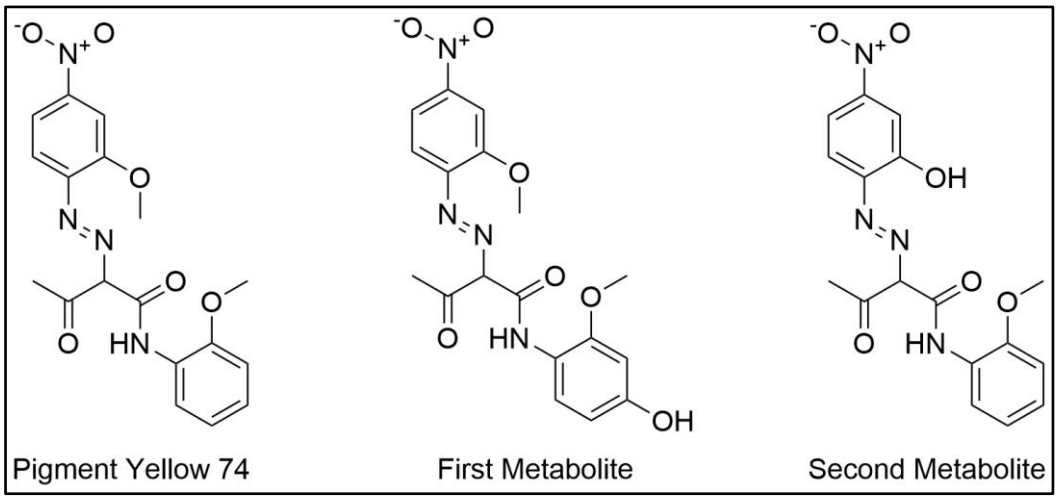



**Figure 1.9: Metabolism of PY74 (Reproduced with permission from [117]).**

Prior research has not clearly determined which fragments result from using laser to irradiate PY74, PY14 and PY65. In practice, laser treatment has not successfully removed yellow pigments, meaning that clearance of yellow pigment remains a challenge. As can be seen from the limited number of studies reported above, further research is required to understand how fragments vary in shape and size when yellow pigments and $TiO_2$ are irradiated by QS laser.

## 1.9 Health concerns due to tattoo ink components

### 1.9.1 Tattoos *in vivo* and *in vitro*

Unlike pharmaceuticals or implants injected into the human body [120], tattoo inks often lack stringent safety regulations. The specific compositions of these inks vary based on the practices of individual manufacturers [8,9]. Additionally, the pigments used in tattoo inks are typically not developed exclusively for tattooing; instead, they were originally intended for industrial applications such as paints, plastics, coatings, or textiles, which generally adhere to lower purity standards [97, 121].

Several studies have been conducted in recent years to examine tattoo inks and their degradation products in *in-vitro* and *in-vivo* skin specimens. Chemicals within tattoo pigments and their breakdown products may present health concerns [58]. These will depend on the composition of the tattoo inks and the concentration of pigments that used during tattooing.

Tattoo ink particles exhibit a wide size distribution, ranging from as small as 5 nm to over 1 μm [37]; hence, inks comprise nanoparticles and microparticles. Because of their high surface-to-volume ratio, nanoparticles can pass through membranes, be carried in the blood, and enter tissue cells, leading to oxidative stress, inflammation, and death [122]. In the production process, carbon black particles initially range in size from 15 to 300 nm, but rapidly agglomerate into irreversible agglomerates of 1-100 μm [37].

The injection of foreign chemicals into the skin during tattooing may cause a toxic or immunological reaction. The timeframe of these hypersensitivity reactions can range from immediately after the tattoo application to several years later, and they can be caused by further tattooing [58, 123]. For example, a 40-year-old man had Candida endophthalmitis [124] and a case of zygomycosis was identified following tattooing [58, 125]. Another patient also experienced an allergic response to three tattoo inks containing pigment yellow 65 a few weeks after tattooing, which affected the entire tattooed area [126]. Aside from the conventional types of responses in tattoo intolerance or allergy, incidences of morphea-like lesions and significant pseudoepitheliomatous hyperplastic lesions have been documented [127].

While clinical case reports highlight a range of adverse reactions following tattooing, *in vitro* studies have also been conducted to further investigate the cytotoxic and sensitisation potential of various tattoo inks under controlled laboratory conditions. The effect of 16 distinct colours of tattoo inks on mitochondrial metabolic activity was investigated using 3D recreated human skin (RHS) [128]. This investigation found that red tattoo ink significantly reduced metabolic activity of RHS to $89.9\% \pm 0.8\%$, indicating significant cytotoxicity [128]. Another study selected five tattoo inks for evaluation using the RHS model to assess their propensity for skin irritation and sensitisation [129]. This work demonstrated that two inks, red and black, elicited a significant release of interleukin-18 (IL-18) into the culture supernatant of RHS compared with the other three tattoo inks, suggesting that these inks may possess sensitisation potential. In addition, tattoo ink was added to the culture media to simulate intradermal exposure. This research found that that tattoo inks cause inflammatory IL-18 to release from HaCaT cells[129]. These data show that inks may cause skin sensitisation, inflammation, and allergic reactions [130].

### 1.9.2 Laser tattoo removal *in vivo* and *in vitro*

Even when the inks' composition is understood, there are obvious risks associated with the production of potentially dangerous particles, from both the carrier and the pigment (i.e. the two components of a tattoo ink). One significant, yet often overlooked hazard in laser treatment of tattoo inks is the formation of potentially harmful degradation products [131].

Studies have shown that laser or sun exposure can degrade tattoo pigments in the skin, potentially raising health concerns about hazardous by-products [36, 40]. Breakdown products, including toxic and carcinogenic substances like benzene, benzonitrile, hydrogen cyanide, and aniline, can form as pigments fragment into smaller particles [36, 40]. The quantity of these degradation products is related to the amount of degraded pigment. Pigment movement within the skin typically stabilises by the time of removal, allowing for the isolation and evaluation of pigments years after tattooing [49].

Several studies have investigated the photodegradation of azo pigments under laser light and natural or simulated sunlight, with findings indicating that light exposure can selectively break azo bonds, resulting in the formation of potentially harmful aromatic amines and other toxic substances present in tattoo inks [16].

Butterfield (2022) summarises the photochemical degradation of numerous azo pigments used in tattoo ink [97, 132]. It is widely recognised that red pigments produce several allergic responses, even when not exposed to laser irradiation. Cardinal red and pigment red 18 are monoazo pigments with azo groups that cleave following light absorption, either by thermal energy or in an electrically excited state. Raising the temperature of azo dyes over 280 °C results in the formation of 3,3′-dichlorobenzidine, a confirmed carcinogen of human lymphocytes [48]. In such materials, laser irradiation should induce a higher localised temperature than 280 °C [48].

In addition to the risks posed by azo-based red pigments, other commonly used tattoo ink components such as carbon black and titanium dioxide ($TiO_2$) have also been implicated in toxicological effects, particularly through mechanisms involving reactive oxygen species (ROS) and photocatalytic activity [77]. The formation of ROS is thought to be a cause of carbon black tattoo toxicity [77]. ROS are extremely reactive chemicals created from $O_2$. These molecules have unpaired electrons, which allows them to undergo oxidative processes that can harm DNA, proteins, and cell membranes. Additionally, the photocatalytic activity of $TiO_2$ can lead to oxidative damage and DNA strand breaks, rendering it genotoxic when exposed to sunlight [103]. In tattoo inks, the white rutile form of $TiO_2$ is commonly recommended [43]. Given its prevalence in most modern tattoo inks and its photocatalytic properties, $TiO_2$ likely plays a role in various phototoxic reactions associated with tattoos [97].

Some immediate effects of laser tattoo removal reported *in vivo* are pain, blisters, crusting, and pinpoint bleeding. These effects are reported more frequently in darker skins and require a high fluence. An acute allergic response in the form of urticarial lesion has been recorded [58, 133]. The most common consequence is pigmentary changes, either hypopigmentation or hyperpigmentation, which appear 4-6 weeks following laser therapy [58]. Kirby *et al.* (2010) found hypopigmentation in 8% and

hyperpigmentation in 22% of individuals with darker skins following laser tattoo removal [133]. After laser tattoo removal of permanent eyeliner, leukotrichia with permanent whitening of the eyelashes was reported [133, 134].

Beyond these common and immediate skin reactions, laser tattoo removal has also been associated with a variety of delayed, pigment-specific, and systemic responses. Wong and Cheung undertook a case report about a 45-year-old female patient who had a major adverse response to both her treated and untreated tattoos after two picosecond laser therapies [135]. Local allergic responses, particularly in response to the red and yellow pigments, might manifest as pruritic papules, nodules, or scaly plaques. Systemic effects have rarely been documented as a result of laser therapy for tattoos. Photoallergic reactions may arise from red or yellow ink, manifesting immediately or after months or years following tattoo removal [58, 63].

In *in vitro* investigations, pig skin has been tattooed with the yellow quinaphthalone PY138 and PO13 followed by laser treatments of this skin, which led to the formation of hexachlorobenzene, phenyl isocyanate, benzene, 3,3′-dichlorobenzidine, and aniline [136]. The chronic carcinogenic laser degradation products were tested for cytotoxicity and genotoxicity using human keratinocyte (HaCaT) and fibroblast (BJ) cell lines, assessed via lactate dehydrogenase (LDH) release and LDH content in the remaining adherent cell layer. The results showed that 3,3′-dichlorobenzidine suppressed growth and dramatically elevated LDH release beginning at 10 µM after 24 hours [136]. It also induced DNA double strand breaks in cell lines [136]. Another research project reported that laser irradiation of copper phthalocyanine created hydrogen cyanide, particularly relevant given its high toxicity to human HaCaT cells and observed significant impact on ATP [137].

## 1.10 Chemical profiling of tattoo inks and their degradation products

A variety of chromatographic and spectroscopic methods, including GC-MS, HPLC, IR, UV-Vis, NMR, and Raman spectroscopy, have been used to examine the chemical composition of tattoo inks and their constituent compounds [42, 138-141].

### 1.10.1 Spectroscopic techniques

Spectroscopic techniques are essential tools for chemical structural identification, providing detailed molecular insights widely applied in the analysis of pigments [142-144]. The analytical profiling of pigments used in tattoo inks plays a crucial role in ensuring product quality and safety. Recent research has demonstrated that when combined with chemometric techniques UV-Vis and FT-IR spectroscopy can effectively estimate pigment content in tattoo inks [141]. This approach enabled the identification of components and impurities in pigments such as PR170, PR 254, and PB15. UV-Vis

spectroscopy proved valuable for monitoring pigment concentration and structural variations, while FT-IR provided insight into functional groups and characteristic fingerprint regions of the spectra [141]. Another study using UV-Vis, IR, and Raman spectroscopy to compare PG36, PG7, and mint green ink confirmed the presence of PG7, rather than PG36 in the tattoo ink [145].

Raman spectroscopy has also proven to be particularly useful for pigment identification due to its non-destructive nature and sensitivity to molecular structure. In a study by Poon *et al*., tattoo pigment analysis was performed using a pig skin tattoo model, allowing for the classification of six specific pigments [42]. The study also noted that rinsing tattoo inks on glass slides led to a strong fluorescent background when exposed to a 632.8 nm He-Ne laser, which interfered with spectral clarity [42].

NMR spectroscopy has been employed to investigate the presence of additives in tattoo inks. Moseman *et al.* conducted a study using NMR spectroscopy to analyse additives in commercial tattoo inks [146]. Glycerol, the most frequently listed additive, was reported in 36 out of 54 ink products. However, NMR analysis detected glycerol in only 29 of the 54 inks, with no observable signals in any of the One Tattoo World inks or in the Solid Ink Lining Black. Additionally, NMR revealed the presence of characteristic peaks for propylene glycol in 15 inks, despite none of the surveyed products listing it as an ingredient. This highlights the utility of NMR in verifying the presence of undeclared or inaccurately reported additives in tattoo inks [146]. These findings highlight the importance of multi-technique spectroscopic methods in accurately characterising both pigments and potential degradation products in tattoo inks [145].

### 1.10.2 Chromatographic techniques

Gas chromatography (GC) and high-performance liquid chromatography (HPLC) are widely recognised analytical techniques for the separation of complex mixtures. GC is particularly effective for the analysis of volatile and thermally stable compounds, whereas HPLC is more suitable for non-volatile, thermally labile, and polar substances[147, 148]. Both techniques are extensively applied in pigment analysis to identify and quantify chemical structures[139, 145] and degradation products in tattoo inks [146, 149-151]. For example, Bauer *et al.* (2025) reported that GC analysis of irradiated green tattoo inks (green concentrate and PG7) revealed the formation of compounds such as 1,4-dichloronaphthalene, trichlorobenzonitrile, and 4,5,6,7-tetrachloro-2,3-dihydro-1H-isoindole-1,4,2 [149]. In addition, GC-MS was used by Høgsberg *et al.* to analyse organic compounds extracted from various coloured tattoo inks [77]. The analysis identified the presence of polycyclic aromatic hydrocarbons such as benzo[b]fluoranthene, pyrene, benzo[a]pyrene, and naphthalene in black tattoo inks [77]. In a study by Cui *et al.* on the photodecomposition of PY74, seven different yellow tattoo inks were examined [116]. The inks were first mixed with water and extracted using methylene chloride.

The resulting samples were then analysed using HPLC with a diode array detector. By comparing the retention times and absorbance spectra of the eluted components to those of authentic standards, PY74 was identified in six out of the seven inks[116]. Finally, a study by Cecchetti *et al.* assessed the chemical degradation of tattoo pigments (using UV-Vis and GC) following laser treatment using five different lasers: Nd:YAG (nanosecond and picosecond, standard and array modes) and a Ruby nanosecond laser [152]. UV–Vis spectroscopy of the treated samples showed an overall decrease in absorption features, indicating pigment degradation. GC analysis of the irradiated samples revealed the formation of several degradation by-products, including butanoic acid, chlorobenzene, and 2,6-dichlorobenzonitrile. Additionally, pigment fragments and siloxanes were commonly produced across all treatments, with hydrocarbons being more abundant following Nd:YAG nanosecond laser exposure [152].

## 1.11 Methods for determining cytotoxicity tattoo inks

Cytotoxicity means a substance is toxic or damaging to cells [153]. Tests with a wide variety of cytotoxic outcomes are currently utilised to assess cellular responses to a harmful substance. The selection of an appropriate assay for cytotoxicity study of a toxicant is influenced by a number of factors, including *in vitro* cell culture models, the substance's physical and chemical characteristics, culture platforms, outcomes, cytotoxicity mechanism, and evaluation or detection techniques[154]. Several *in vitro* cytotoxicity tests have been used and classed based on their objectives. For example, trypan blue, methylene blue staining assay, alkaline phosphatase (ALP) assay, resazurin, and sulforodhamine B assay are used to determine cell numbers. Additionally, LDH assay, Alamar blue, fluorescein diacetate, and Calcein-AM are used for cell viability. For membrane permeability, 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), LDH, annexin, granzyme-based, and caspase-based assays are used [154].

### 1.11.1 Trypan blue assay

The trypan blue stain assay was established in 1975 to detect alive cells and is currently used as a confirmatory test for determining changes in viable cell numbers, induced by a medication or toxin[155, 156]. One of the simplest techniques for measuring live cells is the use of trypan blue stain, which is a large, negatively charged molecule. The rationale behind this test is that living cells have intact cell membranes that reject the trypan blue stain and their cytoplasm will be transparent, while dead cells do not reject the stain with trypan blue and they appear blue[157, 158].

### 1.11.2 MTT assay

Cellular viability is measured using the colorimetric MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) test[159]. It can be utilised for toxic substance hazard evaluation. MTT is a tetrazolium salt (water-soluble yellow dye) quickly absorbed by live cells and converted to purple formazan by mitochondrial succinate dehydrogenase in living cells. MTT reduction occurs exclusively in metabolically active cells (Fig. 1.10), with activity levels used to determine cell viability. Dead cells lose their capacity to convert tetrazolium salts into coloured formazan products. An organic solvent, commonly dimethyl sulfoxide, is used to dissolve insoluble formazan crystal, resulting in a purple-coloured product that is quantified with a spectrophotometer [158-161]. The quantity of formazan generated is directly related to the number of live cells in the sample [158-161].

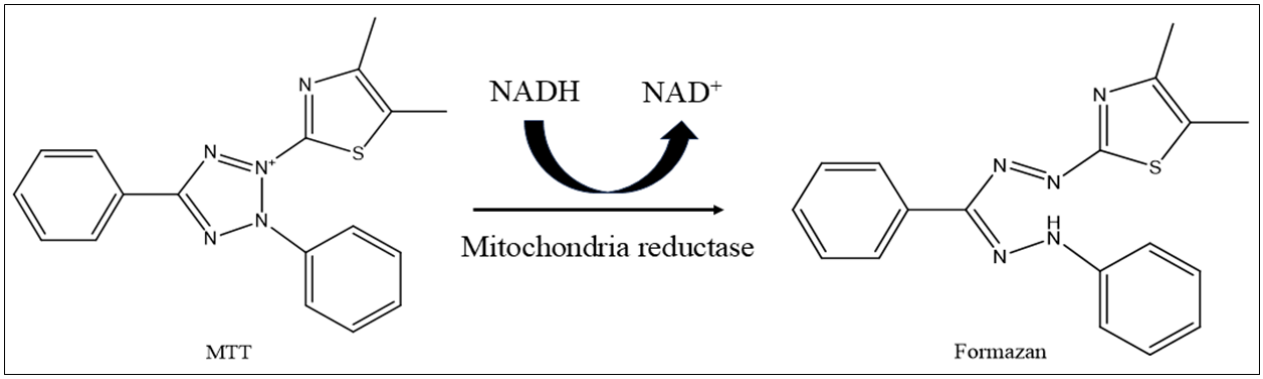



**Figure 1.10: Formation of the formazan by the reduction of MTT [159].**

### 1.11.3 LDH assay

Lactate dehydrogenase (LDH) is an oxidoreductase enzyme present in almost all living cells (including animals, plants, and prokaryotes) released into the cytoplasm during cell lysis [162]. This is a colorimetric cytotoxicity test that assesses membrane integrity [163]. The concentration of LDH is higher in damaged cells relative to normal cells. The activity of LDH is quantified by the conversion of lactate to pyruvate. LDH converts nicotinamide adenine dinucleotide (NAD) to reduced NAD (NADH) and releases $H^+$ ions, which catalyse the reduction process of the tetrazolium salt to produce the coloured formazan molecule, which has an absorbance of 490-520 nm. The oxidation of NADH to $NAD^+$ is determined spectrophotometrically, with absorbance at 340 nm. NADH absorbs more than $NAD^+$ at 340 nm. The amount of colour product generated is dependent on the activity of LDH in the samples [163, 164].

### 1.11.4 CV assay

Crystal violet (CV), also known as triphenylmethane dye, is widely used as a biological stain in human and veterinary medicine, as well as a textile dye in the textile processing industry [165]. The

cationic dye CV, also referred to as gentian violet (impure form), has one dimethylamino group on per phenyl ring [165].

During cell death, adherent cells detach from the culture plate. This property may be utilised to indirectly quantify cell death and to identify changes in proliferation following stimulation with death-inducing chemicals [166]. One straightforward method for detecting cell adhesion is to stain connected cells with crystal violet dye, which binds to proteins and DNA. Cells that die lose their adhesion and are therefore removed from the cell population, lowering the quantity of crystal violet staining in a culture [167, 168]. This protocol presents a simple and accurate screening approach for determining the effect of chemotherapeutics or other drugs on cell survival and growth inhibition [167].

**Cell line types**

Typically, animals are used to assess a substance's toxicity. However, this method is time-consuming, costly, morally contentious, and often of low reliability [169]. These realities compel researchers to endorse alternate techniques [170]. Fundamental cytotoxicity assays use cell cultures [169, 171]. Cell lines are valuable *in vitro* models for the evaluation of harmful impacts generated by chemicals and their use have significantly contributed to understanding of the mechanisms involved in toxicity [154].

The skin serves as our primary barrier against environmental hazards [172]. The determination of cell types for use in *in vitro* cutaneous testing is not straightforward. The skin consists of a variety of cell types, including keratinocytes, fibroblasts, melanocytes, Langerhans cells, mast cells, and Merkel cells. These cells are organised into two distinct tissues: the epidermis, primarily composed of squamous epithelial cells and keratinocytes, and the underlying dermis, largely formed of dermal fibroblasts [172]. Several culture models have been created, including skin explant or organ cultures, traditional (submerged), and air-exposed cell culture [173]. In cytotoxicity studies of tattoo inks *in vitro*, researchers typically select certain cell lines that accurately represent the behaviour of human tissues possibly impacted by tattooing [161]. Table 1.7 shows often utilised cell line types for these studies.

**Table 1.7: Cell Types and Models for Tattoo Ink Toxicity Assessment.**

| Cell/Model Type | Description | Relevance | Reference |
|---|---|---|---|
| **Keratinocytes (HaCaT cells)** | Immortalised human keratinocyte cell line commonly used in | Predominant epidermal skin cells relevant to tattoo ink exposure as | [35, 130, 161] |

| | | | |
|---|---|---|---|
| | dermatological research. | ink is introduced into the epidermis. | |
| **Fibroblasts (NIH-3T3 or primary human dermal fibroblasts)** | Cells that constitute the dermal layer of the skin, where tattoo ink particles are typically found. | Crucial for investigating prolonged ink retention, immunological responses, and tissue reactions. | [174] |
| **Macrophages (RAW 264.7)** | Immune cells involved in the body's reaction to tattoo ink particles. | Provide insight into inflammatory and cytotoxic responses to tattoo ink ingredients. | [175] |
| **Liver Cells (HepG2 cells)** | Cells used to model liver metabolism and potential systemic effects when ink components enter circulation. | Useful for assessing metabolism and systemic impact of tattoo ink components once they reach the liver. | [33, 176] |
| **Human Skin Equivalent Models (3D tissue culture models)** | 3D tissue models that simulate complex skin structures better than monolayer cultures. | Offer more physiologically relevant results compared to monolayer cell cultures, providing insights into interactions within a more realistic skin model. | [177, 178] |

## 1.12 Conclusion

Tattoos have evolved from cultural and ritualistic practices into a widespread form of self-expression and artistry. As the popularity of tattoos continues to grow, it is essential to examine their multifaceted implications, from their historical and cultural significance to their health and safety concerns. This literature review has highlighted key aspects of tattooing, including the history of tattoo inks, their

composition, and the processes involved in creating tattoos. The composition of tattoo inks, once simple and natural, now includes synthetic pigments, metallic additives, and other chemicals that vary widely in quality and safety due to inconsistent regulation.

Despite the cultural and personal significance of tattoos, many individuals experience tattoo regret, fuelling a growing demand for tattoo removal services. Among various methods, laser tattoo removal remains the most effective and commonly used. The theory of selective photothermolysis underpins this technique, allowing targeted destruction of tattoo pigments with minimal damage to surrounding tissues. However, the process is not without complications. During laser treatment, tattoo pigments are fragmented into smaller particles, some of which may enter the lymphatic system or other parts of the body. These fragments pose potential health risks, as some studies have suggested cytotoxic or even carcinogenic effects from both the original inks and their breakdown products.

The lack of standardised regulations governing tattoo inks further exacerbates these concerns. With varying compositions and limited oversight, individuals may unknowingly be exposed to harmful substances during tattooing and removal. This underscores the need for global efforts to establish stringent safety standards for tattoo inks and their use. Public education about the risks of tattoos and their removal, as well as improved training for practitioners, is also essential to minimise health risks.

Health concerns associated with tattoos extend beyond the removal process. The cytotoxicity of tattoo inks themselves, coupled with the potential dangers of laser-induced fragmentation, demands further investigation. Comprehensive toxicological studies are crucial to understanding the long-term effects of tattoo inks and their by-products. Additionally, regulatory frameworks must be designed to ensure safer tattooing practices and to address the emerging challenges of tattoo removal.

This review also highlighted the need for interdisciplinary collaboration among researchers, healthcare professionals, and policymakers to address these complex issues. By understanding the chemical, and biological aspects of tattoos and their removal, stakeholders can develop evidence-based guidelines to protect public health. Ultimately, addressing the safety of tattoos and their removal not only enhances individual well-being, but also supports the sustainable growth of the tattoo industry as a form of artistic and cultural expression.

## 1.13 Research Aim

This project aimed to study the composition and laser degradation products of yellow tattoo inks and pigments, focusing on the roles of key components and resulting toxicity

**Main objectives**

1- **Evaluate the components in commercially available tattoo inks (presented in Chapter 2)**

- Select tattoo inks and their reference pigments
- Analyse the tattoo inks and pigments using suitable analytical techniques
- Compare the ingredient labels on tattoo inks with declaration in the SDS
- Evaluate the potential health implications of undeclared ingredients

2- **Assess the effect of $TiO_2$ on the laser degradation of yellow tattoo inks using wavelength of 532 nm and identify the character of the fragmentations (presented in Chapter 3)**

- Develop a method for dried sample preparation of pigments and $TiO_2$
- Develop a method for laser irradiation
- Develop a GC-MS headspace method to identify volatile degradation products
- Conduct SEM, XRD, and DLS analyses to assess changes in particle shape and size
- Investigate the health implications the laser-induced degradation products

3- **Investigate the impact of melanin pigment on laser degradation of yellow tattoo inks (presented in Chapter 4)**

- Develop a method for dried sample preparation of pigments, inks, and melanin
- Develop a method for laser irradiation
- Conduct headspace GC-MS, SEM, and DLS analyses to identify volatile fragments and morphological changes in the particles

4- **Estimate toxicity of laser degradation products on HaCaT skin cells (presented in Chapter 5)**

- Develop a method for laser irradiation of pigment and ink suspensions
- Prepare serial dilution of pigments and tattoo inks
- Perform cytotoxicity tests on both unirradiated and irradiated pigments and inks
- Develop a GC-MS method for liquid injection analysis to identify the soluble laser fragments
- Evaluate the cytotoxic effects of the identified compounds using relevant *in vitro* assays

A nanosecond laser was used throughout these projects because it is the type commonly available in clinical tattoo removal settings and remains widely used in practice; it is also the laser system available at Flinders University

By conducting this comprehensive investigation, this project aims to contribute to the advancement of laser tattoo removal, making the procedure more effective and safer for patients with various tattoo

types and skin characteristics. Additionally, this research may lead to the development of standardised guidelines for practitioners in the field.

## 1.14 References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# 2 CHAPTER 2: DECODING TATTOO INKS: A MULTI-TECHNIQUE ANALYSIS REVEALS DISCREPANCIES IN INGREDIENT COMPOSITION AND ELEMENTAL CONTENT WHEN COMPARED TO LABEL CLAIMS

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**This chapter was published online September 2025, in: Journal of Environmental Health, Volume 88, pages 1-18, (2025) DOI https://doi.org/10.70387/001c.143999.**

The only alterations are that some figures from supplementary information have been moved to this chapter. Supplementary information for this chapter has been shown in the supplementary section (**Appendix 1**).

**Author contribution statement:**

Batool Aljubran carried out the experimental work, as well as the data analysis for Infrared Spectroscopy (FT-IR), Nuclear Magnetic Resonance (NMR), X-ray Diffraction (XRD), Raman Spectroscopy, Energy-Dispersive X-ray Spectroscopy (EDX-SEM), and Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES). Batool Aljubran also wrote the manuscript with support from Claire Lenehan, Ula Alexander, and Kirstin Ross, who all contributed to supervising the project. All of the authors contributed to experimental design and ideation and approved the final version of the manuscript.

**Four tattoo inks were selected for examination throughout this thesis. Given that previous research has highlighted frequent mislabelling and discrepancies in tattoo ink ingredients, Chapter 2 begins by evaluating the chemical composition of the selected inks. Establishing an accurate understanding of their ingredients is essential, as it provides the foundation for interpreting the results of following experimental studies presented in the following chapters.**

## Abstract

Permanent body art has grown in popularity in recent years, with millions of individuals having black or coloured tattoos. With this comes risk; injecting colouring compounds into the skin has been reported to cause allergies, various skin inflammations, and systemic disorders. Currently, despite the growing number of tattooed individuals, there are few regulations, laws, and safety criteria for tattoo and permanent cosmetic formulations. The goal of this study was to identify the pigments in a set of commercially available yellow tattoo inks. A set of previously unstudied yellow tattoo inks (Lemon Yellow (LY), Golden Yellow (GY), Golden Rod (GR) and Bright Orange (BO)) and reference pigments (Pigment Yellow 14 (PY14), Pigment Yellow 65 (PY65), Pigment Blue (PB15), and Pigment Orange 13 (PO13)), were examined "as received" using a range of techniques, including FT-IR, NMR, XRD, Raman, EDX-SEM and ICP-OES. This work shows that the combined use of these techniques can provide significant insight into the ink composition without the requirement for difficult and time-consuming sample preparation. Results of this study indicate that the ink compositions differed from what was described on the labels. Furthermore, we demonstrated that the tattoo inks tested included additional elements that were not listed as ingredients such as Al, Na, and Si. This raises concerns about the regulation, health impacts, and degradation products of tattoo inks.

## 2.1 Introduction

Body decoration by tattooing has increased in popularity over recent years. It has been reported that 40% of young adults in the United States, and 25% of Australian adults have at least one tattoo [1-4].Tattoo ink suspensions can contain various chemical compounds, including vehicles such as water, glycerine, and other alcoholic derivatives, as well as additives like surfactants, polycyclic aromatic hydrocarbons, nanoparticles, and polymers, along with pigments of varying purity [5-8]. This may include compounds that were designed for use in paints, non-tattoo inks, or plastics.

Throughout history, the composition of tattoo pigments has evolved from natural extracts and metal salts to a mix of inorganic oxides, salts, inorganic pigments and azo dyes [9]. Historically, inorganic compounds like mercury(II) oxide (red), cobalt(II) aluminate (blue), chromium(III) oxide (green), manganese violet, titanium dioxide (white) and iron oxides (brown) tones were used [10-12] and often blended with other organic and inorganic components to enhance colour vibrancy [13]. Nowadays, tattoo ink manufacturers use artificial organic and organometallic pigments alongside inorganic compounds to make tattoo inks [14] with metals still present as chromophores, shading additives, or impurities [6, 12]. However, inorganic pigments based on metal salts are currently used in micropigmentation inks in permanent cosmetics such as permanent eyebrow makeup, eyeliner, and lip colour [12]. This is due to their higher durability against light and heat, better setting capacity, and larger size, which makes their removal more difficult.

Modern inks used for tattooing vary greatly in composition and may contain hazardous ingredients not originally intended for this purpose [15] and may not have a proven track record of safety in tattooing [16, 17]. There are increasing concerns about their effects on human health, including potential carcinogenicity [18, 19]. It has been reported that tattoo inks can also trigger acute allergic reactions immediately or lead to hypersensitivity after long-term exposure [7, 20, 21]. For example, Klügl *et al.* reported that over 70% of 3411 tattooed persons experienced issues with their skin immediately or within a few weeks following their tattoo [22]. Moreover, allergic responses to tattoos, especially with red inks, have been reported to persist for months or years [23].

Given the growing popularity of tattooing and the possibility of dangerous ingredients in tattoo products, regulations are required to reduce the hazards caused by inappropriate tattoo inks [7]. In Australia, tattoo inks are not considered therapeutic materials and are not regulated by the Therapeutic Goods Administration. Instead, the National Industrial Chemicals Notification and Assessment Scheme (NICNAS) regulate the chemicals found in tattoo inks. NICNAS typically does not legislate the import of chemicals used in tattoo ink if those chemicals are listed in the Australian Inventory of Chemical Substances (AICS). however, is known about tattoo ink contamination or adulteration [24],

however there is some evidence of incorrect labelling. In 2016, a NICNAS report into tattoo ink composition advised that certain tattoo inks in Australia were non-compliant with regulations, marketed with incorrect ingredients, or not suitable for use [25]. In addition, according to a survey conducted among tattoo artists in downtown Brisbane and Melbourne Central, tattoo artists were unaware of the ingredients in their inks, or the possible dangers associated with them [26]. In Europe, tattoo inks are regulated by the European Union's General Product Safety Directive. According to this directive, a producer is required to place only safe items on the market, and a comprehensive list of contents must be included on the product label, which is accomplished through classification, labelling, and packaging [15, 27].

In 2011 Hauri reported 34 prohibited pigments in 30 tattoo ink samples throughout their analysis [28]. For example, the pigment green 36 (PG36, C.I. 74265) was declared in three green inks, but the samples were demonstrated to include the prohibited pigment green 7 (PG7, C.I. 74260). Furthermore, a yellow and a blue pigment were stated for one ink, but the ink was again found to contain PG7, not the stated yellow and blue pigments. The ingredient list on a violet ink was evidently incorrect: the ink included the pigment white (titanium dioxide) and the pigment blue 15 (PB15, C.I. 74160), which when combined produce a light blue. The violet colour, on the other hand, was shown to be created with the prohibited pigment violet 23 (PV23, C.I. 51319) [28]. Concerningly, mislabelling and undeclared ingredients continue to persist in commercial tattoo ink formulations. A study conducted by Poon *et al*.(2008) for 190 tattoo inks indicated that 37% included forbidden substances and 53% contained one or more of i) excessive levels of nitrosamine, ii) unreported material, or iii) claimed material that was not found in the ink [10, 29]. In 2021, a decade after the report of Hauri, Wang and co-workers reported that for 50% of the tattoo inks they tested, labelling inaccurately stated at least one pigment component [7]. Furthermore, a recent 2024 study showed significant discrepancies between tattoo ink composition and ingredient labels, especially for the non-pigment (e.g. carrier/vehicle) components [29]. Considering tattoo safety and the possibility of allergic sensitisation, it is likely that some pigments had been removed from the ink formulations due to the EU regulations banning its use in tattoo inks [7, 30].

Identifying pigments in commercial tattoo inks presents a significant challenge due to their complex composition, the diverse combinations of pigments used to achieve subtle colours, poor solubility of pigments in traditional solvents, and other additives present that enhance pigment dispersion [31]. Previous studies have primarily relied on mass spectrometry for pigment analysis. Both Hauri [28] and Wang *et al*. [7] used MALDI-TOF MS to identify pigments in tattoo inks, however this approach has limitations. For example, Wang *et al.* showed that certain pigments were unable to be detected using MALDI-TOF MS due to poor ionisation or low mass [7, 28]. Furthermore, mass spectrometry-based

techniques often require extensive sample preparation, making the analysis time-consuming and challenging for label compliance assessments. Given the complex and varied chemical nature of tattoo ink ingredients, this study aimed to develop a novel approach for pigment identification by utilizing a combination of spectroscopic techniques. Unlike previous studies that primarily analyse tattoo inks after extensive pre-treatment, digestion, or extraction, our approach focuses on examining inks in their dried, untreated state or with minimal sample preparation. Compared to mass spectrometry, spectroscopic techniques such as FT-IR, NMR, XRD, Raman, and EDX enable rapid, minimally-destructive, analysis of pigment molecular structures, crystallinity, and elemental composition with minimal sample preparation requirements [29, 31, 32]. Whilst most prior research uses these techniques in isolation, this work leverages the combined information from spectroscopic and elemental analysis techniques of FT-IR, NMR, XRD, Raman, EDX, and ICP-OES, to rapidly assess the pigment composition of a set of yellow inks that have not been previously examined.

## 2.2 Method

### 2.2.1 Material and instruments

Intenze brand Lemon Yellow (LY) Golden Yellow (GY), Golden Rod (GR) and Bright Orange (BO) tattoo inks were purchased from Tattoo Direct, Victoria, Australia. Pigment yellow 14 (PY14, C.I. 21095) (97% purity), pigment yellow 65 (PY65, C.I. 11740) (98% purity), pigment white ($TiO_2$, C.I. 77891) (99.5% purity), pigment blue 15 (PB15, C.I. 74160) (technical grade), pigment orange 13 (PO13, C.I. 21110) (technical grade), and barium sulfate ($BaSO_4$, C.I. 77120) (99%) were purchased from AK Scientific, Union City, California. Methylene chloride (99.9% purity) was purchased from RCI Labscan, Australia.

Except for the tattoo inks, all chemicals utilised in this study were not purified further. Tattoo inks were pipetted onto microscope slides and dried in open air at ambient temperature for 48 hours prior to characterization. To extract the pigments from liquid tattoo inks, approximately 25 mg tattoo ink was added to a 50 mL conical glass tube containing 2 mL water. The contents were mixed vigorously and extracted three times with 15 mL methylene chloride. The methylene chloride extracts were combined, dried, and analysed as dry tattoo ink extracts.

### 2.2.2 Instrumental analysis

Fourier Transform Infra-Red Spectroscopy (FT-IR) was used to generate an infrared spectrum of the pigments and dried inks using a Perkin Elmer Spectrum 100 FT-IR spectrophotometer equipped with an attenuated total reflectance (ATR) diamond crystal, in the range 400–4000 $cm^{-1}$ with a resolution

of 4 $cm^{-1}$. The energy of electromagnetic radiation is expressed in wavenumbers, and the intensity is expressed as a percentage of transmittance.

Solid state $^{13}$Carbon nuclear magnetic resonance ($^{13}$CNMR) experiments were undertaken using a Bruker Avance III 400 MHz spectrometer operating at 100 MHz for $^{13}$C Chemical shifts are relative to adamantane. Approximately 100 mg pigment sample/dried ink extract was placed in a Bruker 4 mm rotor and spun at 5 kHz. $^{1}$H-$^{13}$C cross-polarisation magic angle spinning spectra were recorded using an acquisition time of 18.4 ms, a recycle delay of 2 s, a contact time of 4 ms with a 50% ramp and decoupling during acquisition (Spinal 64). Sideband suppression was achieved using the standard TOSSa sequence.

X-ray diffraction (XRD) was recorded for pigment samples and dried tattoo inks. Data were collected using a Bruker Advanced D8 diffractometer with Co Kα ($\lambda$ = 1.7889 Å, 2$\theta$ = 10-90°, time per step = 0.5 second). All samples were ground to a fine powder with a mortar and pestle before being loaded onto an XRD sample stage.

Raman spectra were collected using a Horiba Scientific Xplore Plus Raman spectrometer at both 786 nm and 532 nm. The analysis was carried out at from 200 $cm^{-1}$ to 3000 $cm^{-1}$ wavenumbers, with the laser intensity reduced using 10% and 25% filters. For each sample, 12 scans of 20-second pulses were recorded. In all cases dried pigment/ink powder was placed onto a glass slide and mounting the slide on the Raman spectrometer's sample holder for analysis.

Scanning electron microscopy (SEM) was undertaken with a FEI F50 inspect system equipped with an Octane Pro energy dispersive X-ray (EDX) detection system. Samples were prepared by directly spreading pigment powder onto sticky carbon tabs. The working distance was 10 mm, and the acceleration voltage was 10 kV. PY14, GR, and GY inks were coated with platinum with a thickness of about 2 nm to increase their electrical conductivity.

Inductively coupled plasma-optical emission spectrometry (ICP-OES) was conducting using a Perkin Elmer Optima 8000 ICP-OES. Prior to analysis, around 100 mg of the dried ink samples were digested in 5 mL of $HNO_3$ using a microwave digestor. After digestion, the samples were diluted to 50 mL in MQ water, giving a $HNO_3$ concentration of 10%, and an aliquot was diluted twice in ultrapure water (18 MΩ), giving a 5% $HNO_3$ concentration. Finally, the samples were filtered using a 0.45-um nylon filter prior to analysis. Calibration standards (5 ppb to 1 ppm) were prepared in 5% aqueous $HNO_3$.

## 2.3 Results and discussion

### Ink characterisation

### Reported ingredients

As prior research has shown that tattoo inks ingredients are sometimes misidentified on the label [7, 29, 31, 32], this research examined the reported ingredients (according to manufacturer's label and the provided Safety Data Sheet (SDS)) of the four inks used in this study. Table 2.1 shows the SDS ingredient lists are for inks as of 2018, 2022 and 2023, and the declared ingredients on the label. As can be seen, there are some differences in reported ingredients on the SDS over this time. Inks used in this research were purchased in 2019, so it could be expected that the reported ingredients on the label would match those of the SDS from 2018. Despite this, there are some discrepancies between the label and the 2018 SDS. For example, the label for LY ink indicates that it contains PY65 (Fig. 2.1a), but it is not mentioned in the 2018 SDS. Similarly, PY14 (Fig. 2.1b) is not listed as an ingredient on the label of LY ink, but it is listed in the 2018 SDS. The labels for GR and GY inks reported that they contain PO13 (Fig. 2.1c), however PO13 was not listed on the 2018 SDS. Finally, PB15 (Fig. 2.1d) is reported on the label and in the SDS for LY in 2018, 2022, but it is no longer listed on the 2023 SDS. Similarly, there were discrepancies on other ingredients such as barium sulfate that is not listed on the label of GY an BO inks.

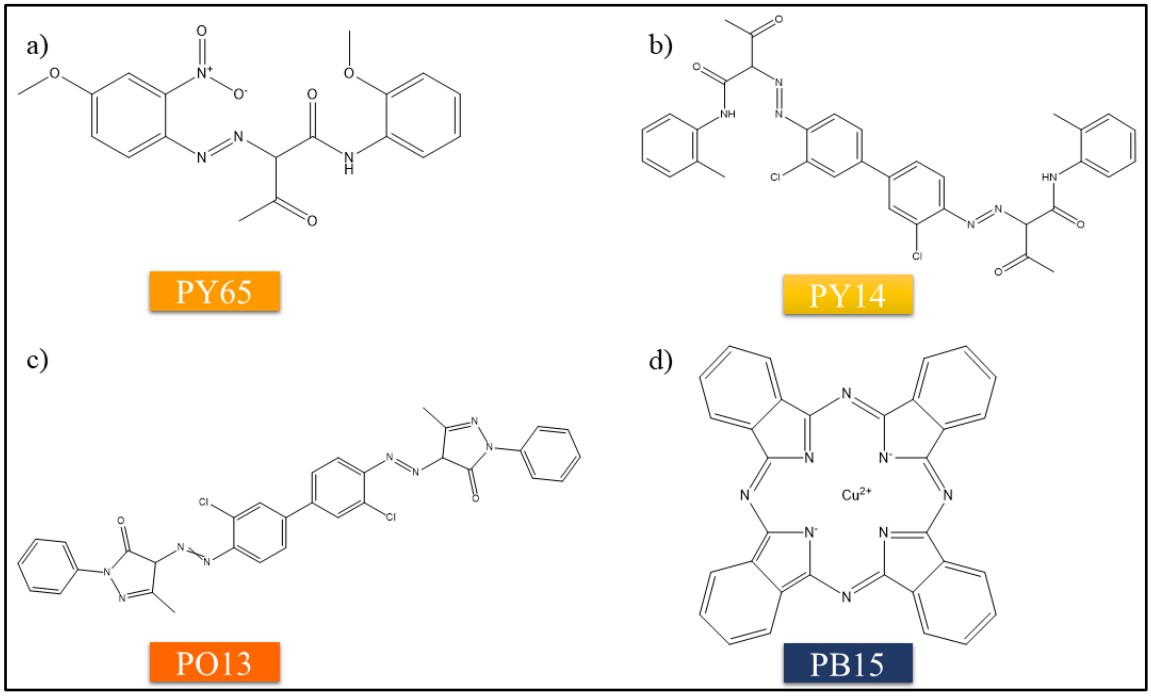


**Figure 2.1: A schematic representation of the pigments that are reported to be present in the LY, GY, GR, and BO tattoo inks.**

**Table 2.1: The reported ingredients of tattoo inks used in this study according to the manufacturers label and the SDS [33].**

| Tattoo ink | INTENZE Lemon Yellow | | | | | INTENZE Golden Yellow | | | | | INTENZE Golden Rod | | | INTENZE Bright Orange | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Declaration ingredients* | SDS | | | Confirmed in the study | Declaration ingredients* | SDS | | | Confirmed in the study | Declaration ingredients* | SDS** | Confirmed in the study | Declaration ingredients* | SDS | | | Confirmed in the study |
| | | 2018 | 2022 | 2023 | | | 2018 | 2022 | 2023 | | | | | | 2018 | 2022 | 2023 | |
| **$TiO_2$** | x | x | x | x | ✓ | x | x | x | x | ✓ | - | - | - | x | x | x | x | ✓ |
| **$BaSO_4$** | x | x | x | x | ✓ | - | x | x | x | ✓ | - | - | - | - | x | x | x | ✓ |
| **PB15** | x | x | x | - | ✓ | - | - | - | - | - | - | - | - | - | - | - | - | - |
| **PY65** | x | - | - | - | × | - | - | - | - | - | - | - | - | - | - | - | - | - |
| **PY14** | - | x | x | x | ✓ | x | x | x | x | ✓ | x | x | ✓ | x | x | x | x | ✓ |
| **PO13** | - | - | - | - | - | x | - | - | x | × | x | x | × | x | x | x | x | ✓ |
| **Aqua** | x | x | x | x | - | x | - | x | x | - | x | x | - | x | x | x | x | - |
| **Glycerine** | x | x | x | x | - | x | - | x | x | - | x | x | - | x | x | x | x | - |
| **Hamamelis Virginiana extract** | x | x | x | x | - | x | - | x | x | - | x | x | - | x | x | x | x | - |
| **Isopropyl alcohol** | - | x | x | | - | - | - | x | x | - | - | x | - | - | x | x | x | - |

* (according to label on bottle inks that were purchased in 2019)

** Golden Rod ink has the same composition according to the three SDS in 2018, 2022, and 2023.

**Pigment characterisation**

Reference pigments, dried inks (D-ink) and ink-extracts (E-ink) were compared using FT-IR, NMR, XRD, Raman, EDX and ICP-OES in order to identify the likely pigments in the inks. Results of these analyses are presented below.

### 2.3.1 FT-IR spectra

Fig. 2.2 depicts the FT-IR spectra of pigments obtained from ink extracts (E-LY, E-GY, E-GR, and E-BO) and reference pigments (PY14, PY65, PB15 and PO13) between 1900 $cm^{-1}$ to 600 $cm^{-1}$. The full range of the spectra (4000 $cm^{-1}$ to 550 $cm^{-1}$) and assignment of functional groups to the FT-IR spectra can be found in (Fig. SI 2.1 and Table SI 2.1). PO13 had characteristic peaks at 1653, 1493, 1371, 1331, 1235, 1144, 1044, 998, 907, and 682 $cm^{-1}$. These peaks were not observed in the ink-extracts indicating that none of the ink-extracts contained PO13, or if they did it present at levels below the instrumental limit of detection. Similarly, PY65 had distinctive absorption peaks at 1546, 1302, 1187, 1135, 1030, and 763 $cm^{-1}$ that were not observed in the ink-extracts. This indicates that PY65 was not present in any of the inks, or if so, it was present at levels below the instrumental limits of detection. The IR spectral of PB15 shows several peaks at 1612, 1464, 1421, 1331, 1287, 1166, 1119, 1087, 901, 877, 778, and 725 $cm^{-1}$. The absence of these peaks in LY ink indicated that the FT-IR spectra could not confirm the existence of this pigment in this ink. PY14 had peaks at 1670, 1515, 1360, 1245, 1171, 950, 860, 782, 750, and 619 $cm^{-1}$ were correlated to the presence of C-Cl, and N-C=O bonds [32]. All four ink-extracts yielded FT-IR spectra that appeared very similar to the spectra from PY14, indicating that they likely contained PY14. FT-IR spectra from the dried inks (D-LY, D-GY, D-GR, D-BO) were consistent with those of the extracted inks (Fig. SI 2.2).

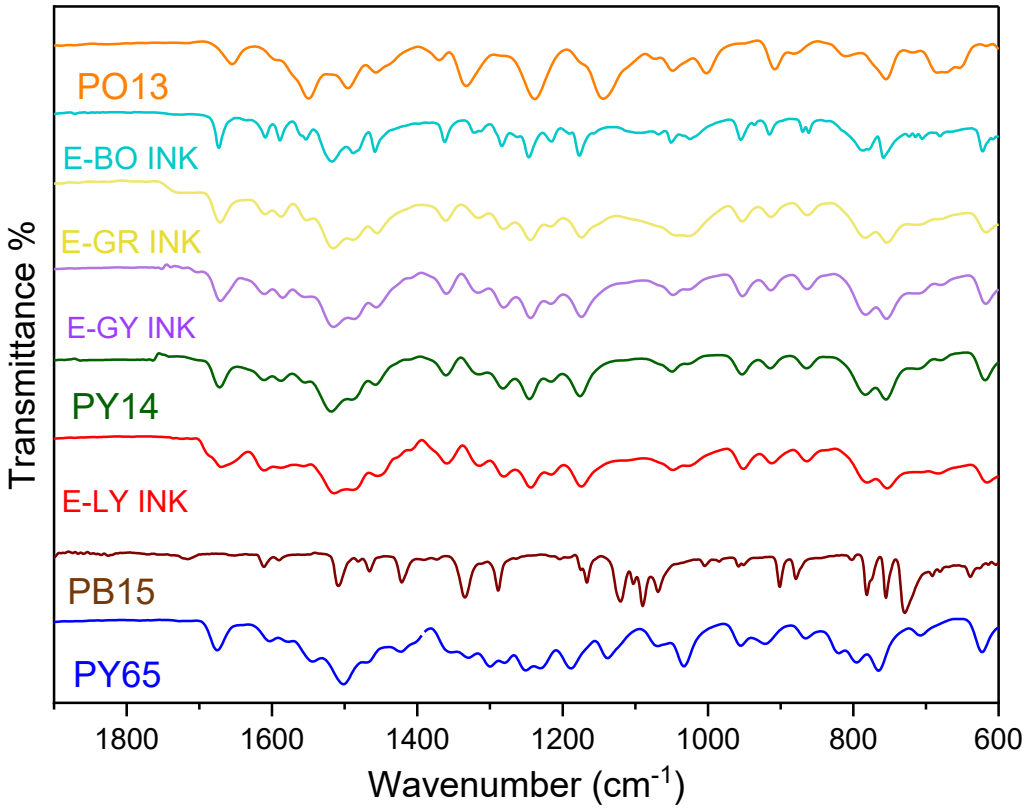


**Figure 2.2: FT-IR spectrum of pigments and inks obtained using the Perkin Elmer Spectrum 100 FT-IR. A magnification of the IR spectra is in the 1900-600 $cm^{-1}$ range with a resolution of 4 $cm^{-1}$ to demonstrate the characteristics more accurately. FT-IR spectra comparison of inks and pigments reveals the presence of PY14 instead of PY65 in the LY ink. GY, GR, and BO inks did not have PO13.**

### 2.3.2 NMR

In case the pigments were at concentrations that were below the limits of detection for the FT-IR study, NMR analysis was also undertaken. Fig. 2.3 shows the $^{13}C$ NMR spectra of PY14, PY65, and PO13, along with dried inks from LY, GR, BO, and GY. NMR spectra of PB15 were not collected due to paramagnetic characteristics of copper that generate local magnetic fields which might interfere with NMR measurements. There were distinct peaks in the PY65 at approximately 38 ppm, and 58 ppm, which associated to the presence of methyl groups ($CH_3$-O, $CH_3$-C=O). Peaks at 145 ppm and 155 ppm were correlated to the presence of C-$NO_2$ and Ar-O groups, which were only found in PY65 [34]. These peaks were not found in any of the inks, suggesting that none of them contained PY65. Moreover, PY14 had peaks between 130 and140 ppm and at 20 ppm and 30 ppm that can be attributed to the presence of the C-Cl and methyl group (C-$CH_3$ and O=C-$CH_3$). These peaks were identified in all inks which confirmed the presence of PY14 in them. In addition, NMR characterisation did not confirm the presence of PO13 in LY, GY and GR inks. This is because PO13 had distinguishing peaks between 150 and 160 ppm that were not detected in these inks. There was also a slight chemical shift of the peak at 12 ppm, which was assigned to the $CH_3$ group in PO13. The presence of PO13 in BO ink was consistent with the manufacturer's ingredients with small quantities

as identified in the NMR spectra. These NMR spectra for the three pigments were consistent with those reported in the literature [35].

The presence of PY14 was confirmed by FT-IR and NMR analysis in all three inks. The results from FT-IR and NMR analysis clearly show that LY ink contains PY 14 instead of PY 65. This contradicts the manufacturers label claim that LY ink contains PY65 (not PY14) but is consistent with the declaration in the SDS which indicates PY14. Furthermore, the presence of PO13 in both GY and GR inks could not be confirmed, as the FT-IR and NMR spectra did not contain the characteristic peaks expected from PO13. The absence of PO13 in the GY ink is consistent with the SDS for the ink (at time of purchase) and indicates that once again the ink bottle may have been mislabelled. The absence of PO13 from GR ink is in contrast with the label and SDS for this ink. Alternatively, the amount of PO13 used in these inks may have been too low for detection by this instrument (i.e., less than 2 mg of PO13 in 100 mg of dried tattoo ink). This was confirmed by the control experiment to identify the limits of detection of the NMR instrument (Fig. SI 2.3).

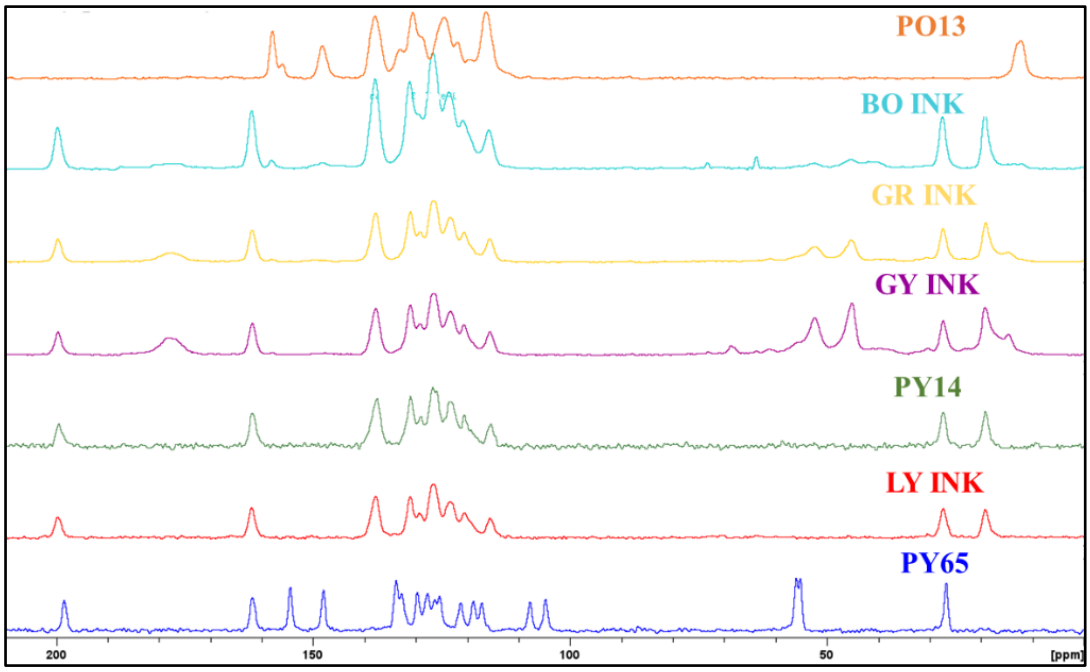


**Figure 2.3: Solid state $^{13}C$ NMR spectrum of reference pigments (PY14, PY65 and PO13) and inks (LY, GY, GR and BO). Spectral analysis confirms the presence of PY14 in all inks, PO13 is in BO ink, while PO13 is absent in GY and GR inks. NMR spectra also confirmed that LY ink did not contain PY65.**

### 2.3.3 XRD

XRD analysis was carried out to confirm the FT-IR and NMR results along with verifying the existence of inorganic ingredients. The XRD diffraction pattern of all inks revealed amorphous and crystalline phases (Fig. 2.4). The vehicles and organic pigments are associated with the amorphous phases of the inks. As shown in this figure, the diffraction pattern of D-LY ink is similar to PY14 but

not to PY65 or PB15, further confirming the presence of PY14. Furthermore, when comparing the XRD pattern of PO13 with that of GY and GR inks, the peaks differed, which was predicted and corresponded with the IR and NMR findings.

$TiO_2$ has been recognised as one of the most common crystalline oxide peaks in both D-BO and D-GY inks. The intensity of $TiO_2$ peaks, however, was greater in D-BO ink than in D-GY ink. This might be because the quantity of $TiO_2$ in the D-GY ink was smaller than that in the D-BO ink, and this is according to the clarification on the manufacturer's website in the SDS of these inks [33]. However, the absence of the $TiO_2$ peaks in D-LY ink suggests that the amount of $TiO_2$ was smaller than the level detectable by XRD. These results of analysing XRD data on pigments and inks were compared with those in the reference [6]. Possible presence of $BaSO_4$ in BO ink was evidenced by low intensity peaks in the XRD however this could not be confirmed using XRD alone as some of these overlapped with $TiO_2$ peaks.

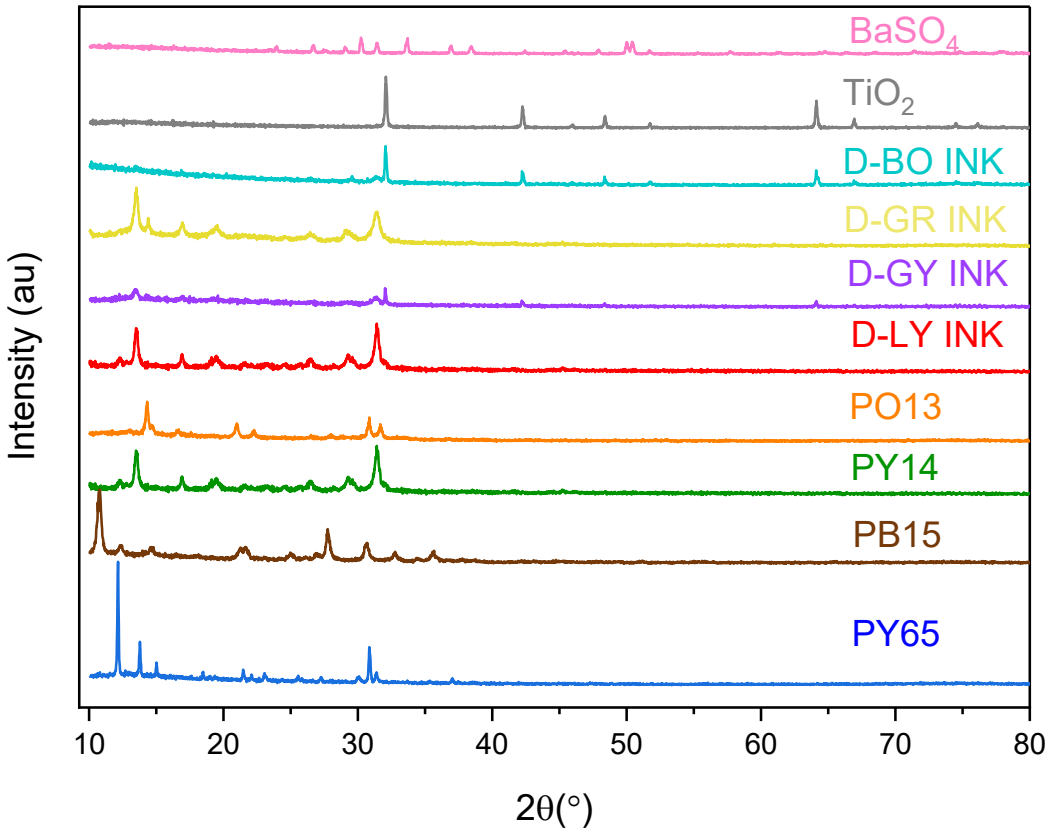


**Figure 2.4: XRD data analysis of organic and inorganic pigments, where theses spectra compared with LY, GY, GR, and BO inks. $TiO_2$ was identified in BO and GY inks.**

### 2.3.4 Raman spectra

Furthermore, Raman analysis was conducted to confirm the previous instrument examination data and the presence of PY65, PO13, PB15, and the other inorganic ingredients ($BaSO_4$ and $TiO_2$) (Fig. 2.5). The full range of the spectra (200 $cm^{-1}$ to 2500 $cm^{-1}$) can be found in Fig. SI 2.4. A study of the

Raman spectra of LY ink revealed that PY 14 is a prominent component and PY 65 is not present. The challenge in resolving these separate pigments for this specific shade of ink can be due, primarily, to the quantity of PY14 vs. PB15 (Fig. 2.6), which causes signals from the PY14 to overwhelm those from the PB15. Many aspects of the comparatively narrow PY14 spectrum corresponded with and overlapped with the few peaks (e.g., 1598.5 $cm^{-1}$) derived from PB15 due to similar structural vibrations. For example, the normally noticeable 1332 $cm^{-1}$ C-C bond stretching in PB15 [36] is hidden by a rather weak feature in PY14 at 1310 $cm^{-1}$. Additionally, the allocated peaks from PB15 at 1414 and 1136 $cm^{-1}$ were shifted to 1456 and 1148 $cm^{-1}$, respectively. While the BP15 peak of 584 $cm^{-1}$ was masked in D-LY ink. Therefore, Raman spectra could not confirm the presence of PB15. Furthermore, the Raman spectra of GY and GR inks were consistent with previous characterisation (e.g., FT-IR, NMR, and XRD) studies, confirming the absence of PO13 in these inks. This is because PO13 had a peak at 1588 $cm^{-1}$ and this peak was shifted to1600 $cm^{-1}$ in the GY and GR inks. The Raman spectra of $TiO_2$ and $BaSO_4$ revealed two distinct peaks at 441$cm^{-1}$ and 603$cm^{-1}$ (Fig. SI 2.6), which were not observed on the Raman spectra of dried inks at the same region. This could be because the quantity of elements was inadequate to be detected by Raman analysis. Therefore, EDX were carried out.

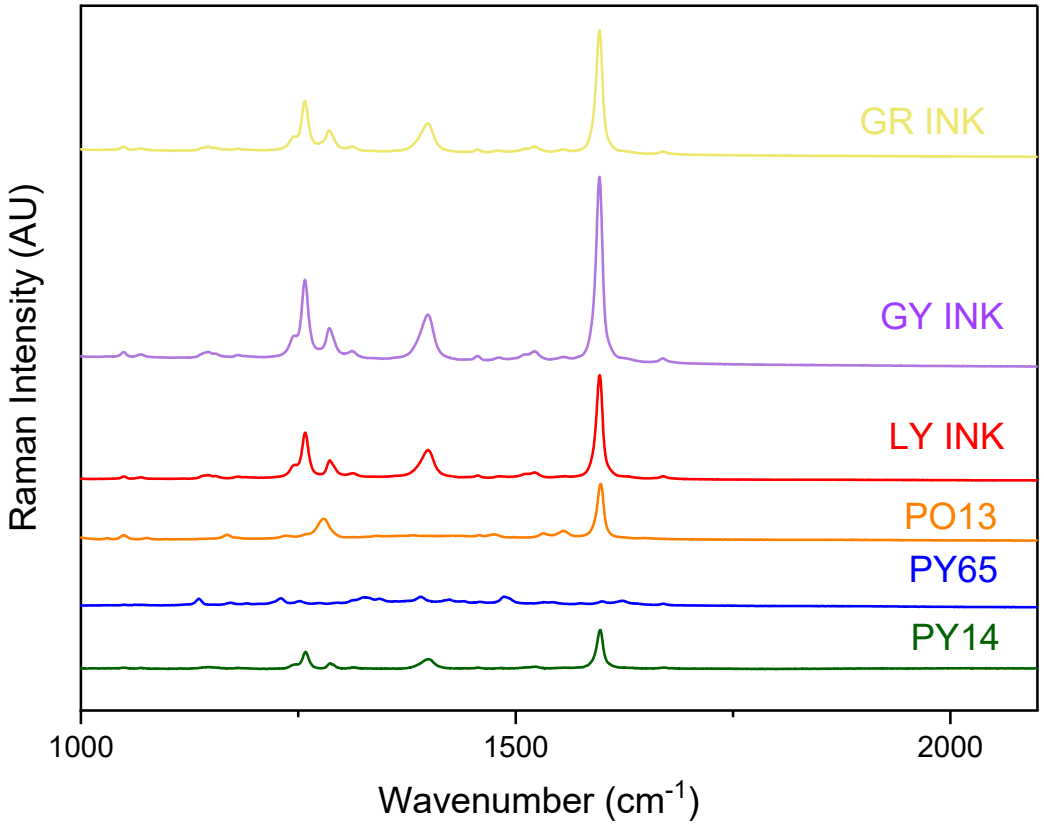


**Figure 2.5: Baseline-corrected Raman spectra of LY, GY, GR inks and PY14, PY65, PO13.**

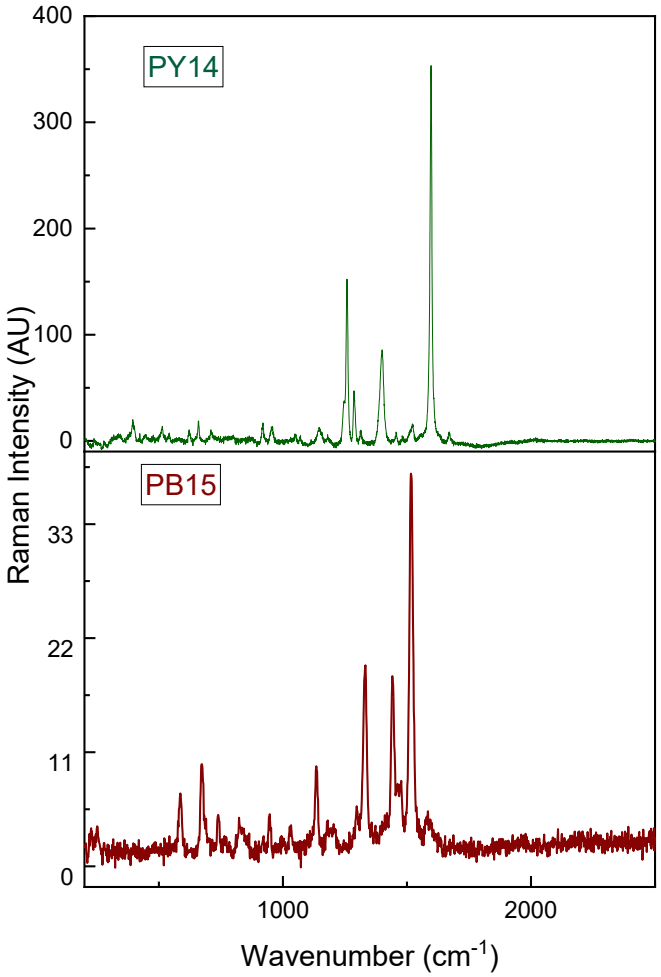


**Figure 2.6: Baseline-corrected Raman spectra of PB15 and PY14. That Raman intensity for both pigments differed which clarified the overlap between the peaks in the LY Raman analysis.**

### 2.3.5 EDX

The pigments and dried inks were analysed using EDX to identify the element composition (Table 2.2 and Fig. SI 2.6-10). As expected, the pigment reference samples contained C, N, O, Cu, Ba, S and Cl. The presence of Cl in the D-LY ink spectrum is a further proof that PY14 is present in this ink. It was expected that copper (from PB15) and barium (from $BaSO_4$) would have been observed in LY and GY inks. However, the EDX did not show elemental copper and barium in the case of either ink. Interestingly, the EDX data indicated that the stated inks contained impurities not listed on the product label. For example, the D-GY ink had excessive levels of Na, and the D-BO ink had a small amount of Al and Si. EDX analysis was consistent with XRD data and revealed that D-BO ink contains a higher concentration of Ti than D-GY inks, which have the same ingredients according to the label on the ink's bottle. Carbon, oxygen, and nitrogen contents were all high in all the inks analysed. The carbon tape holder, which was not entirely covered by the inks or pigments, is responsible for the high intensity peaks of carbon in the spectra. The existence of components revealed in the SEM and EDX examinations was confirmed by XRD analysis, but in different quantities.

**Table 2.2: Element composition analysis of pigments and inks using EDX provides supportive evidence of the presence of certain pigments in inks and establishes a match between LY ink and PY14 pigment. The (%) represents the average atomic percentages from surface analysis of three spots on a sample.**

| Element (%) | C | N | O | Cl | Cu | Ti | Ba | S | Si | Na | Al |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **PY14** | 66 | 19 | 14 | 2 | - | - | - | - | - | - | - |
| **PY65** | 62 | 20 | 14 | - | - | - | - | - | - | - | - |
| **PB15** | 76 | 18 | 6 | - | 2 | - | - | - | - | - | - |
| **PO13** | 70 | 21 | 5 | 2 | - | - | - | - | - | - | - |
| **$BaSO_4$** | - | - | 48 | - | - | - | 30 | 19 | - | - | - |
| **$TiO_2$** | 4 | 3 | 70 | - | - | 23 | - | - | - | - | - |
| **D-LY** | 76 | 11 | 7 | 3 | - | 3 | - | - | - | 3 | - |
| **E-LY** | 70 | 16 | 8 | 2 | - | - | - | - | - | - | - |
| **D-GY** | 65 | 9 | 18 | 2 | - | 4 | - | - | - | 12 | 0.3 |
| **E-GY** | 65 | 21 | 10 | 2 | - | - | - | - | - | - | - |
| **D-GR** | 67 | 20 | 10 | 2 | - | - | - | - | 1 | - | - |
| **E-GR** | 69 | 16 | 11 | 1.5 | - | - | - | - | - | - | - |
| **D-BO** | 60 | 5 | 20 | 2 | - | 10 | - | - | 0.7 | - | 0.8 |
| **E-BO** | 63 | 5 | 18 | 3 | - | - | - | - | - | - | - |

## ICP-OES

XRD and EDX exhibited limited sensitivity when detecting small amounts of Ti, Cu, and Ba. Consequently, ICP-OES proved valuable in verifying the existence of these elements (Table 2.3). ICP-OES analysis was conducted to assess tattoo inks, with ink samples subjected to acid digestion (utilizing $HNO_3$ and $H_2O_2$) in an Ethos UP microwave. It should be noted that titanium cannot really be digested in $HNO_3$ (it requires HF), so those results are not accurate for quantitative analysis.

However, it is clearly shown that GY, LY, BO inks contain Ti, and this was consistent with the EDX and XRD data. Only LY ink contained concentrations of Cu that could be properly measured by the ICP-OES. The presence of Ba in LY and BO inks was confirmed. However, a closer look at the spectra shows that GY contain Ba, although below the limit of quantification. The detection of Ba could be just a match to the SDS of GY and BO and not to the label.

**Table 2.3: ICP-OES data of GY, GR, LY, and BO tattoo inks confirm the presence of PB15, $TiO_2$, and $BaSO_4$ in inks.**

| Sample ID | Ba 455.403 (mg/g) | Cu 327.393 (mg/g) | Ti 334.940 (mg/g) |
|---|---|---|---|
| **Golden Yellow** | Trace | - | 0.0565 |
| **Golden Rod** | - | - | - |
| **Lemon Yellow** | 0.0062 | 0.0035 | 0.0525 |
| **Bright Orange** | 0.0058 | - | High quantity |

## 2.4 Health implications related to tattoo ink

The inconsistency between the ingredients reported on the SDS and the experimental data highlights a significant issue, namely that these manufacturer-provided sheets cannot be relied upon for a comprehensive and accurate characterization of tattoo ink components. They also fall short in accurately reporting of the quantities of each ingredient present. From both consumer and medical perspectives, ingredient mislabelling is a critical concern due to its potential health implications.

This study found PY14, PY65, PB15, PO13 in tattoo inks that are prohibited under European Union Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) regulations in 2015 [7, 28, 37, 38]. These pigments have been banned because they contain substances such as polycyclic aromatic hydrocarbons, metals, and primary aromatic amines (PAAs), all of which pose toxicological risks to human health [29]. Pigments such as PY14 and PO13 have been detected in skin biopsies with reported adverse effects, albeit less frequently. Chronic allergic reactions were the most common type of adverse response observed in these skin samples [38, 39]. PY14 contains an azo functional group, raising particular concerns due to its potential to release PAAs. Recent research by Lachenmeier *et al.* (2023) found that red and yellow tattoo pigments emitted significant levels of PAAs [40]. Furthermore, there has been a documented case where a patient experienced an allergic reaction to tattoo inks containing PY65 (Intenze brand), affecting all tattooed areas [41].

Research on the metabolic breakdown of tattoo inks beneath the skin remains limited, resulting in a substantial knowledge gap in this area [38]. Pigments identified in this study are known to degrade under sunlight or during laser irradiation (such as that used in tattoo removal), raising additional health concerns due to the potential formation of toxic by-products. For example, *o*-toluidine, a known human carcinogen, has been identified as a decomposition product of various organic pigments [38, 42]. According to the literature, compounds derived from commonly used pigments, such as PO13, are classified as sensitizers by both manufacturers and the European Chemical Agency. Notable examples include the carcinogens aniline and 3,3'-dichlorobenzidine, which are degradation products of various pigments [38, 43, 44]. The disazopyrazolone pigment PO13 has also been shown to affect cytokine release in reconstructed human skin models [45]. Another pigment, PB15, is listed in Annex 1 of Germany's cosmetics code, which restricts its use in tattoo inks [46]. PB15 pyrolysis can produce hazardous substances such as hydrogen cyanide, benzene, and 1,2-benzenedicarbonitrile [46]. Concerns also extend to $TiO_2$, a potential human carcinogen, possibly due to the formation of reactive oxygen species that may lead to lung cancer [47]. Barium, present in tattoo inks as $BaSO_4$ to brighten dark colours [48] and act as a stabilizer, may not pose a major issue itself, however, soluble impurities can induce severe effects, including respiratory paralysis, cardiac arrest, or death [7, 49].

## 2.5 Conclusion

In summary, the pigment composition of a set of previously unstudied yellow tattoo inks were investigated in this study. Characterization using IR, NMR, XRD, Raman, EDX, and ICP-OES, showed that the pigments in the commercially available inks tested differed from what was described on the label. The ink characterization indicates the existence of PY14 in the LY ink, which differs from the PY65 mentioned on the bottle label. According to both the label and the SDS, PO13 was predicted to be present in the GY and GR inks, but none was detected. Additionally, Na, Si, and Al were found in the stated inks, but none of these elements have been reported either on the label or in the SDS. The significant discrepancies between the actual and labelled ingredients of tattoo inks raise serious health concerns, underscoring the need for stricter regulations and more accurate labelling to ensure consumer safety. The integration of different techniques for analysis provides a broader understanding of ink composition, while removing the necessity for intricate and time-consuming sample preparation.

## 2.6 Acknowledgement

B.A. extends deepest gratitude to the following organizations for their invaluable support and contributions to this research: King Faisal University, Ministry of Education, Saudi Arabia, for their generous support and encouragement throughout the duration of this study. We acknowledge the

facilities and technical assistance of Microscopy Australia, established under the National Collaborative Research Infrastructure Strategy, through the South Australian Regional Facility, Flinders Microscopy and Microanalysis (FMMA), Flinders University. We also acknowledge the facilities and the scientific and technical assistance of the South Australian node of the Australian National Fabrication Facility (ANFF) under the National Collaborative Research Infrastructure Strategy, located at Flinders Microscopy and Microanalysis, Flinders University. Finally, the authors acknowledge Assoc Prof. Martin Johnston (Flinders University) for his invaluable assistance with the NMR analysis.

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# 3 CHAPTER 3: CHALLENGES IN LASER TATTOO REMOVAL: THE IMPACT OF TITANIUM DIOXIDE ON PHOTODEGRADATION OF YELLOW INKS

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**This chapter was published online 06 March 2025, in: Archives of Toxicology, Volume 99, pages 1-15, (2025) DOI https://doi.org/10.1007/s00204-025-03989-2**

Link: https://link.springer.com/article/10.1007/s00204-025-03989-2

The only alterations are that some figures from supplementary information have been moved to this chapter. Supplementary information for this publication has been shown in the supplementary section (**Appendix 2**).

**Author contribution statement:**

Batool Aljubran carried out the experimental work and the data analysis for gas chromatography-mass spectrometry (GC-MS), scanning electron microscopy (SEM), energy-dispersive X-ray spectroscopy (EDX), dynamic light scattering (DLS), and X-ray diffraction (XRD). Batool Aljubran also wrote the manuscript with support from Claire Lenehan, Ula Alexander, and Kirstin Ross, who all contributed to supervising the project. All authors contributed to experimental design and ideation and approved the final version of the manuscript.

**In previous chapter, the compositional analysis of four tattoo inks (LY, GY, GR, and BO) revealed that three of them (LY, GY, and BO) contained $TiO_2$, while GR did not. Building on these findings, the present Chapter (3) investigates the role of $TiO_2$ in the photothermal response of these inks under laser irradiation. Additionally, the organic pigment (PY14) was identified in all four inks, which provided further rationale for its selection as the focus of this chapter's study.**

# Abstract

As tattoos have grown increasingly popular, there has been an increase in their removal. This is commonly achieved using laser treatments. However, certain tattoo inks are resistant to removal using laser methods because of their composition. This includes the removal of yellow pigments and tattoo inks containing titanium dioxide ($TiO_2$). This research examined a series of yellow pigments (PY14, PY74, PY65) and tattoo inks, pre-and-post irradiation, with a QS Nd:YAG laser irradiation at 532 nm. The pigments and products were analysed using a range of techniques, including EDX-SEM, DLS, XRD and GC-MS. Results of this study indicate that the presence of $TiO_2$ alters the laser degradation process of the pigments studied, with observable changes to particle morphologies, particle size and evolved volatile products. In addition, some of the degradation products were identified to be potentially harmful to the human body.

## 3.1 Introduction

Tattooing is an increasingly common practice that involves injecting tattoo inks into the skin to create a permanent mark or a visual design [1]. Notably, despite the growing popularity of tattoos, there is also a growing demand for their removal as people experience tattoo regret [2, 3]. With the development of improved technology and treatment approaches, tattoo removal processes continue to improve [4, 5]. Laser tattoo removal is presently the most commonly used approach, often using quality switched neodymium-doped yttrium aluminium garnet (QS Nd:YAG), which seems to be successful due to selective photothermolysis of the chromophores [3, 6-8]. The presence of residual tattoo colour is one of the most important indicators of the effectiveness of laser tattoo removal and helps to assess how much pigment remains in the skin, which guides further treatment decisions [9].

Tattoo ink is made up of insoluble pigments suspended in a solvent-based binder along with additives and preservatives that are used to stabilise it and avoid microbiological degradation [10]. Even when the inks' ingredients are understood, there are dangers associated with laser removal. This includes the creation of potentially dangerous particles, both from the pigment itself, and the medium (i.e., the two components of a tattoo ink) [7, 8, 11-13].

Moreover, it has been recorded that the ingredients and colours of tattoo inks contribute to the effectiveness of laser removal procedures, potentially influencing the number of treatment sessions [12, 14]. For example, dark coloured tattoo inks can be efficiently removed with a limited number of treatments using laser such as picosecond alexandrite laser [5]. Light colours such as yellow and white pigments, on the other hand, have been found to be difficult to completely eliminate, since the existing accessible laser wavelengths are not well absorbed by the pigments [15, 16]. In addition, *in vitro* investigations showed that the wavelength of the yellow ink's absorption peak (440 nm, 470 nm, and 485 nm) do not match the current wavelengths that are available in QS laser systems [5, 9, 16]. One study has indicated that using a QS Nd:YAG laser with a nanosecond pulse duration can be effective to remove yellow tattoo inks pigments. However, the complete removal of these yellow inks remains challenging and uneven [9, 17]. Yellow ink had traditionally been a recalcitrant colour to remove using the QS laser instrument, until the development of the picosecond lasers, which effectively remove this ink colour [2, 9].

Certain tattoos might be resistant to treatment not only due to poor absorption of the laser's radiation by the tattoo pigment, but also due to oxidative-reductive alterations in some metals when excited by laser light. One example is the phenomenon where tattoos containing iron oxides tend to darken when

treated with high-powered laser therapy [18-21]. Titanium dioxide ($TiO_2$) is commonly used to enhance the brightness of various coloured tattoo inks such as blue, yellow, green, and purple [22, 23]. The presence of $TiO_2$ has been widely reported to make tattoo removal more difficult, and in most clinics, laser removal of tattoo ink based on $TiO_2$ is not achievable [15, 19, 24]. Tattoos containing $TiO_2$ have been stated to act strangely, with the $TiO_2$ changing colour after the treatment from white to a "filthy" green, dark grey, blue, or pale purple, or not responding to laser light at all [25]. Prior research has indicated that $TiO_2$ can undergo a transformation from white to black under laser treatment [26]. Others have reported that $TiO_2$ is an exceptionally strong substance that is difficult to break down without several laser removal treatments [27]. This is problematic as, in terms of laser removal of $TiO_2$ containing tattoos, it has been noted that "repeated visits usually cause scarring of the skin" [25].

It has been proposed that the reduction of white and other titanium-containing inks causes the blackened colour of irradiated tattoos (Ross *et al*. 2001), and showed a similar effect for a titanium-enriched sunscreen exposed to radiation [23]. This was attributed to the reduction of $Ti^{4+}$ to $Ti^{3+}$, as demonstrated by Torimoto and co-workers [23, 28]. Because of the intense nature of the nanosecond pulses typically employed in laser therapy, even slight absorption of other wavelengths can lead to a darkening response, primarily driven by the reduction of $TiO_2$ within the ink [28]. The impact of $TiO_2$ quantity on the extent of darkening remains uncertain. As many yellow tattoo inks commonly include $TiO_2$ to enhance their brightness, this is also likely contributing to the phenomenon where yellow tattoo inks turned black after laser exposure [29]. This is consistent with a report by Kim and colleagues (2006), who showed that blue tattoo ink containing $TiO_2$ exhibited resistance to Nd:YAG laser therapy [15].

In addition to colour changes, there is limited knowledge on the effects of laser treatment on ink particle size and morphology. Murphy (2018) suggested that ink may be re-aggregated and ejected at high speeds, but no analytical data were offered to back up these claims [30]. Reaggregation of the ink particles would make it potentially increasingly difficult to remove tattoos. It is vital to understand how laser treatments affect ink particles since size and morphological changes in treated inks are likely to occur throughout the removal process.

This research investigated the influence of $TiO_2$ on the laser degradation of pigment yellow, a typical component of tattoo inks, to determine the effect on resulting colour, particle size and morphology. The study purposefully used a simple model, combining the pigment with only $TiO_2$ and exposing it to laser irradiation. This deliberate exclusion of other components mimicked the controlled conditions relevant to laser tattoo removal, specifically aiming to reduce the complexity introduced by other skin components during the process. The study examined yellow pigments, tattoo inks, and the

degradation products produced when these samples are treated with high laser intensities. A comprehensive array of instrumental techniques, including GC-MS, SEM & EDX, XRD and DLS, were employed for the characterization of both unirradiated and irradiated samples. This multidimensional approach facilitated a thorough understanding of the chemical and morphological changes occurring in the studied systems, shedding light on the potential applications and implications of $TiO_2$ in laser-assisted tattoo removal.

## 3.2 Method

### 3.2.1 Material and instruments

Lemon Yellow (LY), Golden Yellow (GY), Golden Rod (GR) and Bright Orange (BO) Intenze® brand inks were purchased from Tattoo Direct, Victoria, Australia. Pigment yellow 14 (PY14) (C.I. 21095) (97%), pigment yellow 65 (PY65) (C.I. 11740) (98%), pigment yellow 74 (PY74) (C.I. 11741) (tech), and $TiO_2$ (C.I. 77891) were purchased from AK Scientific, Union City, California. Methylene chloride (99.9%) was purchased from RCI Labscan, Australia. Methanol (LC MS grade) was purchased from Honeywell, Australia. All chemicals used in this investigation, except the tattoo inks themselves, were used without any further purification. The reported ingredients of tattoo inks used in this study according to the manufacturers label and the safety data sheets is shown in Table 3.1.

**Table 3.1: The ingredients of tattoo inks that used in the projects based on the SDS and the label on the bottles.**

| **Tattoo ink** | **INTENZE Lemon Yellow** | | **INTENZE Golden Yellow** | | **INTENZE Golden Rod** | | **INTENZE Bright Orange** | |
|---|---|---|---|---|---|---|---|---|
| | Declaration ingredients | SDS | Declaration ingredients | SDS | Declaration ingredients | SDS | Declaration ingredients | SDS |
| **$TiO_2$** | x | x | x | x | - | - | x | x |
| **$BaSO_4$** | x | x | - | x | - | - | - | x |
| **PB15** | x | x | - | - | - | - | - | - |
| **PY65** | x | - | - | - | - | - | - | - |
| **PY14** | - | x | x | x | x | x | x | x |
| **PO13** | - | - | x | - | x | x | x | x |
| **Aqua** | x | x | x | - | x | x | x | x |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Glycerine** | x | x | x | - | x | x | x | x |
| **Hamamelis Virginiana extract** | x | x | x | - | x | x | x | x |
| **Isopropyl alcohol** | - | x | - | - | - | x | - | x |

### 3.2.2 Sample preparation and laser experiment setup

The general method used in this research is presented in Fig. 3.1 and chemical structure of reference pigments is shown in Fig. 3.2a-c. Tattoo inks were pipetted onto microscope slides and dried in open air at ambient temperature for 48 h prior to irradiation and/or characterization. Dried inks were scraped from the slide and placed in a GC vial prior to laser treatment. The pigment-$TiO_2$ samples were made by mixing various pigments (PY14, PY74, PY65) in separate GC glass vials with different particle sizes (300 and 500 nm) of $TiO_2$ (rutile form) with a 50:50 w/w % ratio.

Dried tattoo inks and a mixture of PY14, PY74, PY65 with $TiO_2$ were irradiated using a QS Nd:YAG laser (Spectra Physics Quanta-Ray GCR12) at 532 nm. Each sample was exposed to 20 laser pulses (each with a duration of 6 nanoseconds) over a period of around 1-2 minutes. The laser pulses were 157 mJ/pulse over an area of 2 mm diameter (i.e., a fluence of 5 J/cm$^2$). As a negative control, PY14, PY65, PY74 and $TiO_2$ were irradiated individually. The photodegradation products were analysed using a headspace GC-MS to identify volatile fragments. In addition, SEM, DLS, and XRD were used to study the changes in crystal structure, particle shape and size following laser irradiation.

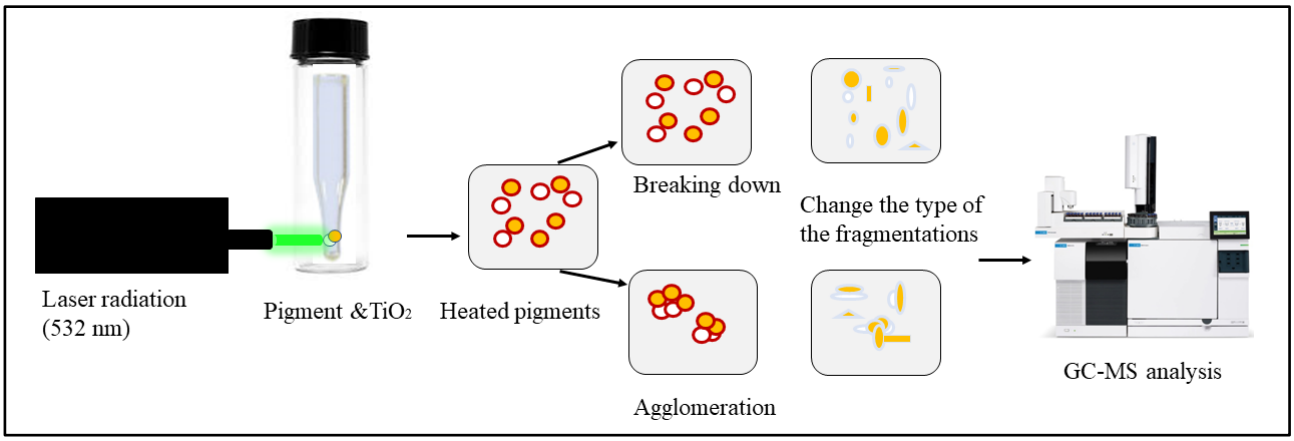



**Figure 3.1: General methodology used in this project (schematic experimental setup).**

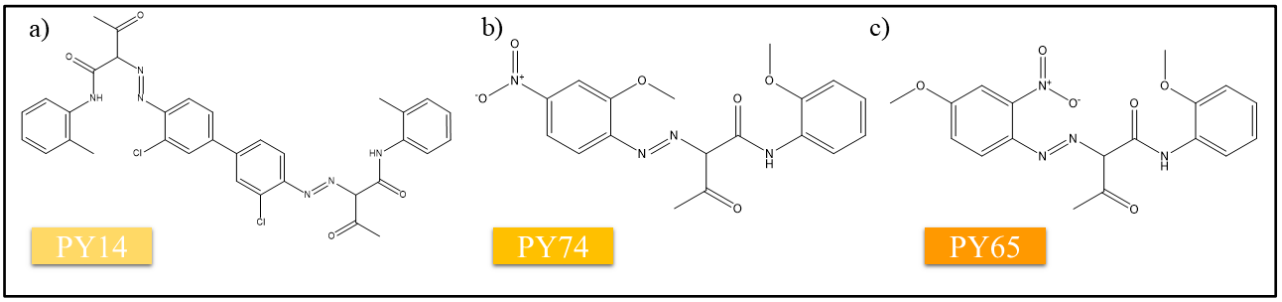



**Figure 3.2: A schematic representation of the chemical structures of a) PY14, b) PY74, and c) PY65.**

### 3.2.3 Instrumental analysis

Headspace gas chromatography mass spectroscopy (GC-MS) analysis was carried out using an Agilent Technologies 7890A GC system with a 5975C inert XL EI/CI MSD Triple Axis Detector and a 7693 sampler. The apparatus was fitted with an Agilent Technologies HP-5MS 5% Phenyl Methyl Siloxane column (29.4 m x 250 µm x 0.25 µm) with a $He_2$ mobile phase at a flow rate of 3.5 mL/min. The headspace injection volume was 5 µL. The GC was operated in isothermal mode with an oven temperature of 40 °C and an inlet temperature of 180 °C throughout the analysis. The MS ion source and quadrupole temperatures were set at 230 °C and 150 °C, respectively. All m/z values between 40 and 500 were taken in scan mode. The presence of volatile hazardous chemicals in the irradiated pigments and inks or the eluted compounds was identified by comparison of the mass spectra using the NIST database and comparison to literature [8, 31-35]. To help determine the identity of the organic volatile compounds, the retention index was measured using the Kovats approach from the headspace GC-MS analysis of standard alkanes (C5-C9) [36-39].

Scanning electron microscopy (SEM) was undertaken with a FEI F50 inspect system equipped with an Octane Pro energy dispersive x-ray (EDX) detection system. Pigment samples were prepared by directly spreading pigment powder onto sticky carbon tabs. The working distance was 10 mm, and the acceleration voltage was10 kV. PY14, PY74, GR, and GY inks were coated with platinum with a thickness of about 2 nm to increase their electrical conductivity.

Dynamic light scattering (DLS) measurements were achieved using a Malvern Nano Zeta Sizer apparatus equipped with a 5 mW HeNe laser and a Peltier temperature control system. Backscattering detection at an angle of 173° was used to determine the hydrodynamic size and size distribution of pigments, inks and $TiO_2$ samples. This configuration is less vulnerable to multiple scattering effects and dust than the 90° geometry. Measurements were taken at 20 °C and were repeated three times.

Before DLS measurements, all dispersions were prepared by dilution of the pigments with deionized water or methanol, followed by 30 minutes of sonication at 40 kHz, at a ratio of 0.1 mg pigment to 1 mL of solvent.

X-ray diffraction (XRD) was recorded for pigment samples and dried tattoo inks. Data were collected using a Bruker Advanced D8 diffractometer with Co Kα (λ = 1.7889 Å, 2θ = 10–90°, time per step = 0.5 second). All samples were ground to a fine powder with a mortar and pestle before being loaded onto an XRD sample stage.

## 3.3 Results and discussion

PY14,$TiO_2$, PY14-$TiO_2$ and dried ink samples were irradiated with a 532 nm QS Nd:YAG laser as outlined in section 3.2.2. Visibly, the pigment and ink samples were observed to change colour from yellow (pre-exposure) to a brownish green (post-irradiation) (Fig. 3.3). The $TiO_2$ samples were observed to turn slightly grey after irradiation. This contrasts with prior reports that have indicated that $TiO_2$ turned black post irradiation [26]. This is because the study used a 532 nm laser, however $TiO_2$ does not absorb radiation at this wavelength. Samples were characterised pre-and-post-irradiation using GC-MS, SEM, DLS, and XRD to examine changes in sample chemistry, composition, morphology, and particle size. Results from these analyses are discussed below.

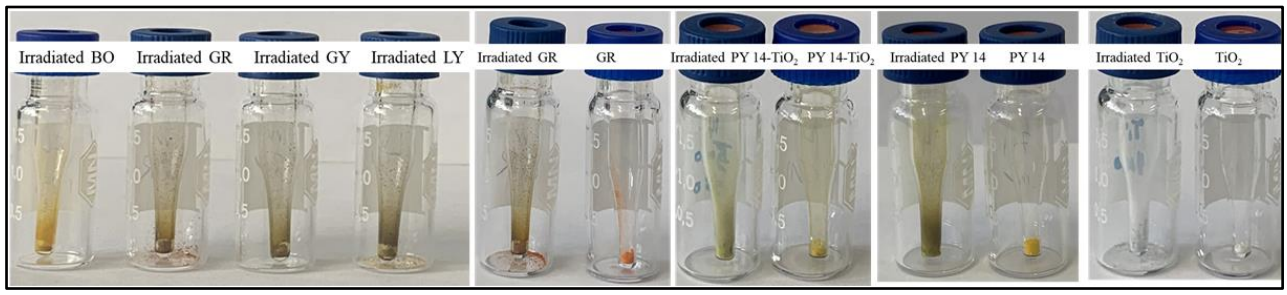



**Figure 3.3: Photographs of pigments (PY14, $TiO_2$) and inks (LY, GY, GR, BO) pre- and post-irradiation.**

### 3.3.1 GC-MS

Headspace GC-MS was carried out to identify volatile breakdown products resulting from the laser irradiation of reference pigments, pigment-$TiO_2$ mixtures and inks. Fig. 3.4a shows the GC-MS chromatograms of irradiated PY14, and two irradiated PY14-$TiO_2$ mixtures with different $TiO_2$ particle sizes. The GC-MS chromatograms of tattoo inks post-irradiation (LY, GY, GR, BO) are shown in Fig. 3.4b. The GC-MS chromatograms of the irradiated empty vial, unirradiated $TiO_2$, unirradiated PY14, and unirradiated inks did not exhibit any peaks (see supporting information Fig. SI 3.1), confirming that the volatile compounds resulted from the irradiation process. Table 3.2 provides details on fragmentation products identified in the samples post-laser treatment including

retention time and primary mass losses for each component. Notably, 1,3-butadiyne benzene and toluene were present across all samples. In addition, dried inks produced 2-propenoic acid-ethyl ester, methyl methacrylate, and styrene, while PY14 produced 3-butenyl methacrylate, and a peak at 1.7 minutes that was tentatively assigned as either *N,N'*-dimethyl-1,2-bis(aminooxy)ethane, or methyl 1-dideutrero-2-propenyl ether. Neither of these are readily attributed to fragmentation of the pigment or rearrangement of volatile fragments, and further work is required to confirm the identity of this peak. Both PY74 and PY65 generated 2-propenenitrile whereas benzyl alcohol was formed from PY65.

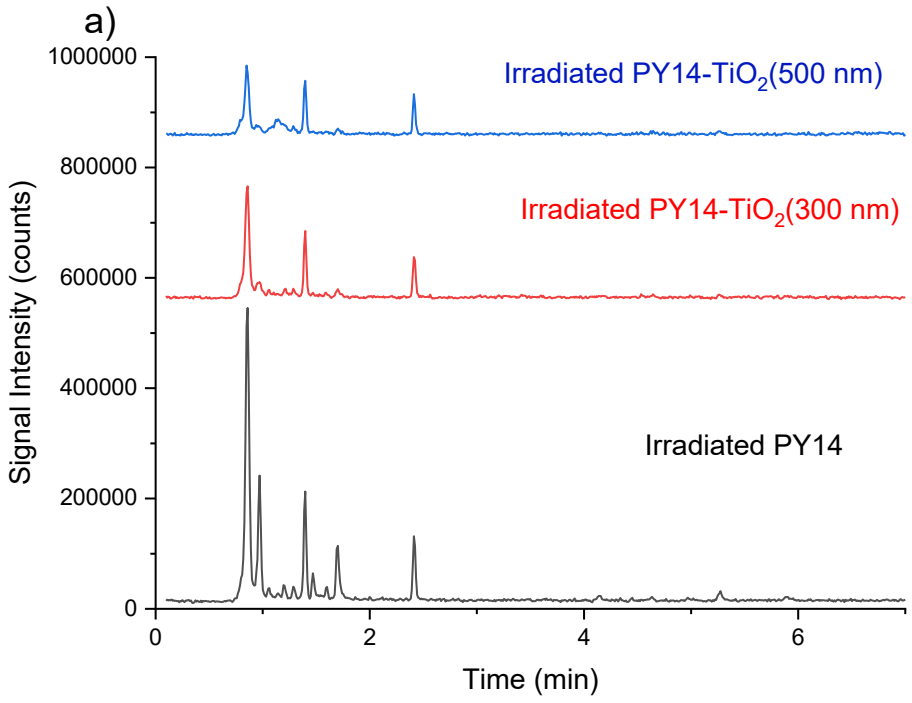
The presence of benzene, toluene, and styrene is consistent with previous studies on the laser irradiation of suspensions containing pigment blue 15, pigment green 7 and 36, pigment yellow 138, pigment orange 13, pigment violet 19, as well as pigments red 170 and 245 [31, 32, 34, 35, 40]. 2-propenenitrile was reported to be formed by thermal chain scissions of the binders, solvents, and additives of Edding felt-tip pen inks [41]. The formation of the remaining compounds (1,3-butadiene, 2-propenoic acid-ethyl ester, methyl methacrylate, 3-butenyl methacrylate, benzyl alcohol) from the dried inks and pigments has, to our knowledge, not previously been reported. These products could potentially be attributed to the impurities present in the pigments during the manufacturing process or other ingredients in the tattoo inks which could later degrade under laser irradiation. Additionally, the intense energy from the laser might induce rearrangement or fragmentation of the pigment molecules themselves, leading to the formation of new compounds not originally present in the pigment. The variation in the products formed implies an influence of the ink matrix on fragmentation during laser irradiation, which is changed when the ink is partially removed from the vehicle layer and the pigment aggregated.

When the GC-MS chromatograms from irradiated dried tattoo inks were compared to that of the irradiated organic reference pigments, the inks exhibited higher intensity peaks. It is hypothesized that the particles in the inks have a greater specific surface area compared to agglomerated pure pigments, leading to altered response rates to the laser light.

The number of peaks observed in the GC-MS chromatogram from the irradiated PY14-$TiO_2$ mixtures was decreased when compared to irradiated PY14. Fragments observed in irradiated PY14 at 0.92, 1.458, and 1.702 min were absent from the GC-MS chromatograms of the PY14-$TiO_2$ mixtures. Additionally, a reduction in peak intensity was observed in the chromatograms of irradiated PY14-$TiO_2$ mixtures when compared to the irradiated PY14. This appears to be dependent on the $TiO_2$ particle size, with mixtures of PY14 and 500 nm $TiO_2$ having lower intensities than the mixture containing 300 nm $TiO_2$. In addition to these changes, an additional peak with low intensity at 1.144

min was detected as a photodegradation product from irradiated PY14-$TiO_2$ but not in the irradiated PY14. Based on the MS data, this could be attributed to either benzamide or benzonitrile. Both are potential degradation products based on the structure. Benzonitrile was reported as a laser decomposition product from PO13 [34]. Similar changes in the GC-MS chromatograms were observed for PY75 and PY65 when irradiated in the presence of $TiO_2$ (Fig. SI 3.2). These results are consistent with previous research that stated that $TiO_2$ alters the photodecomposition of PO13 [42], and are likely to be attributable to the presence of $TiO_2$ and its capacity to absorb or reflect laser light, which changes how laser light interacts with these yellow pigments. The presence of $TiO_2$ may reduce the energy of light available to breakdown the PY14, hence reducing the intensity of the volatile fragments in the GC-MS peak decreases. Additionally, this might result in novel interactions between volatile compounds produced during laser irradiation and the rearrangement of the volatile fragments, which would produce new molecules.

The effect of $TiO_2$ on laser degradation of inks was verified by comparing the GC chromatograms of GR and BO inks (Fig. 3.4b). These inks have a very similar ingredient list, only differing in that GR does not contain $TiO_2$, whilst BO is listed as containing $TiO_2$ (25%, according to the label). In BO ink, the intensity of the peaks at 1.635 and 1.722 min from 2-propenoic acid-ethyl ester and methyl methacrylate were reduced significantly. Furthermore, the signal intensity of additional fragments was lower in BO ink than in GR ink. This result provides further evidence to suggest that the presence of $TiO_2$ alters the way the inks interact with laser light and reduces amount of fragmenting.



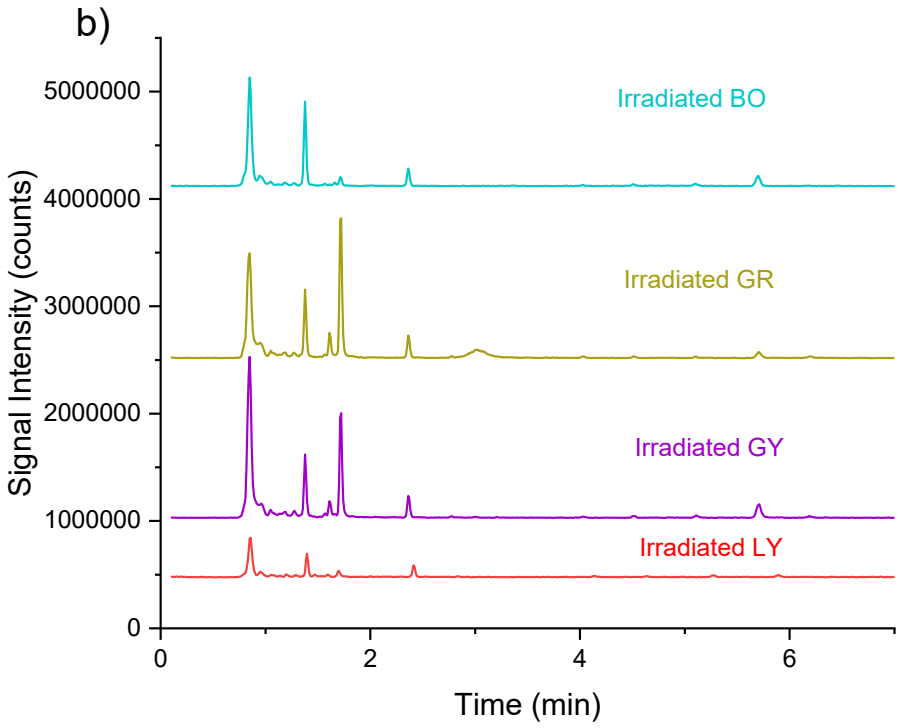



**Figure 3.4: GC-MS chromatogram of irradiated a) PY14, PY14-$TiO_2$ and b) dried LY, GY, GR and BO tattoo inks.**

**Table 3.2: The volatile fragmentation products released during laser irradiation of pigments and inks were evaluated along with their retention times, retention indices, and major mass losses. *The volatile fragments were tentatively identified.**

| Retention time (min) | Compound | Main fragments (m/z) | Observed | | | | | | | | | | Retention index |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | PY 14 | PY 74 | PY 65 | $TiO_2$ | | | LY | GY | GR | BO | |
| | | | | | | PY 14 | PY 74 | PY 65 | | | | | |
| **0.849** | 1,3-butadiyne | 50, 49, 48 | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | - |
| **0.920** | 3-butenyl methacrylate | 69, 54, 51 | ✓ | - | - | - | - | - | - | - | - | - | 594.29 |
| **0.953** | 2-propenenitrile | 53 | - | ✓ | ✓ | - | ✓ | ✓ | ✓ | - | - | - | - |
| **1.144** | benzamide/benzonitrile | 103, 76, 50 | - | - | - | ✓ | - | - | - | - | - | - | 631.37 |
| **1.397** | benzene | 78, 50 | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | 667.25 |
| **1.635** | 2-propenoic acid-ethyl ester | 99, 55 | - | - | - | - | - | - | - | ✓ | ✓ | - | 768.33 |
| **1.70** | *N, N'*-dimethyl-1,2 bis(aminooxy) ethane | 120, 74 | * | - | - | - | - | - | * | - | - | - | - |
| **1.70** | methyl 1-dideutrero-2-propenyl ether | 74 | * | - | - | - | - | - | * | - | - | - | - |
| **1.722** | methyl methacrylate | 100, 69 | - | - | - | - | - | - | - | ✓ | ✓ | - | - |

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **2.412** | toluene | 91, 65, 51 | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | 770.86 |
| **5.701** | styrene | 104, 78, 51 | - | - | - | - | - | - | ✓ | ✓ | ✓ | ✓ | 891.27 |
| **7.208** | benzyl alcohol | 108, 78 | - | - | ✓ | - | - | - | - | - | - | - | 931.74 |

### 3.3.2 SEM

PY14, $TiO_2$, PY14-$TiO_2$ mixtures and tattoo inks were examined pre-and-post-laser irradiation via SEM to ascertain any morphological changes (Fig. 3.5). As can be seen, prior to irradiation, the SEM images of $TiO_2$ showed cuboid rectangular shapes with edges and corners, typical of $TiO_2$, and consistent with previous study [43] (Fig. 3.5a). Irradiated rutile $TiO_2$ nanoparticles revealed a change in morphology when compared to unirradiated $TiO_2$. There are some agglomerated, larger spherical nanoparticles, long rods as well as tiny nanoparticles in this sample (identified in the Fig. 3.5b). Rather than simply breaking apart the $TiO_2$ the laser irradiation appears to have a heating and melting impact on $TiO_2$ particles. Laser-induced fragmentation of larger particles leads to the formation of smaller particles. As the irradiation proceeded, the smaller nanoparticles remaining might be heated and melted at high temperatures above 1000 ºC [44, 45]. These shape alterations could be caused by laser photon energy absorption (including non-linear absorption and heating or melting of the nanoparticles) causing alterations in their phase and crystalline structure. Unirradiated PY14 consisted of finely aggregated powders, however, post-irradiation the PY14 sample was reorganised into thinly layered smooth sheets of 2-6 μm in length (Fig. 3.5c and d). Interestingly, the SEM images of the irradiated PY14-$TiO_2$ show that the particles had agglomerated, with the TiO2 appearing to be "glued" onto the PY14 surface as smaller, irregularly shaped particles (Fig. 3.5e and f). Additionally, it appears $TiO_2$ forms clusters around PY14 particles. This may play a role in preventing the fragmentation of PY14 into smaller particles and contribute to the decreased signals in the GC-MS which are presented in Fig. 3.4a.

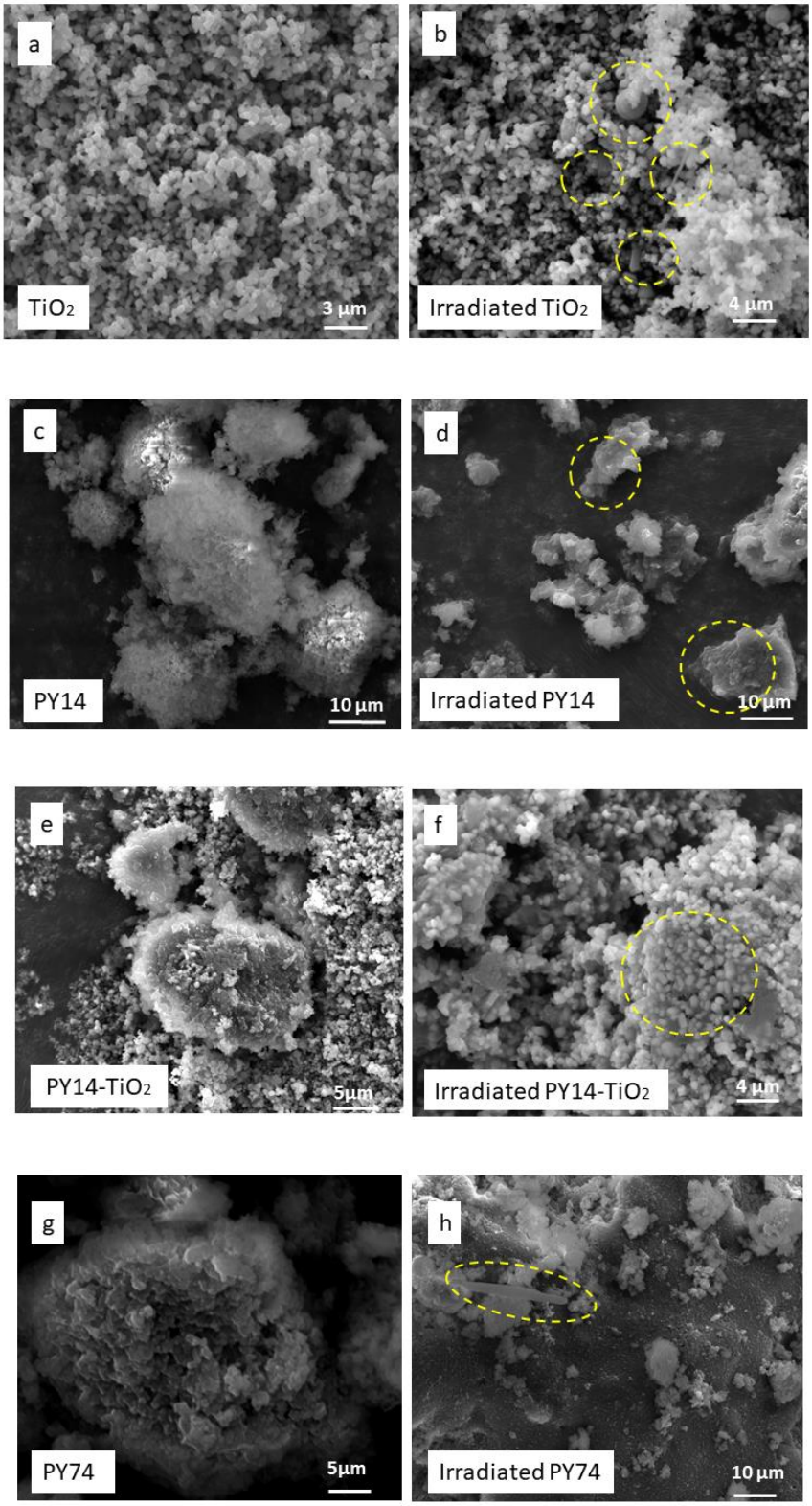
Similar results demonstrating the effect of the photoactivity of $TiO_2$ were also observed in the SEM images of irradiated PY74-$TiO_2$ and PY65-$TiO_2$. In comparison with the unirradiated mixture of PY74-$TiO_2$ (Fig. 3.5i), the irradiated mixture demonstrated that $TiO_2$ agglomerated around PY74 particles as a coating layer (Fig. 3.5j). It is interesting to note that PY65 exhibits unique micro-morphologies including cubes, rods, spheres, and undefined-shaped microcrystals (Fig. 3.5k). The

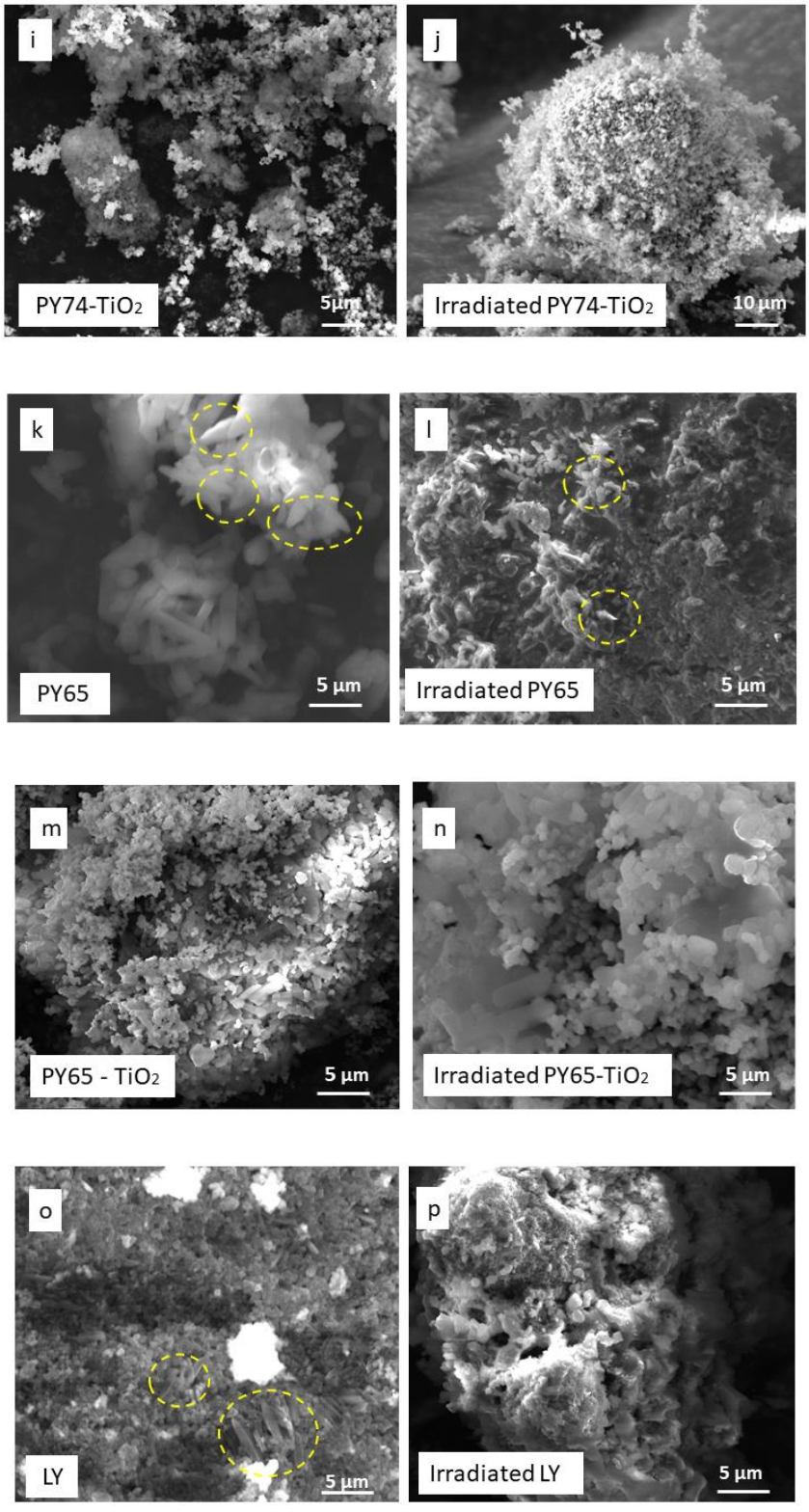
smoothness was also seen in the irradiated PY65-$TiO_2$, with more intense melting and a vacuous look with small floating particles in between (Fig. 3.5n). In addition, SEM of the irradiated PY65-$TiO_2$ showed $TiO_2$ particle adhesion on the surface of PY65. The adhesion of $TiO_2$ to the pigment's surface supports the hypothesis of the photoactivity of white pigment upon exposure to laser light, causing the pigments to heat up and agglomerate with these yellow pigments. EDX was carried out to distinguish between yellow pigments and $TiO_2$ particles (Fig. 3.6).

Tattoo inks are a combination of pigments and other components, therefore, their morphologies vary depending on their content, and various particle shapes and sizes show up in the same sample [46]. Smoothness of surface was the shared feature between GY, GR, and BO inks (Fig. 3.5q, s, and u). However, SEM analyses are performed on dried ink samples, and the behaviour of the particles in solution may differ, i.e., the removal of solvent may increase aggregation and agglomeration in the original inks [47]. After irradiation, damage to the smoothness of the dried inks' surface was observed. Pits formed that extended to the entire surface, with the remaining intact surface creating islands between them and the structure became more porous. Following laser irradiation, fibre structures sized in the micrometer range and arranged in a random structure of LY ink were melted (Fig. 3.5o and p). Other characteristics of the irradiated tattoo ink sample include coalescing blocks and punctured regions with wide holes (the formation of cavities and pores). Moreover, it has been reported that Cu (found in PB15) is a promoter and one of the ingredients in LY ink, therefore, particle agglomeration may occur [48]. In this work, irradiated BO ink had a different morphology compared with the GY and GR inks (Fig. 3.5r, t, and v). SEM images of irradiated BO inks revealed greater heterogeneity, with spheres and tiny particles aggregated on the ink's surface. An EDX investigation of the irradiated sample revealed that the surface alterations were due to a large amount of $TiO_2$ appearing on the surface (Fig. 3.7). This is likely due to a higher concentration of $TiO_2$ in the ink, when compared with the GR and GY ink.

The laser treatment indicates that the shape and size alteration of inks and pigments particles occurred by particle melting. If this is the case, the measured laser fluence threshold for the transition should correspond to the fluence at which the particles' temperature surpasses the melting point of pigment [49]. According to the literature, the optical properties of a pigment, specifically its colour and hiding power, are determined by the shape and size of its particles [50]. Therefore, the darkening of inks and yellow pigments (Fig. 3.3) could be a result of the alterations of the size and the morphology after irradiation with laser. The mechanism during irradiation that results in morphological changes is most likely connected to the temperature at the focusing location. It has been assumed that all irradiated pigments are broken into smaller particles, and as a result, laser removal is generally a successful method in the tattoo removal process. However, in the presence of $TiO_2$, the particle size appears to

increase, making the removal process difficult. The aggregation phenomena are presumably the combined effects of heating and beam penetration, impacting both the pigment and the carrier in tattoo inks at the same time.




i
PY74-TiO2
5µm
j
Irradiated PY74-TiO2
10 µm
k
PY65
5 µm
l
Irradiated PY65
5 µm
m
PY65 - TiO2
5 µm
n
Irradiated PY65-TiO2
5 µm
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5 µm
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Irradiated LY
5 µm

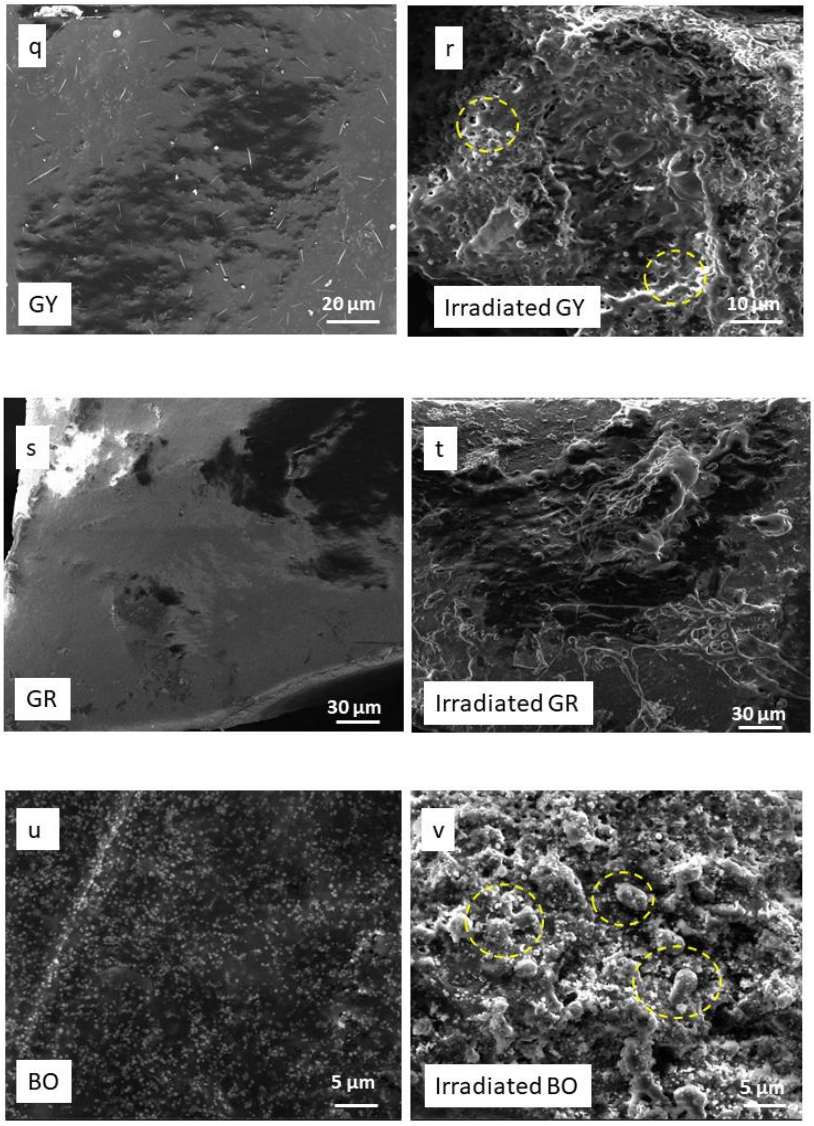


**Figure 3.5: SEM images at different magnifications of unirradiated and irradiated PY14, PY74, PY65, $TiO_2$, mixtures of pigments with $TiO_2$, and dried tattoo ink. This shows the change in the morphology of pigments and inks after laser irradiation. $TiO_2$ aggregates around pigments.**

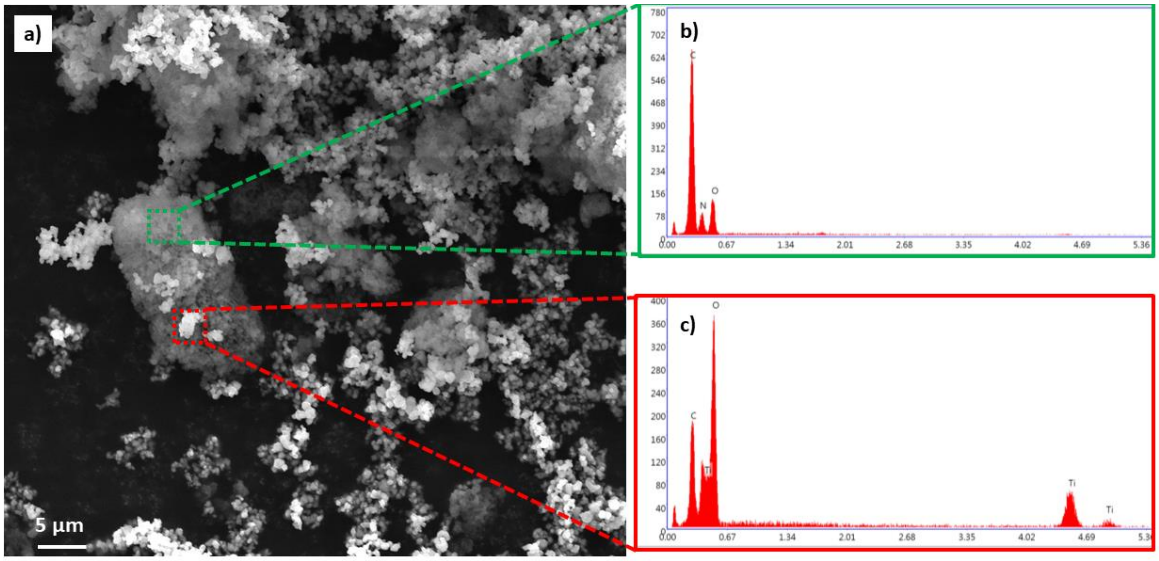


**Figure 3.6: a) SEM image of unirradiated mixture of PY74 -$TiO_2$, b) and c) EDX scan of the mixture to distinguish between yellow pigments and $TiO_2$ particles.**

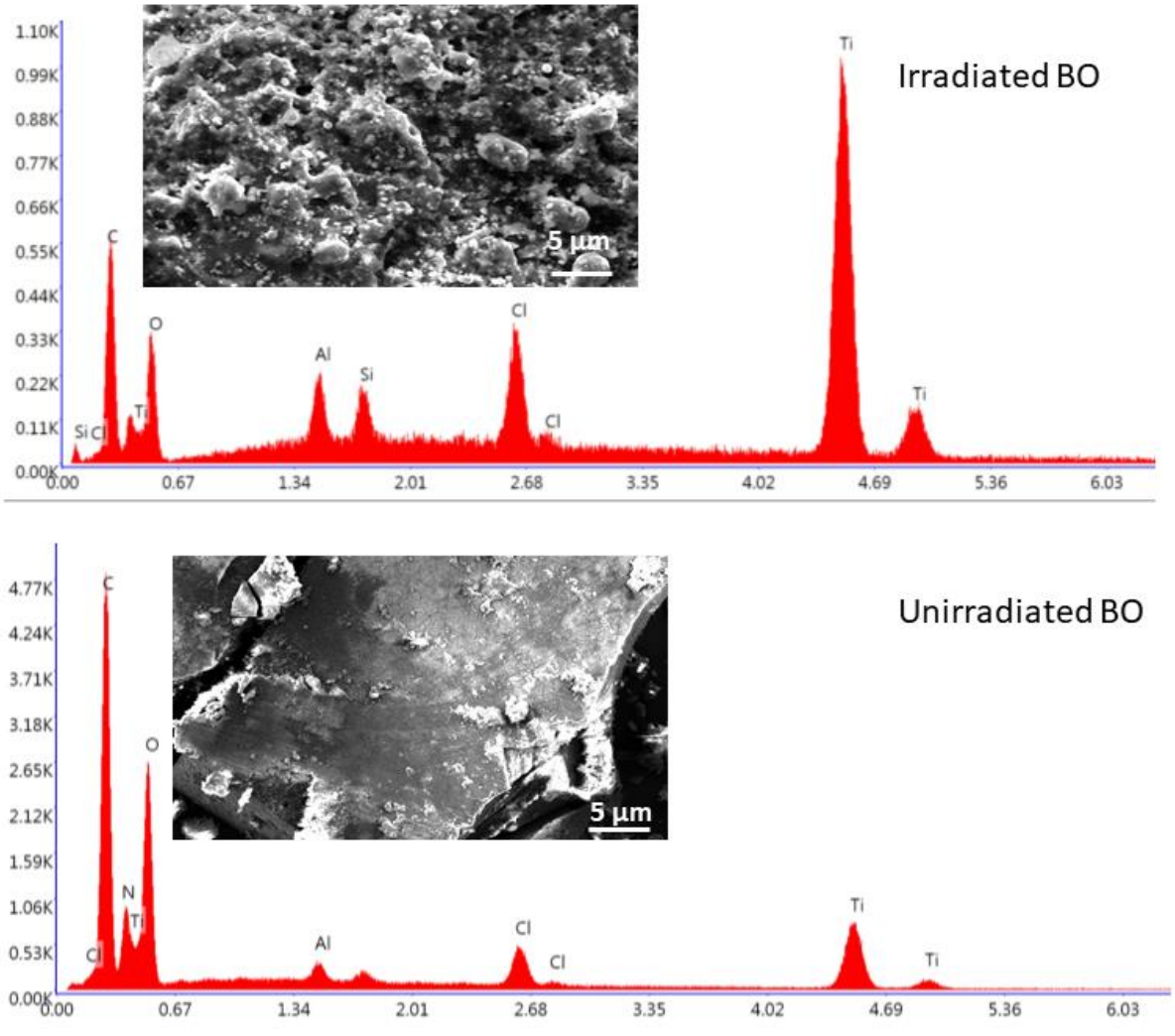


**Figure 3.7: EDX analysis surface of unirradiated and irradiated BO ink**

### 3.3.3 DLS

The effect of laser irradiation on particle size was explored using DLS. The nanosized diameter of suspensions of unirradiated and irradiated PY14, PY14-$TiO_2$, and ink particles was examined (Fig. 3.8 and 3.9 and Table S13.1). As can be seen from Fig. 3.8, the DLS measurements provides the overall average size and shows that 300 nm $TiO_2$ agglomerated to a large particle size of around 473±7 nm after laser irradiation. However, PY14 particles fragmented from 705±39 nm (unirradiated) into smaller particles with a diameter of around 301±20 nm when irradiated. For $TiO_2$ pigment mixtures, the irradiated mixtures had a larger size of around 461±29 nm when compared to irradiated PY14 and were similar in size to the irradiated $TiO_2$. This research indicates that the $TiO_2$ plays a major role in the overall final particle size of irradiated PY14-$TiO_2$ mixtures. It is hypothesized that this phenomenon is linked to reduced absorption of laser light by PY14 in presence of $TiO_2$ which hinders fragmentation into smaller particles during treatment. Furthermore, considering the elevated local temperatures greater than 1000 ºC during laser removal [44, 45], it is more probable that the particles melt and form larger particles.

These findings align with SEM results showing increased particle sizes post-irradiation forming spherical shapes. Notably, while SEM identified higher particle sizes than DLS measurements, this discrepancy may be attributed to differences arising from agglomerate dispersion within the DLS solution.

A similar trend was also observed for PY65 and PY74. Laser irradiation of PY65-$TiO_2$ resulted in a size shift of the particles from 272 ±16 nm (irradiated PY65) to 455±13 nm. Similarly, DLS analysis showed that irradiated PY74-$TiO_2$ exhibited a significant change in hydrodynamic diameter from 165±21 nm (irradiated PY74) to 334 ±14 nm, attributed to varying amounts of $TiO_2$ present (Fig. SI 3.3).

Prior to irradiation, LY, GY, and GR have similar particle sizes of around 164±35 nm, with BO having a larger average particle size of around 329±20 nm. The larger particle size of BO is likely due to the large quantity of $TiO_2$ compared with the remaining inks which had no $TiO_2$ (GR) and smaller quantities (LY and GY) (Table 3.1). All of the inks had larger particle sizes after irradiation, however the magnitude of the change varied. Interestingly, BO ink showed only a small increase in the ink's particle size after laser treatment, which could support the hypothesis that the presence of $TiO_2$ in high quantities limits the effect of the laser on particle breakdown and resulted in aggregates of this tattoo ink. A larger increase in particle size was observed for GY and GR inks following laser irradiation. Further suggesting that the small amount of $TiO_2$ (1–10%) has less effect on the particle size. It would be expected that if the particle size from the ink was just dependent on the pigment, a

smaller particle size would result after irradiation. However, the inks comprise both pigment and $TiO_2$ along with a number of other vehicles. These vehicles may also be playing a role.

The DLS data support the SEM observations, particularly concerning agglomerates, even though large agglomeration structures may exceed the detection range of DLS. For irradiated pigments and tattoo inks, both SEM and DLS indicate heterogeneous structures with diameters ranging from 250 to 800 nm. In the case of the DLS technique, as per Mie's theory, where scattering intensity follows a power-law relationship with radius size, it is plausible to infer that larger aggregates could potentially mask smaller structures that might be less than 100 nm in size [51].

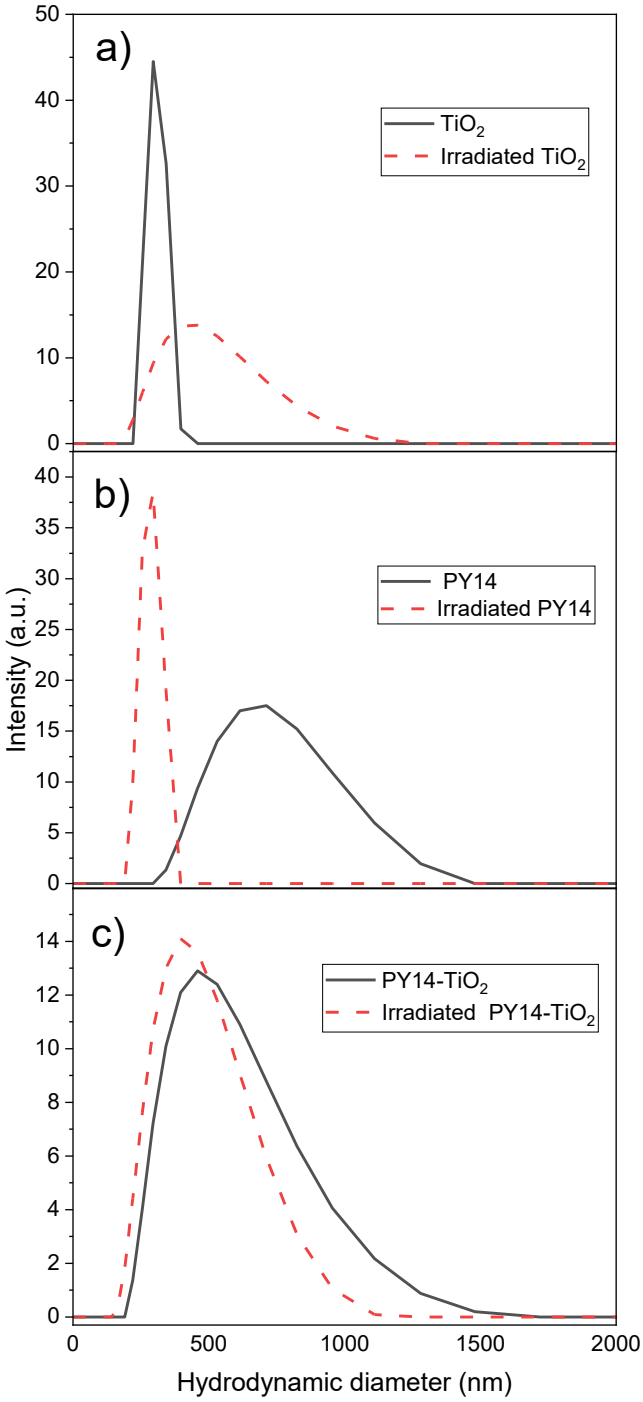


**Figure 3.8: DLS data of unirradiated and irradiated a)$TiO_2$, b) PY14, and c) PY14-$TiO_2$. Comparison of the particle size of irradiated pigments with and without $TiO_2$, showing the particle size of irradiated pure pigments is smaller than irradiated PY-$TiO_2$**

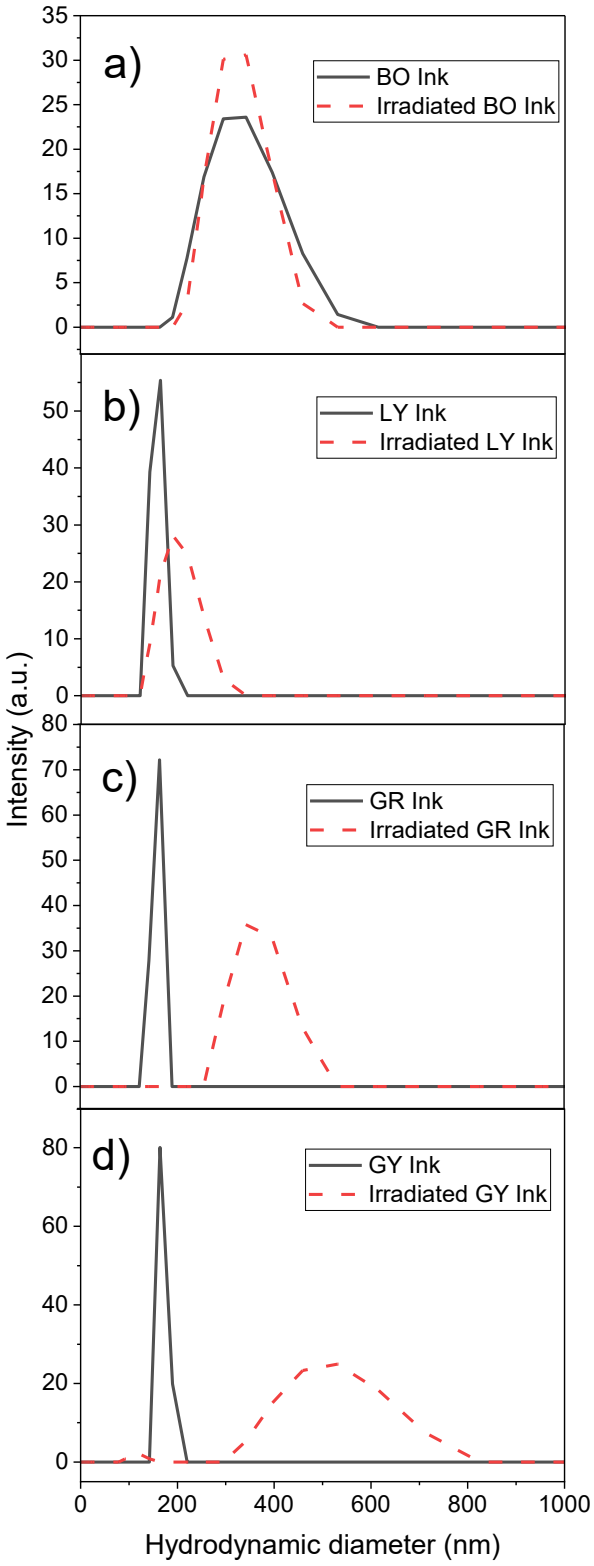


**Figure 3.9: DLS data of unirradiated and irradiated a) BO, b) LY, c) GR, and d) GY inks.**

### 3.3.4 XRD

XRD analysis was conducted on both unirradiated and irradiated pigments and inks to explore the potential relationship between pigment darkening, molecular composition alterations, binding

medium degradation, and changes in phase structures. The XRD analysis of irradiated $TiO_2$ and PY14 indicated a rearrangement of the crystal structure, as evidenced by decreased intensity of diffraction peaks (Fig. 3.10a and b). LY, GY, and GR inks behaved similarly (Fig. SI 3.4). In contrast, the XRD data of BO ink (Fig. 3.10c) showed more pronounced high-intensity peaks after laser irradiation. This increase in intensity is likely due to $TiO_2$'s arrangement towards the surface of the BO ink after exposure to 532 nm laser light, as shown in the SEM images (Fig. 3.5v). The SEM images, together with the XRD results, suggest that $TiO_2$ undergoes photoactivity or photoreaction upon laser light exposure, potentially indicating a structural transformation from rutile to anatase [26, 52].

The findings from this study support the hypothesis that pulsed laser irradiation induces changes in the crystalline structure and phase of the pigments. Chemical or photochemical redox processes may contribute to colour changes in inorganic and metallo-organic pigments. Given the high local temperatures (>1000 ºC) generated during laser removal [44, 45], these reactions are more likely to occur, which could explain the common darkening of tattoos post-treatment. Additionally, local colour changes might result from redox reactions involving photocatalytic $TiO_2$ and copper phthalocyanine pigments, such as Blue 15 and Green 7. Organic pigments could decompose into colourless or coloured fragments through chemical and/or photochemical reactions, which in some cases may pose health risks [21, 23]. The most compelling evidence of the thermal effect is the observed colour shift and pigment darkening following laser irradiation, likely driven by heat-induced changes in the crystal lattice structure of each pigment.

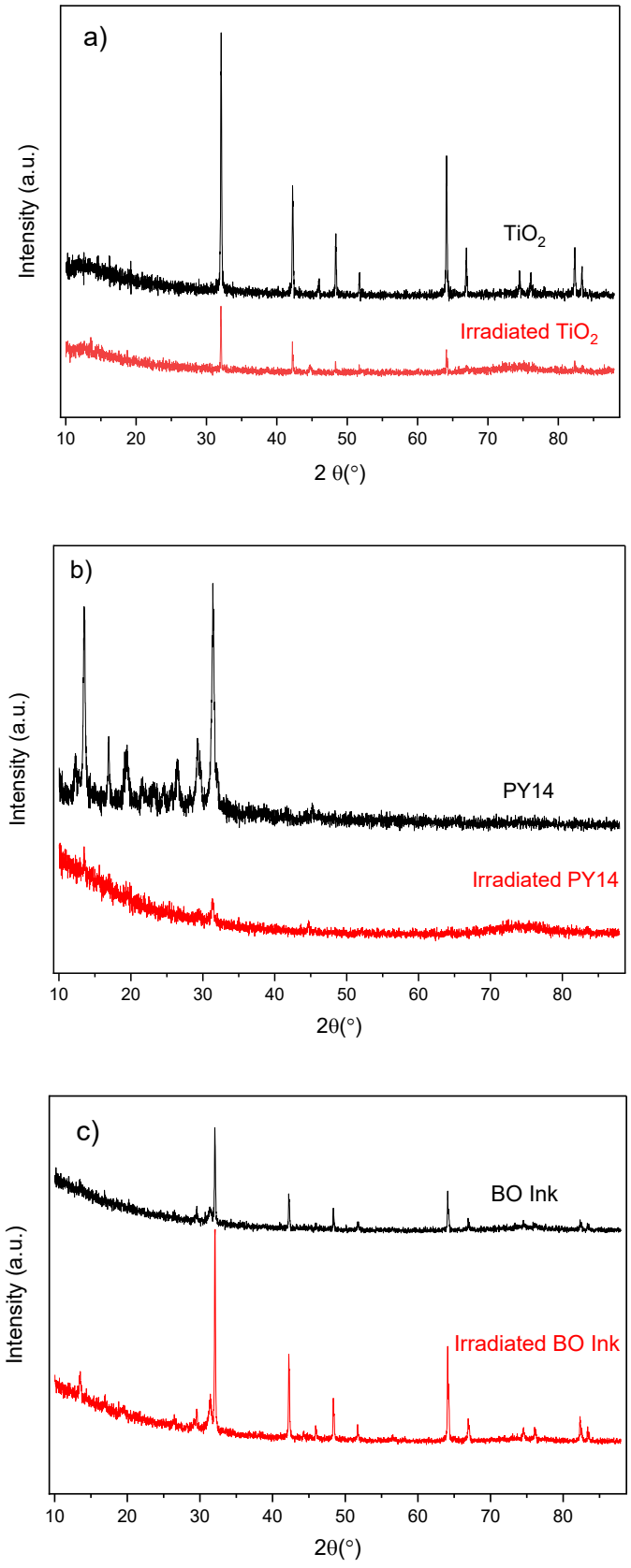


**Figure 3.10: XRD analysis of unirradiated and irradiated a) $TiO_2$, b) PY14, c) BO Ink. $TiO_2$ and PY14 presents how the crystal structure change after laser irradiation. BO ink surface analysis shows high intensity peaks of $TiO_2$ after laser irradiation.**

## 3.4 Potential health concerns

This study raises significant health concerns related to the *in-vivo* formation of potentially toxic molecules and aggregates upon the laser irradiation of pigments and tattoo inks. GC-MS analysis identified several volatile fragments, which all have potential health effects as outlined in their safety data sheets obtained from Chemwatch [53]. These health hazards are summarised in the supporting information (Table. 3.3). For example, compounds such as 2-propenenitrile, benzene, methyl methacrylate, toluene, and styrene are known to induce toxicity/harm (H311, H312), irritation (H315) and allergic reactions (H317) upon contact with the skin. Furthermore, benzene is classified as carcinogenic (H350), methyl methacrylate is recognised for causing severe eye damage (H319) and is also a skin irritant, while toluene may harm the unborn child (H361d) and can cause organ damage with prolonged or repeated exposure (H373). These hazardous chemicals are typically encountered through inhalation or skin contact. This research indicates that these toxic compounds may be formed within the body during laser treatment, adding a new route of exposure. There is limited understanding of the risks from this new route of exposure when compared to exposure through skin and respiratory routes. In any case there is concern that *in-vivo* generation of these compounds could result in more significant health effects. Importantly, during laser irradiation the compounds are likely concentrated at the treatment site, which could potentially lead to significant localised damage. Following this they may be distributed through the body through the blood and lymphatic systems. The level of hazard will depend on their likelihood to be excreted from or accumulate within the body. In addition, results from this study illustrates that the presence of $TiO_2$ makes laser pigment removal more challenging, thus additional sessions may be required. This would result in repeated exposure to these volatile hazardous fragments, but at a lower dose per session.

In addition to the formation of volatile compounds, DLS results shows laser irradiation of PY14, PY74 and PY65 led to the formation of smaller nanoparticles. These substances could be redistributed in the body and cause harmful or even carcinogenic responses [54, 55]. SEM of the irradiated pigments also showed that the produced morphologies were highly diverse, with some of them potentially hazardous when in touch with or expelled from the skin. For example, the fibre and needle-shaped structures observed in unirradiated PY65, unirradiated LY, irradiated PY74 and irradiated $TiO_2$, might be harmful [8, 56]. It is known that the toxicity of fibrous nanoparticles rises as their aspect ratio increases [57]. Aspect-ratio-dependent toxicity is commonly observed in the lungs. Nanofibers approximately 150 nm in thickness and 2, 5, or 10 μm in length are indicative of lung cancer, mesothelioma, and asbestosis, respectively [56, 57]. It has been reported that Needle-shaped

nanoparticles demonstrate greater toxicity than spherical nanoparticles due to enhanced endocytic processes, higher internalisation rates, and increased adhesiveness to target cell surfaces [55, 58].

In contrast, DLS showed an increase in particle size after irradiation of tattoo inks containing $TiO_2$ and pigment-$TiO_2$ mixtures. This was supported by SEM images which revealed altered morphologies, including large aggregates. When tattoo inks agglomerate rather than decompose during laser therapy, significant toxicity issues may emerge. Initially, if the ink particles aggregate into larger clusters, the body might encounter difficulties to removing them. Larger aggregates are less likely to be transported via the bloodstream or lymphatic system [46, 59], which means they can stay in the body for extended periods. Over time, these aggregate particles may gradually release hazardous decomposition products into surrounding tissues or the circulation, potentially resulting in localised or systemic poisoning. For example, release of polycyclic aromatic hydrocarbons (PAHs) from black and yellow inks has been reported [13]. Prolonged exposure to such carcinogens could increase cancer risk.

Additionally, if the body detects the aggregated particles as foreign, it might trigger a prolonged immune response [60]. Larger particles may persist in the skin or lymphatic system, perhaps resulting in chronic local irritation or inflammation. It has been reported that nanoparticle aggregation has the potential to cause inflammatory lung diseases in people [61]. In addition, agglomerated carbon nanotubes have been shown to be more hazardous than well-dispersed carbon nanotubes, and longer nanotubes in dimension have been observed to be more toxic [62, 63].

$TiO_2$ nanoparticles have been extensively studied for their toxicity, and their small size and high surface area are often associated with increased reactive oxygen species (ROS) generation, which can lead to oxidative stress and cellular damage [64]. Studies have shown that nanoparticles exhibit distinct toxicological profiles based on their size, shape, and surface characteristics [65, 66]. While smaller $TiO_2$ particles have been linked to higher cytotoxicity, the aggregation of $TiO_2$ into larger clumps, as observed in our study, could modify their interactions with cells or tissues. Larger aggregates may be less efficiently taken up by cells but could contribute to local toxicity through prolonged retention or deposition in tissues, potentially inducing inflammatory responses or cellular damage upon sustained exposure. Additionally, the aggregation of titanium dioxide ($TiO_2$) nanoparticles on the surface of the BO ink following laser treatment not only alters the surface morphology but may also have implications for the material's toxicity.

Depending on the distribution/elimination of the irradiation products within the body, the cumulative effect of repeated exposure could pose serious health risks to individuals undergoing tattoo removal.

More study is needed to examine the *in-vivo* formation of these compounds and fate within the body. Additionally, this study only examined a small number of inks. Further work is required to examine a broader range of inks in order to comprehensively assess the likely toxicity of tattoo ink irradiation products within the body.

**Table 3.3: The toxicological evaluation and the hazard statement* were made according to ECHA database and Chemwatch.**

| Retention time (min) | Compound | Hazard Codes* | Hazard category | Description |
|---|---|---|---|---|
| **0.849** | 1,3-butadiyne | H220 | 1A | Extremely flammable gas. |
| | | H280 | Compressed gas liquefied gas dissolved gas | Contains gas under pressure may explode if heated |
| **0.920** | 3-butenyl methacrylate | NA | NA | NA |
| **0.953** | 2-propenenitrile | H225 | 2 | Highly Flammable liquid |
| | | H301 | 3 | Toxic if swallowed |
| | | H311 | 3 | Toxic in contact with skin |
| | | H315 | 2 | Causes skin irritation |
| | | H317 | 1, 1A, 1B | May cause an allergic skin reaction |
| | | H319 | 2/2A | Causes serious eye damage |
| | | H331 | 3 | Toxic if inhaled |
| | | H335 | 3 | May cause respiratory irritation |

| | | | | |
|---|---|---|---|---|
| | | H350 | 1, 1A, 1B | May cause cancer |
| | | H361 | 2 | Suspected of damaging fertility or the unborn child |
| | | H411 | 2 | Toxic to aquatic life with long lasting effects |
| **1.144** | benzamide | H302 | 4 | Harmful if swallowed. |
| | | H341 | 2 | Suspected of causing genetic defects. |
| | benzonitrile | H227 | 4 | Combustible liquid. |
| | | H302 | 4 | Harmful if swallowed. |
| | | H312 | 4 | Harmful in contact with skin. |
| **1.397** | benzene | H225 | 2 | Highly flammable liquid and vapour. |
| | | H304 | 1 | May be fatal if swallowed and enters airways. |
| | | H315 | 2 | Causes skin irritation. |
| | | H319 | 2/2A | Causes serious eye irritation. |
| | | H336 | 3 | May cause drowsiness or dizziness. |
| | | H340 | 1, 1A, 1B | May cause genetic defects. |
| | | H350 | 1, 1A, 1B | May cause cancer. |
| | | H360Fd | 1, 1A, 1B | May damage fertility |
| | | H372 | 1 | Causes damage to organs through prolonged or repeated exposure. |

| | | | | |
|---|---|---|---|---|
| | | | | Suspected of damaging the unborn child. |
| | | H401 | 2 | Toxic to aquatic life. |
| **1.635** | 2-propenoic acid-ethyl ester | NA | NA | NA |
| **1.702** | N, N'- dimethyl-1,2 bis(aminooxy)ethane | NA | NA | NA |
| **1.70** | methyl 1-dideutreio-2-propenyl ether | NA | NA | NA |
| **1.722** | methyl methacrylate | H225 | 2 | Highly flammable liquid and vapour. |
| | | H315 | 2 | Causes skin irritation. |
| | | H317 | 1, 1A, 1B | May cause an allergic skin reaction. |
| | | H319 | 2/2A | Causes serious eye irritation. |
| | | H335 | 3 | May cause respiratory irritation. |
| | | H336 | 3 | May cause drowsiness or dizziness. |
| **2.412** | toluene | H225 | 2 | Highly flammable liquid and vapour. |
| | | H302 | 4 | Harmful if swallowed. |
| | | H304 | 1 | May be fatal if swallowed and enters airways. |
| | | H315 | 2 | Causes skin irritation. |
| | | H319 | 2/2A | Causes serious eye irritation. |
| | | H336 | 3 | May cause drowsiness or dizziness. |

| | | | | |
|---|---|---|---|---|
| | | H361d | 2 | Suspected of damaging the unborn child. |
| | | H373 | 2 | May cause damage to organs through prolonged or repeated exposure. |
| **5.701** | styrene | H226 | 3 | Flammable liquid and vapour. |
| | | H302 | 4 | Harmful if swallowed. |
| | | H315 | 2 | Causes skin irritation. |
| | | H319 | 2/2A | Causes serious eye irritation. |
| | | H332 | 4 | Harmful if inhaled. |
| | | H351 | 2 | Suspected of causing cancer. |
| | | H361d | 22 | Suspected of damaging the unborn child. |
| | | H373 | 2 | May cause damage to organs through prolonged or repeated exposure. |
| **7.208** | benzene methanol | H302 + H332 | 4 | Harmful if swallowed or if inhaled. |
| | | H319 | 2/2A | Causes serious eye irritation. |

*Association between hazards code and toxicity of fragments produced by laser treatment of inks was according to globally harmonized system of classification and labelling of chemicals (GHS*).http://www.unece.org/fileadmin/DAM/trans/danger/publi/ghs/ghs_rev08/ST-SG-AC10-30-Rev8e.pdf.*

## 3.5 Conclusion

In conclusion, this study investigated the effects of laser treatment on tattoo inks, specifically focusing on pigments, dried inks, and mixtures of PY14, PY74, PY65 with $TiO_2$. The results revealed a diverse

range of degradation products, along with variations in morphology and size. The laser degradation process was significantly influenced by the presence of $TiO_2$, with different aggregate morphologies forming when pigment-$TiO_2$ mixtures and $TiO_2$-containing inks were irradiated. Additionally, the presence of $TiO_2$ altered the chemical profile of volatile degradation products detected by GC-MS, along with reducing their concentration, suggesting that $TiO_2$ may decrease the extent of degradation. This effect is likely due to $TiO_2$ aggregating around pigment particles, thereby limiting the availability of the pigment for degradation. Moreover, the aggregation of $TiO_2$ with pigment particles following laser irradiation may impede the efficacy of laser tattoo removal, as the formation of larger particle could reduce their clearance by the body's natural removal mechanisms. These findings provide insight into the challenges and toxicity associated with the laser removal of tattoos and underscore the need for further research and technological advancements in tattoo removal techniques.

## 3.6 Acknowledgements

B.A. extends deepest gratitude to the following organizations for their invaluable support and contributions to this research: King Faisal University, Ministry of Education, Saudi Arabia, for their generous support and encouragement throughout the duration of this study. We acknowledge the facilities and technical assistance of Microscopy Australia, a company established under the National Collaborative Research Infrastructure Strategy, through the South Australian Regional Facility, Flinders Microscopy and Microanalysis (FMMA), Flinders University. We also acknowledge the facilities and the scientific and technical assistance of the South Australian node of the Australian National Fabrication Facility (ANFF) under the National Collaborative Research Infrastructure Strategy, located at Flinders Microscopy and Microanalysis, Flinders University.

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# 4 CHAPTER 4: ANALYTICAL INVESTIGATION OF MELANIN'S IMPACT ON THE LASER FRAGMENTATION AND MORPHOLOGY OF YELLOW TATTOO PIGMENTS

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**This chapter was accepted by the journal of Photochemical & Photobiological Sciences.** The only alterations are that some figures from supplementary information have been moved to this chapter. Supplementary information for this chapter has been shown in the supplementary section (**Appendix 3**).

## Author contribution statement:

Batool Aljubran carried out the experimental work, as well as the data analysis for gas chromatography-mass spectrometry (GC-MS), scanning electron microscopy (SEM), energy-dispersive X-ray spectroscopy (EDX), and dynamic light scattering (DLS). Batool Aljubran also wrote the manuscript with support from Claire Lenehan, Ula Alexander, and Kirstin Ross, who all contributed to supervising the project. All of the authors contributed to experimental design and ideation, discussion on data analysis, manuscript preparation and approved the final version of the manuscript.

**The previous chapter explored how the existence of $TiO_2$, presents significant challenges during laser tattoo removal. $TiO_2$ was shown to contribute to reduced laser absorption, and led formation of potentially harmful by-products, all of which complicate the efficacy and safety of the removal process. While these findings shed light on the chemical and physical barriers introduced by certain ink components, laser tattoo removal can also be hindered by intrinsic skin characteristics particularly melanin. Therefore, the following chapter aimed to investigate the role of melanin, especially in individuals with darker skin tones, and how its high optical absorption competes with tattoo pigments during laser treatment. This exploration aimed to uncover how melanin contributes to reduced laser effectiveness, increased risk of adverse effects, and further complicates treatment strategies for safe and successful tattoo removal.**

# Abstract

The popularity of tattooing has increased the demand for effective tattoo removal methods. Laser techniques are standard, but their efficacy can vary, particularly for individuals with darker skin tones due to melanin interference. This study aims to investigate the role of melanin in the effectiveness of QS Nd:YAG laser treatments on tattoo inks and to provide experimental data on their interaction. A 532 nm QS Nd:YAG laser treatment was used on yellow tattoo inks, reference pigments, and pigment-melanin mixtures. The degradation products, along with their morphology and particle size, were examined using GC-MS, SEM, and DLS. The results reveal that melanin significantly absorbs 532nm laser light, hindering the degradation of yellow pigments and inks. The presence of melanin reduces volatile fragment formation, promotes ink conglomerates, and increases ink particle size. The study's *in-vitro* nature, which may not reflect live skin interactions. This paper examines yellow pigments and inks only, at 532nm. It does not address what may happen for different colours or at other laser wavelengths. The presence of melanin plays a crucial role in laser tattoo removal efficacy, indicating the need for tailored approaches to improve treatment outcomes for individuals with darker skin tones.

## 4.1 Introduction

Tattoos, an art form with a history spanning thousands of years, have become increasingly prevalent in contemporary social and cultural trends [1]. Alongside the rise in tattoo popularity, there has been a corresponding increase in instances of tattoo regret, leading to a significant surge in demand for tattoo removal over the past decade [2-4]. Laser therapies are the standard method for tattoo removal due to their superior efficacy compared to other techniques [5-7]. The laser tattoo removal process involves targeting tattoo ink particles with short and intense laser pulses that fragment the pigments into smaller components that are subsequently removed by lymphatic system processes [8]. The breakdown of tattoo ink particles is achieved by passing laser light through the epidermis and subsequent photothermolysis of the tattoo pigments [7, 9]. The mechanism behind this approach is believed to involve inducing mechanical stress via thermal mechanisms during treatment sessions [5, 10].

Although using lasers for tattoo removal process is a well-established protocol, it is far from being the simple and ideal procedure for successful tattoo removal that many operators assume [2]. According to the literature, multiple laser treatments are often required to achieve successful tattoo removal, making it challenging for people with limited time. In addition, the number of treatments necessary for a specific patient remain undetermined and depend on three major considerations: the laser settings used (e.g. wavelength, pulse energy, pulse duration, spot size), the skin pigmentation, and tattoo-dependent parameters, which include the style, depth, and size of the tattoo along with the inks used [11].

Skin pigmentation can have a significant impact on the efficacy of the tattoo removal procedure [12]. As reported in the literature, patients who have lighter skin tones (Fitzpatrick skin types I to III) can be treated with higher fluences and smaller spot sizes. On the other hand, low fluences and larger spot sizes are recommended to treat patients with darker skin tones, including Fitzpatrick skin types IV to VI [13, 14]. In addition, it has been claimed that by applying longer wavelengths, longer pulse durations, and more effective cooling devices, laser treatments can safely and effectively treat individuals with darker skin types [15-17]. Furthermore, it has been reported that removing tattoos from darker-skinned people is often difficult, and it is thought that this may be complicated by the presence of melanin pigments [9, 18].

Melanin in the epidermis acts as a competing chromophore for the absorption of laser light used for tattoo removal [16, 19]. Natural melanin, which includes eumelanin, pheomelanin, and neuromelanin, is created in biological systems through a sequence of enzymatic processes that begin with L-tyrosine. Synthetic melanin is a melanin-like substance created by the enzymatic or chemical oxidation of various monomers [20]. Melanin chromophores can absorb laser radiation, and are reported to heat and

cool faster than larger structures including blood vessels [21, 22]. This can cause cell damage, dyspigmentation, blistering, and scarring [14, 19]. As a result of melanin, there will be an increase in the laser light required to breakdown the intended target pigment's chromophore in darker skin, leading to an increase in the number of laser treatment sessions [11].

QS 694 nm ruby laser, 755 nm alexandrite laser, and QS Nd:YAG (1064 nm and 532 nm) lasers are commonly used in tattoo removal. However, the use of 532 nm and 694 nm laser light is not advised for dark skinned peoples, as epidermal melanin absorbs a significant portion of the laser's energy, making darker-skinned patients more prone to long-term dyspigmentation and other adverse effects [23]. Thus, removing lighter tattoo ink colours will be hindered or more challenging on darker skin due to the presence of melanin [17, 24]. This is challenging for treatment of coloured tattoos, as longer wavelengths have been reported to effectively remove blue and black tattoo colours [23, 25], but red, orange, and yellow colours are best treated with 532nm laser light.

The main objective of this study was to investigate the impact of melanin pigments on the irradiation process of yellow pigments and inks, specifically focusing on changes in fragment size and morphology. The QS Nd:YAG laser treatment was applied to a mixture of tattoo inks and melanin pigments. Both natural and synthetic melanin pigments were utilized due to their shared characteristics such as strong light absorption and free radical quenching ability [20]. It was necessary to verify the results using melanin pigments extracted from animal models before extrapolating them for research involving human epidermal melanin. Laser radiation targeted the yellow pigment present in the ink, aiming to eliminate any effects from other components within the ink vehicle while enabling comparisons with pure ink properties.

## 4.2 Method

### 4.2.1 Material and sample preparation

Lemon Yellow (LY), Golden Yellow (GY), Golden Rod (GR) and Bright Orange (BO) Intenze® brand inks were purchased from Tattoo Direct, Victoria, Australia. Pigment yellow 14 (PY14) (C.I. 21095) (97%), pigment yellow 65 (PY65) (C.I. 11740) (98%), and pigment yellow 74 (PY74) (C.I. 11741) (tech) were purchased from AK Scientific, Union City, California. Methanol (LC MS grade) was purchased from Honeywell, Australia. Synthetic melanin (SM) (M863) powder, produced by oxidation of tyrosine with hydrogen peroxide, and extracted melanin (EM) (M2649) (99%) from *Sepia officinalis* were purchased from Sigma-Aldrich, Australia. All chemicals used in this investigation, except the tattoo inks themselves, were used without any further purification. Samples

were placed in standard 2mL wide-mouth GC vials equipped with a 200 µL small volume insert (Rowe scientific,VV0055).

### 4.2.2 Sample preparation and laser experiment setup

The overall approach employed in this study is depicted in Fig. SI 4.1 and the chemical structure of the pigments tested is shown in Fig. 4.1a-c. Tattoo inks were pipetted onto microscope slides and dried in open air at ambient temperature for 48 h prior to irradiation and/or characterization. Dried inks were scraped from the slide and placed into a GC vial prior to laser treatment. Unadulterated samples of pigments (PY14, PY65, PY74), inks (BO, GR, LY, GY), and melanin (EM, SM) were used as controls. Prepared mixtures were made by combining melanin (EM, SM) with the different pigments (PY14, PY65, PY74) or dried inks (BO, GR, LY, GY) in individual GC glass vials. The ratio of the combination was 50:50 by mass.

Control samples and prepared mixtures were irradiated using a QS Nd:YAG laser (Spectra Physics Quanta-Ray GCR12) at 532 nm. Each sample was exposed to 20 pulses (with a duration of 6 nanoseconds) over a period of around 1-2 minutes. The laser pulses were 140-135 mJ/pulse over an area of 2 mm diameter (i.e., a fluence of 4.4 – 4.2 J/cm$^2$). The photodegradation products were analysed using a headspace GC-MS to identify volatile fragments. In addition, SEM and DLS were employed to investigate changes in particle shape and size following laser irradiation.

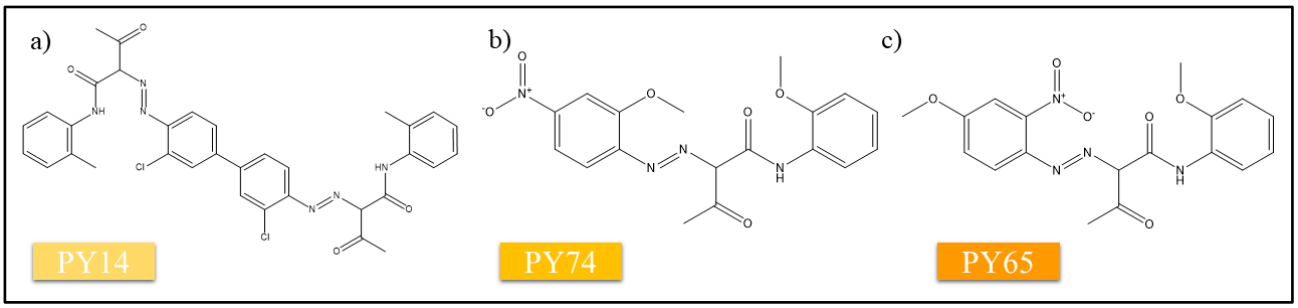



**Figure 4.1: A schematic representation of the chemical structures of a) PY14, b) PY74, and c) PY65**

### 4.2.3 Instrumental analysis

Scanning electron microscopy (SEM) was undertaken with a FEI F50 inspect system equipped with an Octane Pro energy dispersive X-ray (EDX) detection system. Pigment samples were prepared by directly spreading pigment powder onto sticky carbon tabs. The working distance was 10 mm, and the acceleration voltage was 10 kV. PY14, PY74, GR, and GY inks were coated with platinum with a thickness of about 2 nm to increase their electrical conductivity.

Headspace gas chromatography mass spectroscopy (GC-MS) analysis was carried out using an Agilent Technologies 7890A GC system with a 5975C inert XL EI/CI MSD Triple Axis Detector and a 7693 sampler. The apparatus was fitted with an Agilent Technologies HP-5MS 5% Phenyl Methyl Siloxane column (29.4 m x 250 µm x 0.25 µm) with a $He_2$ mobile phase at a flow rate of 3.5 mL/min. The headspace injection volume was 5 µL. The GC was operated in isocratic mode with an oven temperature of 40 °C and an inlet temperature of 180 °C throughout the analysis. The MS ion source and quadrupole temperatures were set at 230 °C and 150 °C, respectively. All m/z values between 40 and 500 were taken in scan mode. Using the NIST database and published references [4, 26-29], the presence of volatile hazardous substances in irradiated pigments and inks or eluted compounds were determined. The Kovats technique was used to assess the retention index (RI) of the organic volatile chemicals [30-33]. This was done by analysing the headspace of standard alkanes (C5-C9) using GC-MS.

Dynamic light scattering (DLS) measurements were achieved using a Malvern Nano Zeta Sizer apparatus equipped with a 5 mW HeNe laser, a Peltier temperature control system. Backscattering detection at an angle of 173° was used to determine the hydrodynamic size and size distribution of pigments and inks samples. This configuration is less vulnerable to multiple scattering effects and dust than the 90° geometry. Measurements were taken at 20 °C and were repeated three times. Before DLS measurements, all dispersions were prepared by dilution of the pigments with deionized water or methanol at a ratio of 0.1 mg pigment to 1 mL of solvent, followed by 30 minutes of sonication at 40 kHz.

## 4.3 Results and discussion

Pigments, inks, melanin, and melanin-ink/pigment mixtures were irradiated with a 532 nm QS Nd:YAG laser as outlined in section 4.2.2. Mixtures and controls were analysed using SEM, DLS, and GC-MS to determine whether the presence of melanin led to changes in the morphology and chemical composition of the mixtures. SEM and DLS were used to investigate the effect of laser irradiation on particle shape and size while headspace GC-MS was employed to identify any volatile fragments. Results presented in this manuscript focus on PY14, EM, SM, BO and GR. Additional tests were conducted on PY65, PY74, LY, and GY inks, and the corresponding results are provided in the supporting information. All pigments behaved similarly, as did the inks.

### 4.3.1 SEM

Melanin, pigment, ink and melanin-ink/pigment mixtures were analysed by SEM pre-and-post laser irradiation to examine morphological changes resulting from the irradiation process. Fig. 4.2 to 4.5

show the SEM images of EM, SM, PY14, GR, and BO. The SEM images of PY74, PY65, LY, and GY are presented in (Fig. SI 4.2 to SI 4.6).

As shown in Fig. 4.2a-f, laser irradiation induced a noticeable variation in the surface morphology of irradiated melanin when compared to the unirradiated samples. Unirradiated EM exhibited aggregates with smooth spherical granules ranging from 100 to 200 nm in diameter, consistent with previous studies [34, 35]. Following irradiation, the EM cluster had an irregular surface, indicating the photoactivity of melanin towards laser light (Fig. 4.2a-c). Similarly, changes upon irradiation were observed for SM. Irradiation caused the SM to develop a structure resembling smashed ice, contrasting with the pre-irradiation shape of SM particles which was spherical shape (Fig. 4.2d-f). These results contrast with previous studies reporting that a 755 nm laser does not affect the morphology of melanin pigment. and instead acts as a heat source in picosecond laser treatments [36]. Our study used short pulses of laser light at a wavelength of 532 nm rather than 755 nm and it is clear that the absorption of 532 nm light by melanin particles results in changes in their morphologies as observed in the SEM images.

Irradiation of PY14 resulted in melting and agglomeration of PY14 particles, primarily on the top layer of the surface (Fig. 4.3 a and d). This is likely due to the limited penetration of the laser beam in this area. To target deeper pigment deposits, additional treatment sessions or higher fluences would be required, similar to what was used in our previous research where increasing fluences from 4.1 to 5 J/cm$^2$ resulted in reaching deeper layers (Fig. SI 4.2) [37]. SEM images of irradiated PY14-EM and PY14-SM (Fig. 4.3e and f) reveal that laser irradiation of these mixtures resulted in amorphous aggregates of pigment and melanin particles, in contrast to clearly differentiable pigment and melanin deposits in the unirradiated mixtures (Fig. 4.3b and c). This phenomenon is consistent with observations of laser interactions with ink dispersions [26, 27]. Melanin pigment conglomerated on the PY14, resulting in the formation of large clusters, whereas irradiated PY14 without melanin exhibited smoothness from melting pigment particles on its surface. Similar results were observed for the irradiated mixture of melanin pigments with PY74 and PY65 (Fig. SI 4.3 and 4)

Dried GR and BO inks both had relatively smooth surfaces prior to irradiation (Fig. 4.4a and 4.5a) which appeared to melt and form craters upon irradiation (Fig. 4.4d and 4.5d), likely representative of the melting of PY14 particles that used to make these tattoo inks. In contrast SEM images of irradiated ink-melanin mixtures did not exhibit significant appearance of melting (Fig. 4.4e, f, 4.5e, and f). Instead, they showed numerous smaller melanin pigment particles were dispersed or adhered to the ink surface. These melanin particles were significantly smaller and more dispersed than those observed prior to irradiation (Fig. 4.4b, c, 4.5b, and c). It seems that the presence of melanin led to

an alteration in how the laser interacted with ink particles, reducing the breakdown of the inks. Similarly, SEM images of irradiated GY and LY inks supported the hypothesis regarding melanin's ability to absorb laser light since there was less effect observed on these ink surfaces by the laser beam (Fig. SI 4.5, and 6)

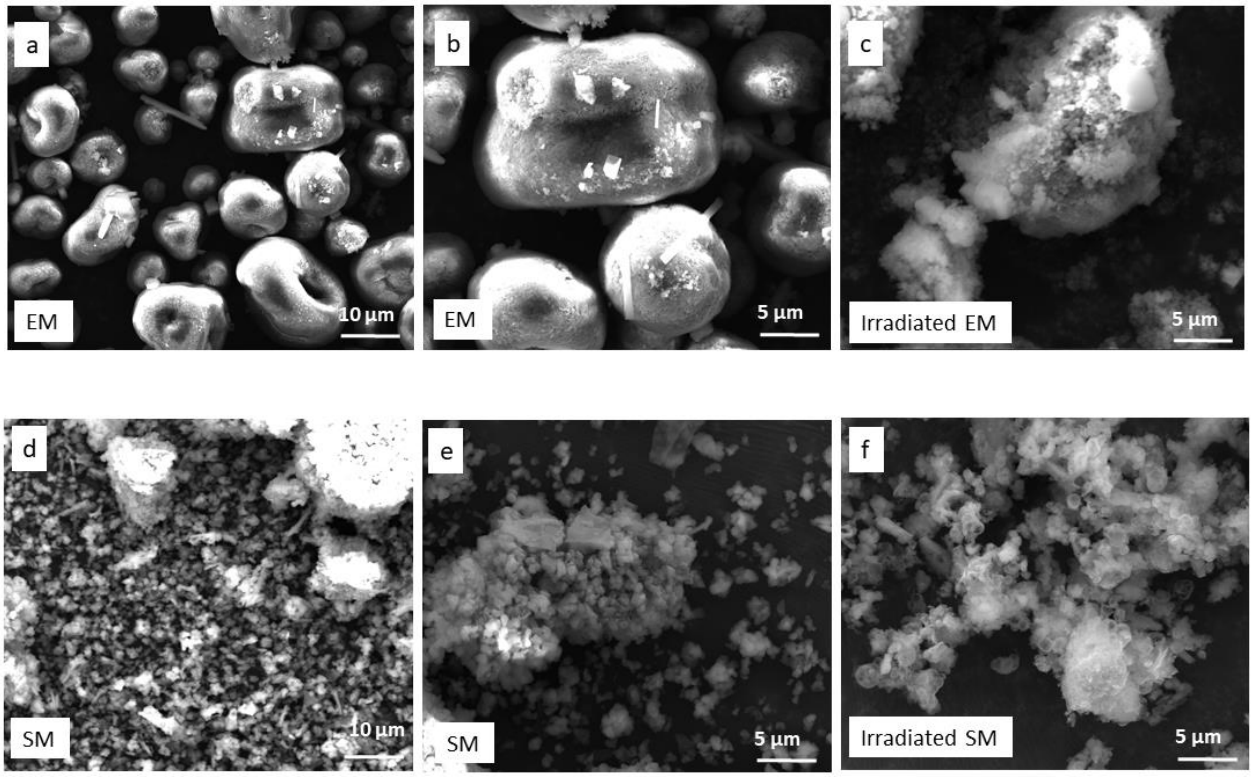
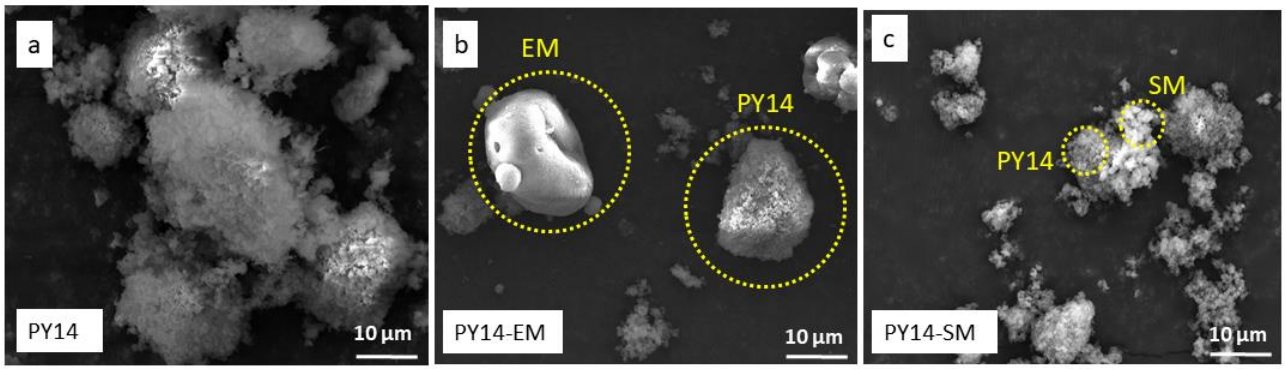



**Figure 4.2: SEM images of unirradiated and irradiated extracted and synthetic melanin samples.**



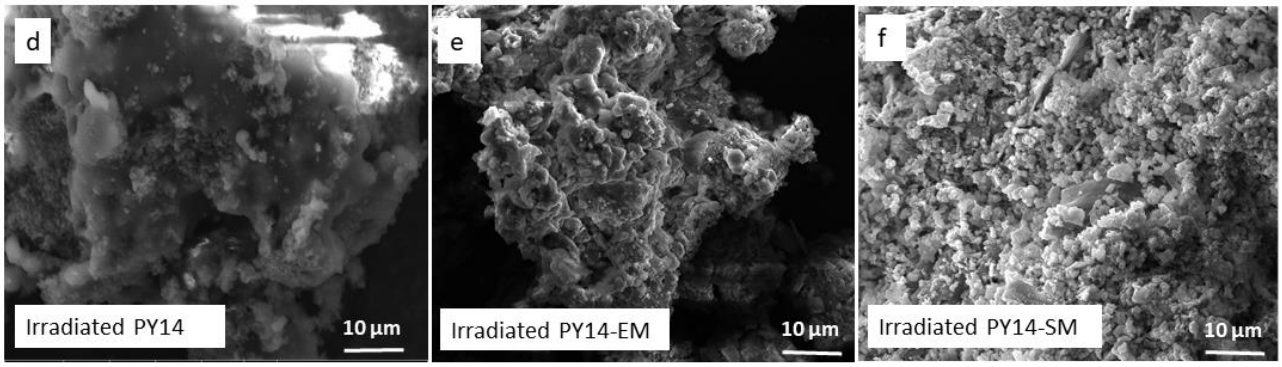



**Figure 4.3: SEM images of unirradiated and irradiated PY14 samples and PY14-Melanin mixtures.**

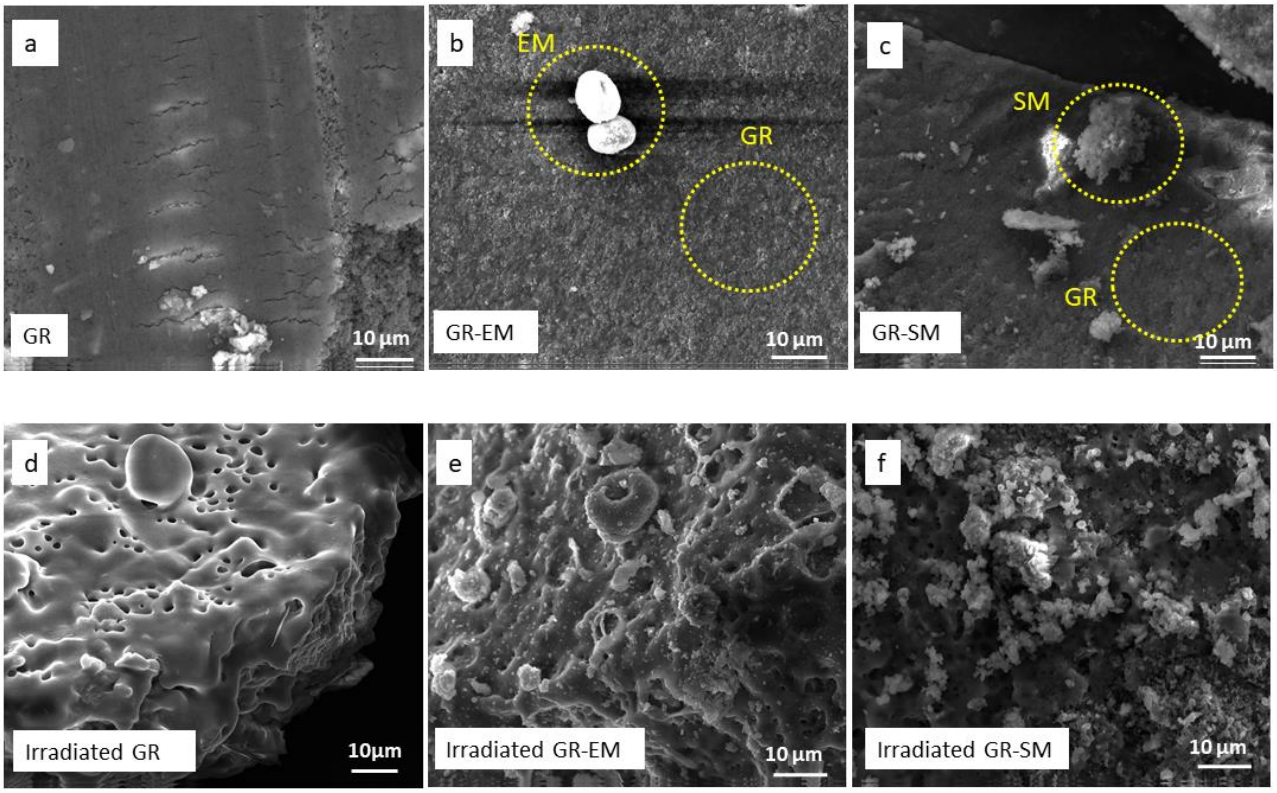
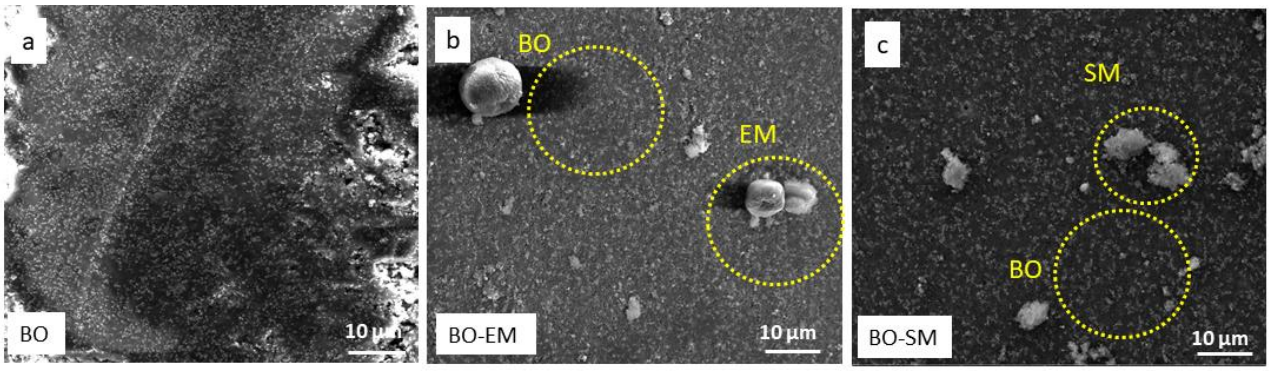



**Figure 4.4: SEM images of unirradiated and irradiated GR samples and GR-Melanin mixtures.**



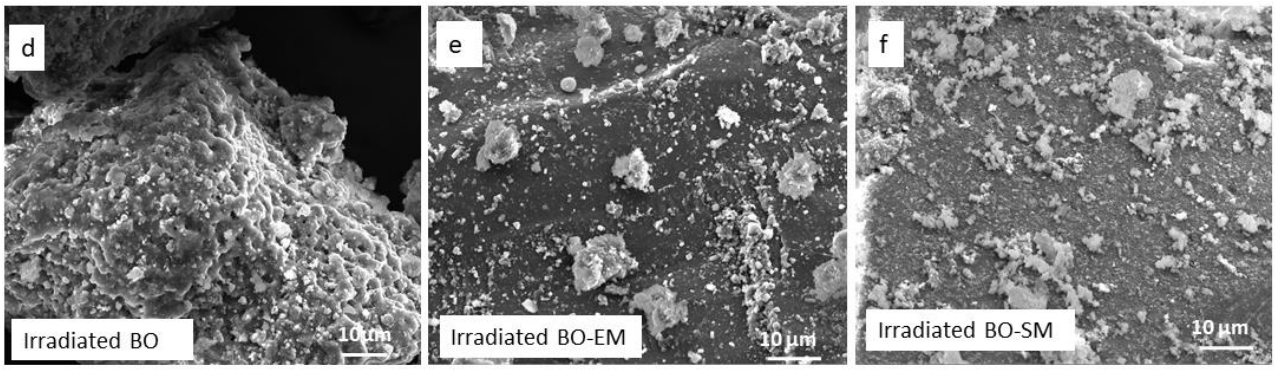



**Figure 4.5: SEM images of unirradiated and irradiated BO samples and BO-Melanin mixtures.**

### 4.3.2 GC-MS

Headspace GC-MS analyses were employed to determine the volatile degradation products formed upon the laser irradiation of reference pigments, inks, and melanin-ink/pigment mixtures. Since SEM data shows that melanin pigments absorbed 532 nm laser wavelength, the GC-MS analysis aimed to investigate potential variations in the volatile degradation patterns of tattoo inks and pigments when exposed to laser treatments, particularly in the presence of melanin.

The GC-MS chromatograms of irradiated EM, SM, PY14, PY14-EM, and PY14-SM are presented in Fig. 4.6a. GC-MS analysis of irradiated PY65 and PY74 are included in the supplementary information (Fig. SI 4.7). The GC-MS results for tattoo inks and ink-melanin mixtures post-irradiation (GR and BO) are shown in Fig. 4.6b and c, with LY and GY data in Fig. SI 4.8.The GC-MS chromatograms for the irradiated empty vial, unirradiated melanin, unirradiated PY14-melanin mixtures, unirradiated inks-melanin mixtures showed no peaks, indicating that the volatile compounds were produced as a result of the irradiation process (Fig. SI 4.9).

The list of fragmentation products from the irradiated samples is shown in Table 4.1, which also includes the retention time and primary mass losses for each component. The major photodegradation products that were produced from all irradiated samples (excepting irradiated SM) were 1,3-butadiyne, benzene, and toluene. Furthermore, chloromethane was detected as a degradation product of EM, while 2-propenenitrile was produced from EM, PY65, PY74, and LY. In contrast, 1-methoxybut-2-yne was formed from PY14. The formation of 2-propenoic acid-ethyl ester, methyl methacrylate, and styrene as additional volatile chemicals was from irradiation of dried tattoo inks.

The detection of benzene, toluene, and styrene aligns with other investigations on the laser exposure of suspensions including pigment blue 15, pigment green 7 and 36, pigment yellow 138, pigment orange 13, pigment violet 19, and pigments red 170 and 245 [4, 27-29, 38, 39]. Chloromethane (observed in

irradiated EM) and methyl methacrylate (observed in irradiated tattoo inks) have not been reported in prior studies that examine the laser irradiation of tattoo inks and pigments. The presence of chloromethane in the irradiated EM sample was unexpected. It was not observed in the unirradiated EM, and melanin does not contain chlorine atoms. We hypothesise that this may result from a small quantity of solvent residues remaining within the extracted melanin powder. Methyl methacrylate was observed in three of the tattoo inks, and one tattoo ink-melanin mixture. This chemical was not listed as an ingredient in the tattoo inks, however this has been reported by others as a binding agent in tattoo ink [28, 40]. Chloromethane and methyl methacrylate were also identified from the pyrolysis GC-MS of Faber-Castell felt-tip pen (AFC136) inks that contain violet dioxazine pigment and pigment red 9 (PR9) [41, 42].

2-Propenenitrile was only observed in irradiated PY65, PY74, and LY ink, that did not contain melanin. It has not been previously reported as a product of laser irradiation of pigments nor in yellow tattoo inks. However, this compound was reported to be formed by thermal chain scissions of the binders, solvents, and additives of Edding felt-tip pen inks [41]. To our knowledge, the formation of the remaining compounds (1,3-butadiyne, 2-propenoic acid-ethyl ester, and 1-methoxybut-2-yne) from the irradiated pigments and inks has not previously been stated.

An overall decrease in the peak intensity of the volatile fragments of the GC-MS chromatograms shows the effect of melanin's capacity to absorb 532 nm laser light and the formation of volatile fragments upon irradiation of yellow pigments and tattoo inks [17, 24]. The low number and intensity of volatile fragments from irradiated melanin-ink/pigment mixtures observed in GC-MS was expected given that the SEM images confirmed that EM and SM absorb laser light which result in the alteration on their morphology.

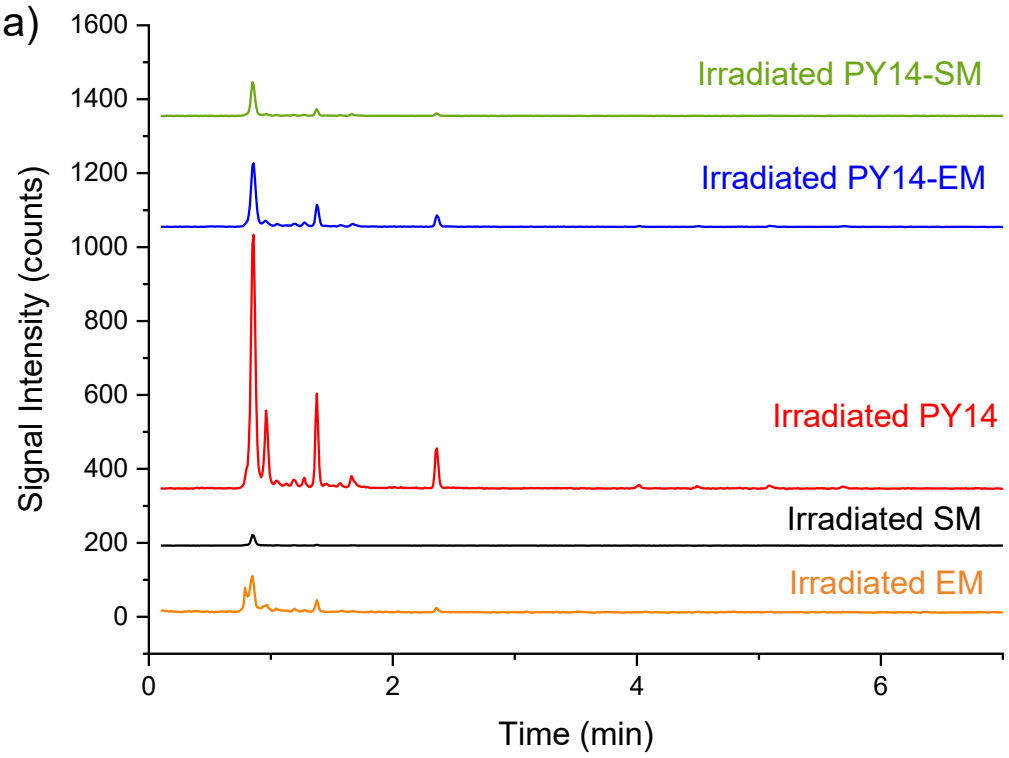
Irradiation of EM and SM with a 532 nm laser revealed distinct interactions with the laser light. The GC chromatogram of irradiated EM displayed three major peaks at low retention times, corresponding to the presence of chloromethane, benzene, and toluene. In contrast, these compounds were absent in the GC chromatogram of irradiated SM. This difference may be attributed to the thermal stability of these melanin pigments. EM appears to be less thermally stable than SM, leading to its breakdown into multiple volatile fragments. Supporting this observation, Pralea *et al*. (2019) demonstrated through thermogravimetric analysis (TGA) that synthetic melanin exhibits higher thermal resistance at elevated temperatures compared to natural melanin. This finding may explain the differing responses to laser irradiation between extracted and synthesized melanin pigments [25, 34].

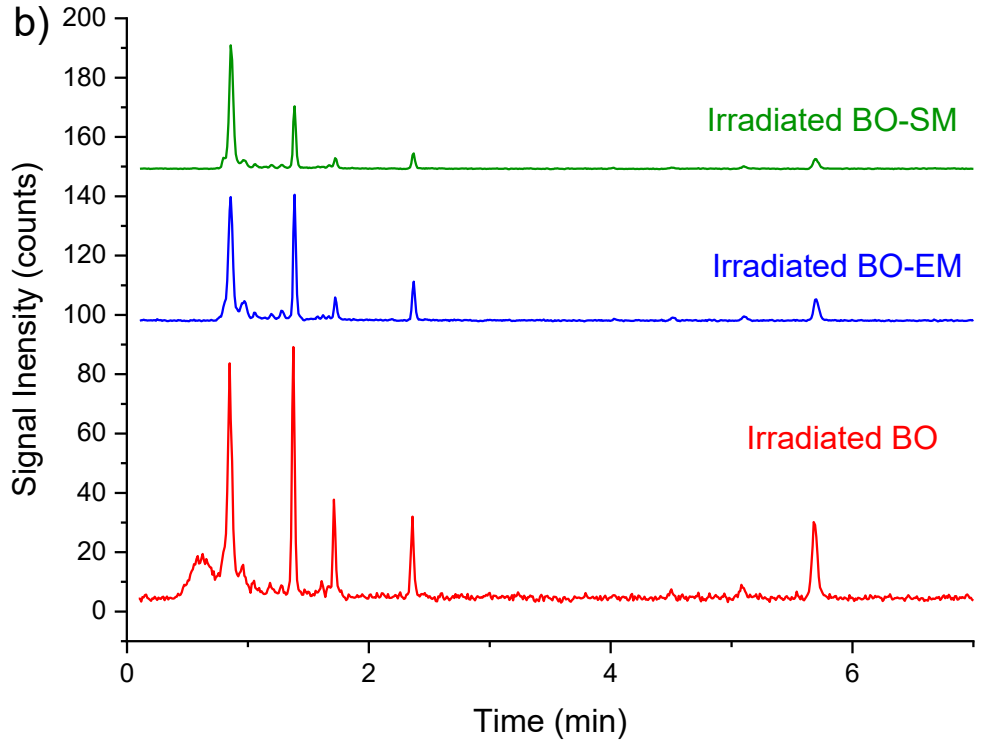
The GC chromatogram of irradiated PY14 revealed volatile degradation products that are associated with the presence of 1,3-butadiyne, 1-methoxybut-2-yne, benzene, and toluene. These fragments were detected in the absence of melanin pigments. Nevertheless, the presence of melanin resulted in a significant decrease in the peak intensity associated with fragments released from irradiated PY14-EM and PY14-SM. Furthermore, the peaks detected at 0.962 and 2.412 min vanished entirely, suggesting the inhibition of PY14 degradation.

The GC-MS analysis of irradiated PY74 and PY65 mixtures with melanin pigments yielded findings similar to those obtained with PY14 (Fig. SI 4.7). Notably, the presence of melanin pigments resulted in a considerable drop in the peak intensity of fragmented products, showing that breakdown of PY65 and PY74 was hindered.

Similarly, the presence of melanin pigments significantly reduced the peak intensity of volatile products resulting from the laser degradation of dried tattoo inks, as observed with the yellow pigments. The interaction between laser light and tattoo inks, on the other hand, varied depending on the variety of their composition. For example, GR, GY, and BO inks all contain PY14, but vary in the quantity of $TiO_2$ present. This variation along with the presence of melanin pigments had an effect on the amount of volatile fragmentation generated after laser irradiation of inks (Fig. 4.6b and SI 4.8) [37]. Furthermore, the composition of LY ink differs from that of the previously stated inks, as it also contains PB15, resulting in a distinct GC chromatogram (Fig. SI 4.8). For example, the peak intensity of 1,3-butadiyne that eluted at 0.849 min from irradiated LY inks was increased in the presence of EM and SM. This might be due to the PB15 which absorbs the 532 nm laser wavelength and degrade to form 1,3-butadiyne (Fig. 4.7).

Volatile chemicals were detected upon laser irradiation of reference pigments or inks, indicating that the presence of melanin in the mixtures did not result in the formation of new volatile compounds under the experimental conditions. However, melanin's strong broadband absorption significantly reduced the intensity of incident light that reaches the yellow pigment, thereby restricting the pigments photodegradation and the formation of degradation products. Notably, some of the detected degradation products were unexpected based on the known chemical structures of the parent molecules. This indicates the involvement of secondary reaction pathways, perhaps resulting from interactions among volatile intermediates produced during the irradiation process.


a)
Signal Intensity (counts)
1600
1400
1200
1000
800
600
400
200
0
Irradiated PY14-SM
Irradiated PY14-EM
Irradiated PY14
Irradiated SM
Irradiated EM
0
2
4
6
Time (min)


b)
Signal Inensity (counts)
200
180
160
140
120
100
80
60
40
20
0
Irradiated BO-SM
Irradiated BO-EM
Irradiated BO
0
2
4
6
Time (min)

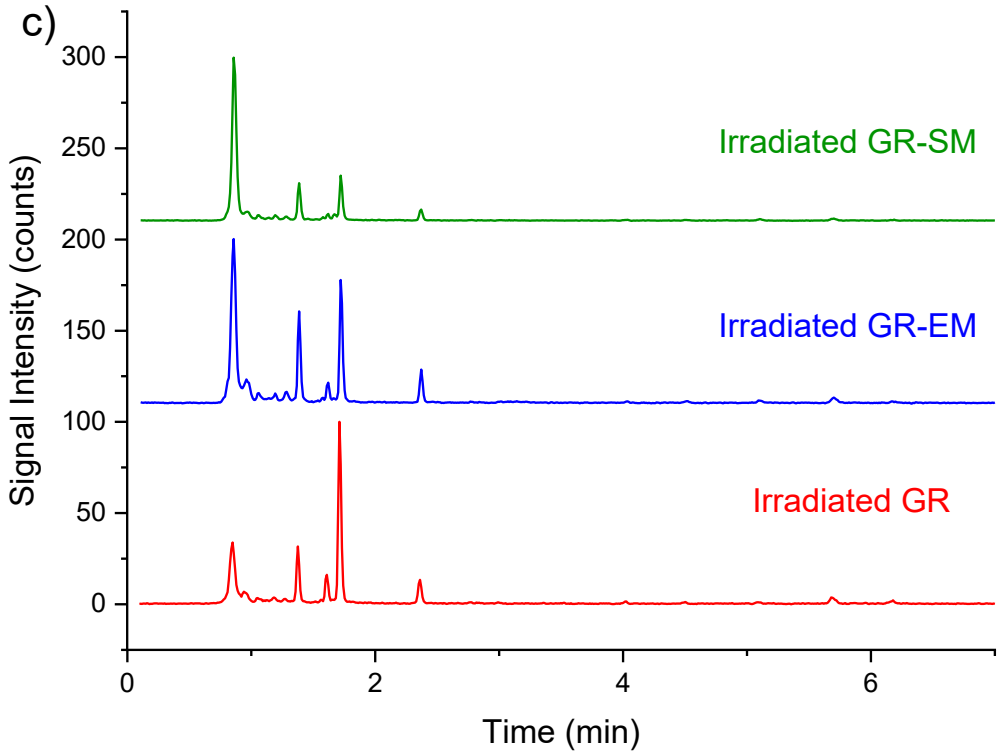



**Figure 4.6: GC-MS analysis of irradiated a) PY14, b) BO, and c) GR with and without melanin pigments.**

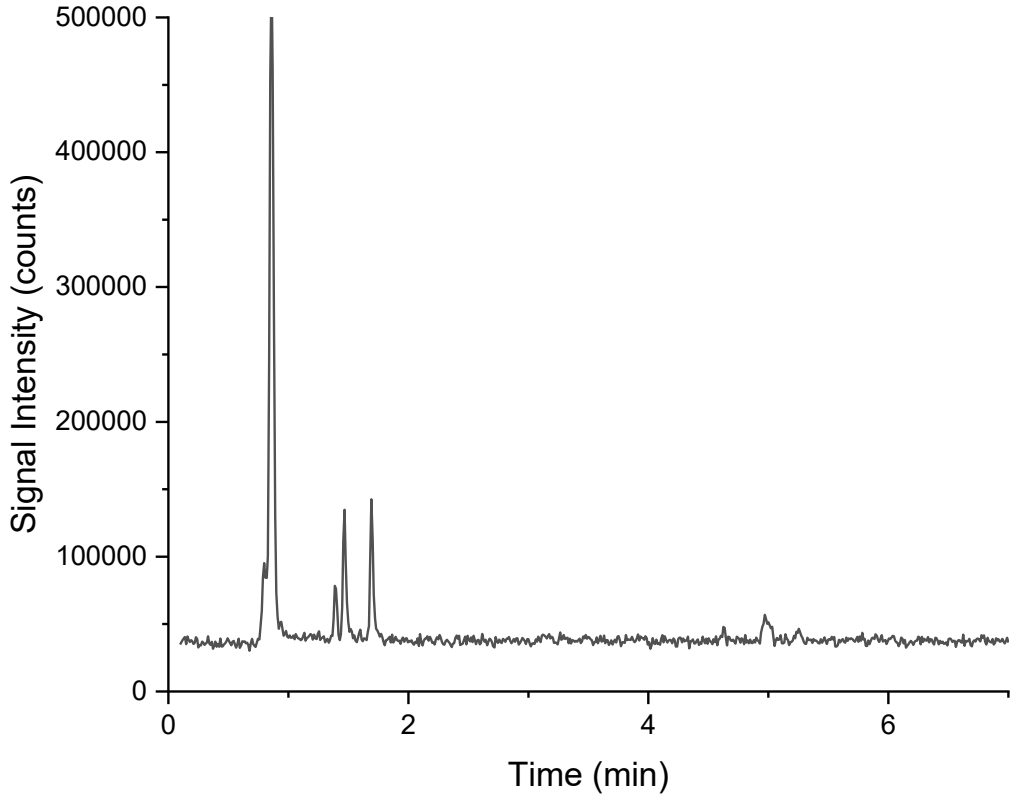



**Figure 4.7: GC-MS analysis of irradiated PB15.**

**Table 4.1: The volatile fragmentation products released during laser irradiation of pigments, inks, as well as the retention times, the retention index, and major mass losses.**

| Retention time (min) | Compound | Main (m/z) | Observed in | | | | | | | | | | | | | | | Retention index |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Non-mixed with melanin | | | | | | | | | Mixed with melanin | | | | | | |
| | | | EM | SM | PY14 | PY74 | PY65 | GR | BO | LY | GY | PY14 | PY74 | PY65 | GR | BO | LY | GY |
| **0.849** | 1,3-butadiyne | 50, 49, 48 | - | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | - |
| **0.809** | chloromethane | 50 | ✓ | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| **0.953** | 2-propenenitrile | 53 | ✓ | - | - | ✓ | ✓ | - | - | ✓ | - | - | - | - | - | - | - | - | - |
| **0.971** | 1-methoxybut-2-yne | 69, 54 | - | - | ✓ | - | - | - | - | - | - | - | - | - | - | - | - | - | 542.04 |
| **1.397** | benzene | 78, 50 | ✓ | | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | 667.25 |
| **1.635** | 2-propenoic acid-ethyl ester | 99, 55 | - | - | - | - | - | ✓ | - | | ✓ | - | - | - | - | - | - | ✓ | 768.33 |

| | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **1.671** | *N*-2-hydroxyethyl urea | 74 | - | - | - | - | - | - | - | - | - | ✓ | - | - | - | - | | | 776.37 |
| **1.702** | *N, N* dimethyl-1,2 bis(aminooxy)etha ne | 74 | - | - | - | - | - | - | - | ✓ | - | - | - | - | - | - | ✓ | - | - |
| **1.722** | methyl methacrylate | 100, 69 | - | - | - | - | - | ✓ | ✓ | - | ✓ | - | - | - | - | - | ✓ | | - |
| **2.412** | toluene | 91, 65, 51 | ✓ | - | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | - | - | ✓ | ✓ | ✓ | ✓ | 770.86 |
| **5.701** | styrene | 104, 78, 51 | - | - | - | - | - | ✓ | ✓ | ✓ | ✓ | - | - | - | - | ✓ | ✓ | - | 891.27 |

### 4.3.3 DLS

In comparison to GC-MS and SEM images, DLS measurements offer additional insights into fragment dimensions and the average size of clusters suspended in methanol post laser treatment. It must be noted that DLS measures particles in suspension, and aggregates may disaggregate in this technique. Thus, the particle sizes measured using DLS may not reflect the SEM results. SEM analyses are performed on dried samples, and the behaviour of the particles in solution may differ, i.e., the removal of solvent may increase aggregation and agglomeration in the original inks [43]. Despite this limitation, DLS provides useful information about the effect of the laser on the particle size and distribution.

DLS analysis of EM and SM before and after laser irradiation reveals varying effects on particle size due to laser light exposure, with EM showing a slight increase from 155±32 nm to 228±33 nm (Fig. 4.8a), while SM remained unaffected (Fig. 4.8b). The particle size of PY14 decreased from 705±39 nm to approximately 344.9±10 nm (Fig. 4.9a).

However, DLS analysis of irradiated PY14-EM indicates a slight decrease in particle size from 460±45 to 325±28 nm, whereas PY14-SM shows a significant increase post-laser treatment from 459±39 to754 ±100 nm (Fig. 4.9b and c). This discrepancy may be attributed to the smaller particle size and lower thermal stability of EM compared to SM; where the lower thermal stability suggests that EM readily absorbs laser light, breaking down into smaller particles forming conglomerates with pigments and tattoo inks on a smaller scale. Conversely, the higher thermal stability exhibited by SM reduces the impact of lasers resulting in larger particles sizes observed for PY14-SM. A similar trend was also observed for PY74 and PY65-melanin mixtures (Fig. SI 4.10).

It is important to note that unirradiated and irradiated EM has a smaller particle size compared to SM, which suggests that SM may have a greater impact in reducing the amount of laser light required to irradiate pigments and inks. This observation aligns with the GC-MS results, where the presence of SM with PY14 and dried inks was associated with a decrease in the formation of volatile fragments, as indicated by the detection of lower peak intensity (Fig. 4.6a).

DLS analysis further indicates that the particle sizes of BO and GR inks increased post laser irradiation (Fig. 4.10a and d), potentially influenced by other ink components. However, this increase was not observed in the presence of EM (Fig. 4.10b and e), likely due to different interactions between ink content and laser light, resulting in reduced particle size but increased nanoparticle agglomeration.

The DLS analysis of irradiated BO-SM and GR-SM (Fig. 4.6c and f) demonstrates a significant rise in particle size attributed to large SM particles' size along with ink particle agglomeration. SM notably impacted PY14 and BO, leading to the largest particles among all irradiated samples.

The DLS findings supported by GC-MS and SEM data suggest melanin's critical role in limiting pigment and ink fragmentation by lasers, resulting in larger clusters formation.

### 4.3.4 Implications of laser tattoo removal on dark skin

These results underscore the challenge posed when removing tattoo pigments from dark skin, which may necessitate additional laser treatment sessions or different treatment conditions for complete removal. It should be noted that laser removal works by breaking down pigments into smaller particles and molecular fragments, which are then transported away from the skin by phagocytosis [44]. Phagocytosis is a mechanism that requires actin polymerisation to take particles larger than 0.5 μm into cells [45, 46]. The target aspects impacting the physical process of phagocytosis that have already been discovered is the morphological characteristics such as shape and dimension or aspect ratio [47]. Target size is considered as a significant factor in determining uptake. Maximum target internalisation takes place in the 1-3 μm size range [48]. Therefore, the DLS analysis indicated that the particle size of irradiated samples is within the range at which phagocytes can engulf these materials, but these measured sizes could be due to the dispersal of agglomerates in the DLS solution, whereas the SEM image shows a larger cluster dimension of irradiated pigments and inks. As a result, the phagocytosis process might be affected by the geometric properties of irradiated pigments and inks with varying morphologies and dimensions. Hence, this could clarify the difficulties of removing tattoo inks from dark skin. The findings demonstrate that treatment of tattooed dark skin using a 532 nm laser wavelength is ineffective and results in poor removal efficacy.

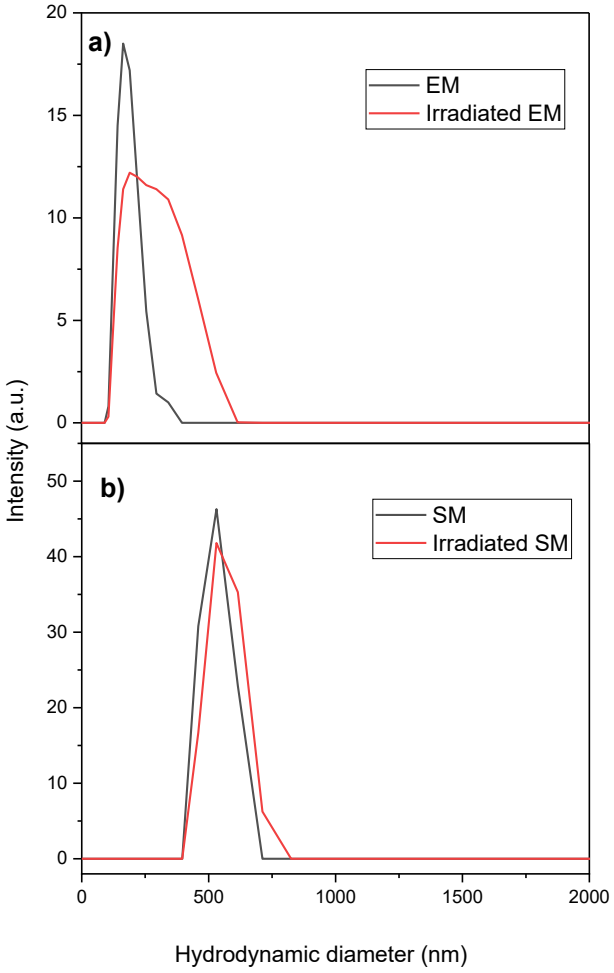



**Figure 4.8: DLS analysis of unirradiated and irradiated a) EM pigment and b) SM pigment.**

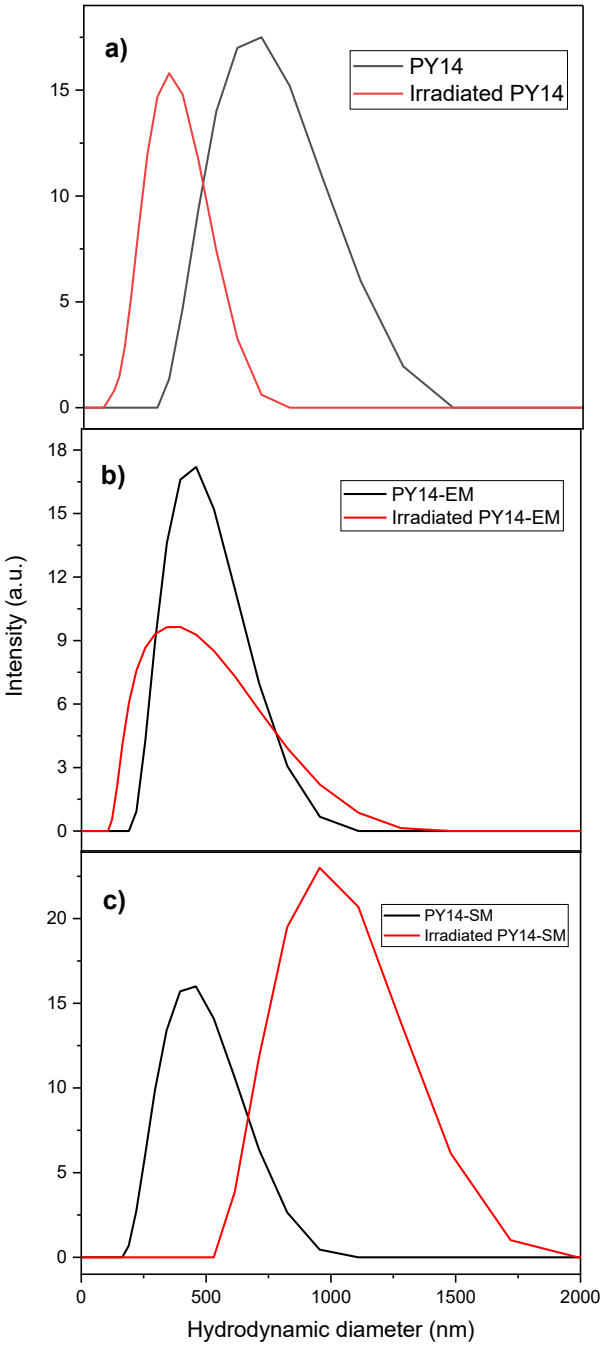



**Figure 4.9: DLS analysis of unirradiated and irradiated a) PY14, b) PY14-EM, and c) PY14-SM.**

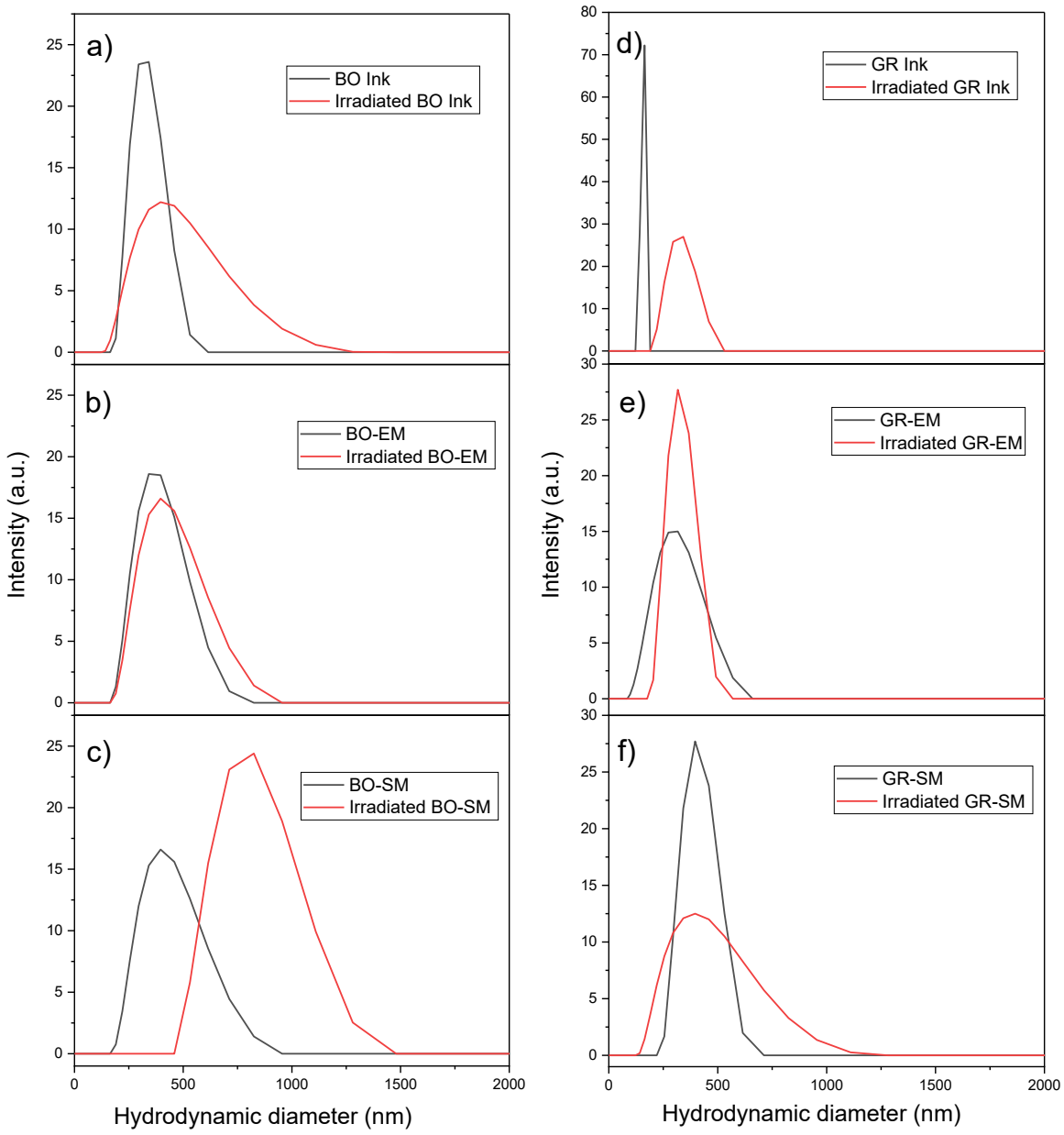



**Figure 4.10: DLS analysis of unirradiated and irradiated a) BO, b) BO-EM, c) BO-SM, d) GR, e) GR-EM, and f) GR-SM.**

## 4.4 Conclusion

In conclusion, this study investigated the response of melanin pigments to 532 nm laser irradiation, a process commonly used in the removal of yellow tattoos. The findings highlight the crucial role of melanin in absorbing 532 nm laser light during the irradiation of yellow pigments and tattoo inks. The observed reduction in peak intensity in the GC-MS chromatograms, along with the presence of numerous agglomerations in SEM images and larger particle sizes in DLS, are indicative of melanin's ability to hinder the breakdown of pigments and inks. These results suggest that melanin pigments significantly influence the behaviour of tattoo inks and pigments under laser treatment, providing insight into why certain tattoo colours are more challenging to remove from darker skin.

## 4.5 Acknowledgements

B.A. extends deepest gratitude to the following organizations for their invaluable support and contributions to this research: King Faisal University, Ministry of Education, Saudi Arabia, for their generous support and encouragement throughout the duration of this study. The authors acknowledge the facilities and technical assistance of Microscopy Australia, a company established under the National Collaborative Research Infrastructure Strategy, through the South Australian Regional Facility, Flinders Microscopy and Microanalysis (FMMA), Flinders University. We also acknowledge the support of the College of Science and Engineering, Flinders University for access to the DLS and GC-MS instrumentation.

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# 5 CHAPTER 5: CHEMICAL AND CYTOTOXIC PROFILING OF TATTOO INK DEGRADATION PRODUCTS GENERATED BY LASER IRRADIATION

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**This chapter was submitted to Journal of Exposure science & Environmental Epidemiology.**

The only alterations are that some figures from supplementary information have been moved to this chapter. Supplementary information for this chapter has been shown in the supplementary section (**Appendix 4**).

## Author contribution statement:

Batool Aljubran carried out the experimental work (cells culture, cells count, cells seeding, MTT assay), and the data analysis for gas chromatography-mass spectrometry (GC-MS) and ultra-violate-visible spectroscopy (UV-Vis), Batool Aljubran also wrote the manuscript with support from Claire Lenehan, Liu-Fei Tan, Ula Alexander, and Kirstin Ross, who all contributed to supervising the project. All of the authors contributed to experimental design and ideation and approved the final version of the manuscript.

**Building on the chemical characterization of laser-induced degradation products, this chapter assesses their potential biological impact to determine whether these compounds pose risks to human skin cells. Chapter 5 investigates the cytotoxic effects of both irradiated and unirradiated inks and pigments on HaCaT skin cells.**

## Abstract:

Tattooing has grown significantly in popularity, leading to increased demand for laser-based tattoo removal. However, limited studies have examined the chemical toxicity of tattoo inks, and their degradation products formed during laser irradiation. This study investigates the cytotoxic and chemical profiles of commonly used tattoo inks (PY14, PY65, GR, BO) before and after exposure to a Q-switched Nd:YAG laser. The toxicological profiles of both unirradiated and irradiated pigments/inks were assessed using the HaCaT keratinocyte cell line. GC-MS and UV-Vis spectroscopy were used to characterise pigments, inks, and their laser-induced degradation products. Irradiated pigments and inks showed increased cytotoxicity compared to unirradiated samples. The MTT assay demonstrated a dose-dependent decrease in cell viability. Unirradiated PY14, PY65, GR, and BO reduced cell viability to around 50%, while irradiated forms caused significant cytotoxicity, with 0% cell viability observed at higher concentrations. GC-MS analysis showed that *o*-toluidine, 2-methoxyphenyl isocyanate, and *o*-toluene isocyanate were predominant laser degradation products. These compounds exhibited considerable toxicity at concentrations over 10 µg/mL. The results highlight potential health risks associated with laser tattoo removal, as laser irradiation of tattoo inks, particularly at higher concentrations, significantly increases cytotoxicity. This may pose risks to skin cells and overall tissue health, emphasizing the need for further evaluation of laser tattoo removal safety.

## 5.1 Introduction

Tattoos are popular among people of all ages, genders and socioeconomic backgrounds [1] with reported prevalence rates ranging from 11.7% to 31.5% in industrialized countries [2]. Tattooing is the permanent application of coloured chemicals under the skin using a solid needle, resulting in the colouring of the skin [3, 4]. Tattoo inks consist of pigments and additional components such as carriers (water, glycerine, and other alcoholic derivatives) and additives (surfactants, polycyclic aromatic hydrocarbons, nanoparticles, and polymers) [3, 5, 6]. It is estimated that, during the tattooing process, large quantities of tattoo ink are deposited in the dermis, ranging between 0.60 and 9.42 mg/cm$^2$ [3, 6, 7].

Considering the growing popularity of tattooing, tattoo inks have been poorly evaluated for human use, as the inks are generally developed for use in printing and painting [8, 9]. It has been reported that tattoo inks have the potential for adverse health effects. For example, they can undergo photochemical degradation into carcinogens and allergens, resulting in health issues and allergic responses, as indicated by clinical and toxicological studies [2, 7, 10]. A limited number of studies have been conducted to evaluate the potential health effects of widely used tattoo inks [11, 12]. Results from these studies show that many harmful compounds included in tattoo inks and their metabolic products might constitute a threat to health, not just for the skin but also for other organs [13]. For instance, black and red tattoo inks have been detected in the liver, indicating that the tattoo pigment was transported from the inked skin via the bloodstream to this key organ of detoxification [14]. Tattoo inks may potentially reach excretion organs via the liver or kidney, and pigment particles from tattoo suspensions have been detected in Kupffer cells [13]. Furthermore, tattoo inks have been shown to have detrimental effects on humans, resulting in skin problems [15], bacterial and viral infections, and eczematous skin reactions [16-18]. Joey *et al.* (2021) found that injecting tattoo inks into rebuilt human skin results in different levels of decreased metabolic activity and histological cytotoxicity [8]. This study also shows that cytotoxicity of tattoo inks was detected as cellular swelling, dermal fibroblast karyolysis, epidermal loss of laminar organization, and karyorrhexis [8]. Inks can be associated with cytotoxicity and oxidative stress, which happen mostly when using inks containing azo pigments, such as red and yellow inks [1, 19].

With the rising number of people getting tattoos, a significant percentage of the population is currently seeking tattoo removal treatment. Tattoo removal can be driven by cultural factors to enhance self-perception or avoid social stigma, or merely that the individual no longer likes their tattoo [20, 21]. A survey conducted between 2017 and 2020 found that tattoo regrets were most prevalent among educated individuals in South America, with sixty percent of the patients requesting tattoo removal services [22]. In addition, fifty-eight percent of tattooed people in Saudi Arabia regretted their tattoo,

and 42.5% attempted to remove it [20]. This study also reported that tattoo regret was linked to symptoms such as localized itching, pain, and infection.

Tattoos are typically removed using laser techniques [23]. The basic concept of laser tattoo removal is selective photothermolysis, in which tattoo ink particles absorb laser light of a particular wavelength and are heated for a shorter period than their thermal relaxation time. Tattoo removal of black and blue tattoos often yields good outcomes, particularly in those with light skin tones. Removing yellow, red, and green tattoos has been shown to be more difficult and typically results in a significantly faded but still visibly present [24]. Laser irradiation fragments tattoo ink into smaller particles, which are engulfed by macrophages, [24-26] stored in lysosomes within skin cells, and cleared via the lymphatic system [27].

The removal of tattoos by laser has raised health concerns due to the creation of potentially hazardous products from both the pigment and the delivery matrix (the two components of tattoo ink) [23, 28]. Previous laboratory based studies have identified compounds such as benzene, toluene, hexachlorobenzene, aniline, and 3,3'-dichlorobenzidine as potential laser degradation products [29, 30]. Furthermore, it has been reported that laser therapy on tattooed skin has the potential to cause an immune system response to the foreign particles that are created, resulting in a range of immunological reactions such as hypersensitivity, allergy, and anaphylaxis [31].

There is limited knowledge of the effects of laser degradation products on skin cell viability, with only one study examining tattoo inks and degraded tattoo inks products effects on skin cells. The work conducted by Aubry *et. al.*[2] found that exposing PO13 to simulated sunlight resulted in the formation of 4((3,3'dichloro-[1,1'biphenyl]−4yl)diazenyl)−5methyl-2phenyl-2,4dihydro-3Hpyrazol-3one (DCBP). Subsequent cell exposure to both photoaged PO13 and DCBP caused a statistically significant decrease in HaCaT cell viability [2]. This study was limited to a single ink, and broad spectrum irradiation with simulated sunlight [2, 18]. In contrast, laser removal uses high-power pulses of discrete radiation, thus the fragmentation process, resulting degradation products and their concentrations may vary from those observed using solar irradiation. Consequently, this study aimed to perform a cytotoxicity assay of a selection of yellow pigments and tattoo inks, along with their laser-induced breakdown products on human skin cell lines, specifically HaCaT skin cells. MTT assays were conducted to evaluate the effects on HaCaT cell viability. Pigments and inks were irradiated and analysed using GC-MS and UV-Vis.

## 5.2 Materials and methods

RPMI-1640 media (R8758), fetal bovine serum (FBS) (SFBS-NZ), Penicillin-Streptomycin (P4333) MEM Non-essential amino acid solution (100×) (M127), and Trypan Blue (15250061) (0.4%) were purchased from Thermo Fisher Scientific. Phosphate buffered saline (PBS) (P3813), Dimethyl sulfoxide (DMSO) (D8418-) (99.7%), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (M2128), hydrogen peroxide ($H_2O_2$) (216763) (30% wt. in $H_2O$), and trypsin-EDTA solution (T4049) were purchased from Sigma-Aldrich (AU). Golden Rod (GR) and Bright Orange (BO) Intenze® brand inks were purchased from Tattoo Direct, Victoria, Australia. Pigment Yellow 14 (PY14) (C.I. 21095) (97%) and Pigment Yellow 65 (PY65) (C.I.11740) (98%) were purchased from AK Scientific, Union City, California. All chemicals were used without any further purification. 2-Methoxyphenyl isocyanate (547078) (98%), *o*-toluene isocyanate (T40703) (99%), and *o*-toluidine (185426) (99%) were purchased for Sigma-Aldrich.

The general method used in this research is presented in Fig. 5.1. The additives and (azo) pigments included in the analysed inks, together with hazard identification, are displayed in Table 5.1 [32-34] .

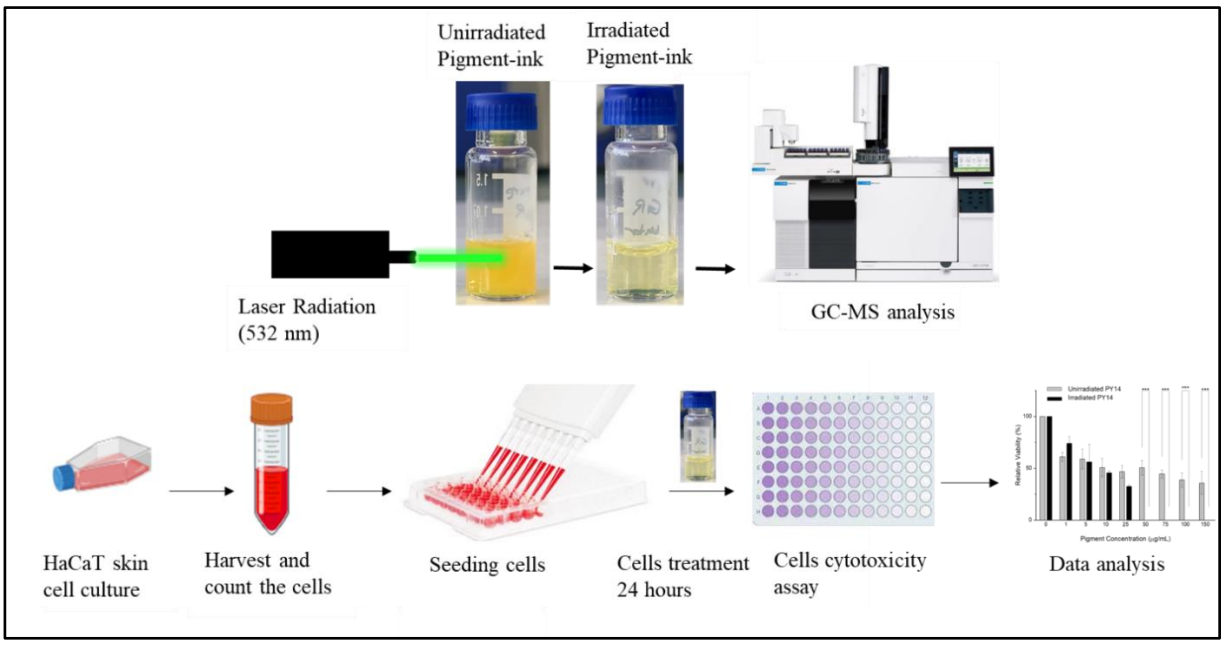


**Figure 5.1: General methodology used in this project (schematic experimental setup).**

**Table 5.1: Commercial tattoo inks and the risks associated with their ingredients.**

| Tattoo ink | Batch number | Chemical listed in ink | C.I.* no. | CAS* | Hazard identification [35] |
|---|---|---|---|---|---|
| **Intenze Golden Rod (GR)** | HCY020O87IMX40 | PY14 | 21095 | 5468-75-7 | Respiratory irritation.<br>Eye irritation.<br>Skin irritation.<br>Possible risks of irreversible effects.<br>May cause cancer.<br>Very toxic to aquatic life. |
| | | PO13 | 21110 | 3520-72-7 | Skin sensitizer.<br>Eye irritant.<br>Respiratory toxicity. |
| | | Glycerine | NA | 56–81-5 | NI |
| | | Hamamelis Virginiana extract | NA | 84 696–19-5 | NI |
| | | Isopropyl alcohol | NA | 67–63-0 | Eye irritant. |
| **Intenze Bright Orange (BO)** | HCY010W139O85IMX40 | PY14 | 21095 | 5468-75-7 | Respiratory irritation.<br>Eye irritation.<br>Skin irritation.<br>Possible risks of irreversible effects. |

| | | | |
|---|---|---|---|
| | | | May cause cancer.<br>Very toxic to aquatic life. |
| PO13 | 21110 | 3520-72-7 | Skin sensitizer.<br>Eye irritant.<br>Respiratory toxicity. |
| Glycerine | NA | 56–81-5 | NI |
| Hamamelis Virginiana extract | NA | 84 696–19-5 | NI |
| Isopropyl alcohol | | 67–63-0 | Eye irritant |
| $TiO_2$ | 77891 | 13 463–67-7 | Suspected carcinogen.<br>Eye irritant.<br>Organ damage upon prolonged/repeated exposure. |

*Abbreviations: CAS no = Chemical Abstracts Service number; C.I. = colour index; NA = not available; NI = none identified.

### 5.2.1 HaCaT cell culture

## Cell lines

The HaCaT cell line, derived from human non-cancerous keratinocytes, was provided by the Department of Medical Biotechnology, College of Medicine and Public Health, Flinders University,

Australia. These cells were cultured using RPMI medium with 10 % Fetal Bovine Serum, 1% non-essential amino acid, and 1% penicillin streptomycin. Cultures were initiated by seeding cells in tissue culture flasks and then maintained in a fully humidified environment at 37 °C with 5% $CO_2$. Subculturing was performed twice a week when the cell confluence reached 80-90 % or higher. The cell concentration and viability of the HaCaT cell culture intended for assay were assessed using a trypan blue staining method, as described below.

## Trypan blue assay

The trypan blue assay is the most frequent way to determine the viability of cells. Trypan blue is a crucial stain that stains nonviable cells (dead cells with a broken membrane), which take up the dye and give the cells a colour that is blue when seen under a microscope, whereas viable cells remain unstained (owing to intact cell membranes) [36].

To prepare the hemocytometer slide for cell counting, 10 µl of the cell suspension was diluted 1:1 with 10 µl of 0.2% trypan blue dye. The mixture was gently mixed, and 10 µl was placed into each chamber. Cells in the four corner squares were counted to calculate the concentration using the following formula:

$$Cell\ concentration\ \left(\frac{cells}{mL}\right) = Average\ number\ of\ cells\ \times DF \times 10^4$$

The average number of cells multiplied by the dilution factor (DF) x $10^4$ yielded the cell concentration per mL. In this experiment, the dilution factor was 2 (due to the 1:1 dilution with trypan blue), and the constant $10^4$ accounts for the volume of one square of the hemocytometer (0.0001 mL).

## Cell treatment

To determine the cytotoxicity of unirradiated and irradiated pigments (PY14, PY65) and tattoo inks (GR, BO) to HaCaT cells, a methyl tetrazolium (MTT) assay was performed. HaCaT cells were seeded into 96-well plates at a density of 1 x $10^4$ cells per well and incubated for 24 hours at 37 °C in 5 % $CO_2$ to allow for cell adhesion. Following incubation, the media was aspirated, and each well was supplemented with 200 µL of the treatment solution, consisting of tattoo ink suspensions diluted in fresh medium. Cells were incubated with the different concentrations of treatment solution of tattoo inks or pigments (ranging from 0.0001-500 µg/mL) for 24 h before initiating bioassays (Table 5.2). The treatment solution was then removed, and the cells were washed twice with 200 µL of PBS to eliminate any remaining treatment solution. Untreated cells served as the negative control (0 dose). All experiments were conducted in triplicate.

**Table 5.2: Preparation of treatment solutions at various concentrations for cell exposure experiments. Stocks were prepared by mixing the pigments or inks with deionized water (1mg/mL).**

| Final Conc. (µg/mL) | Final Conc. (mg/mL) | Stock Used | Stock Volume (µL) | Media Volume (µL) | Total Volume (mL) | Notes |
|---|---|---|---|---|---|---|
| **500** | 0.500 | 100 % stock | 1500 | 1500 | 3.0 | |
| **250** | 0.250 | 100 % stock | 750 | 2250 | 3.0 | |
| **200** | 0.200 | 100 % stock | 600 | 2400 | 3.0 | |
| **150** | 0.150 | 100 % stock | 450 | 2550 | 3.0 | |
| **100** | 0.100 | 100 % stock | 300 | 2700 | 3.0 | |
| **75** | 0.075 | 100 % stock | 225 | 2775 | 3.0 | |
| **50** | 0.050 | 100 % stock | 150 | 2850 | 3.0 | |
| **25** | 0.025 | 100 % stock | 75 | 2925 | 3.0 | |
| **10** | 0.010 | 100 % stock | 30 | 2970 | 3.0 | |
| **5** | 0.005 | 100 % stock | 15 | 2985 | 3.0 | |
| **1** | 0.001 | 1% stock (0.01 mg/mL) | 300 | 2700 | 3.0 | Prepared from 1% intermediate stock |
| **0.5** | 0.0005 | 1% stock | 150 | 2850 | 3.0 | Prepared from 1% intermediate stock |
| **0.1** | 0.0001 | 0.1% stock (0.001 mg/mL) | 300 | 2700 | 3.0 | Prepared from 0.1% intermediate stock |
| **0.05** | 0.00005 | 0.1% stock | 150 | 2850 | 3.0 | Prepared from 0.1% |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | | | | intermediate stock |
| **0.01** | 0.00001 | 0.01% stock (0.0001 mg/mL) | 300 | 2700 | 3.0 | Prepared from 0.01% intermediate stock |
| **0.001** | 0.000001 | 0.001% stock (0.00001 mg/mL) | 300 | 2700 | 3.0 | Prepared from 0.001% intermediate stock |
| **0.0001** | 0.0000001 | 0.001% stock | 30 | 2970 | 3.0 | |

## Inducing oxidative stress to assess the level of protective activity.

As a positive control, cells were exposed to various concentrations of $H_2O_2$ instead of the treatment solution to determine the concentration that reduces cell viability to 40 %. $H_2O_2$ at concentrations of 100 μm and 60 μm was used as the positive control (Fig. 5.2).

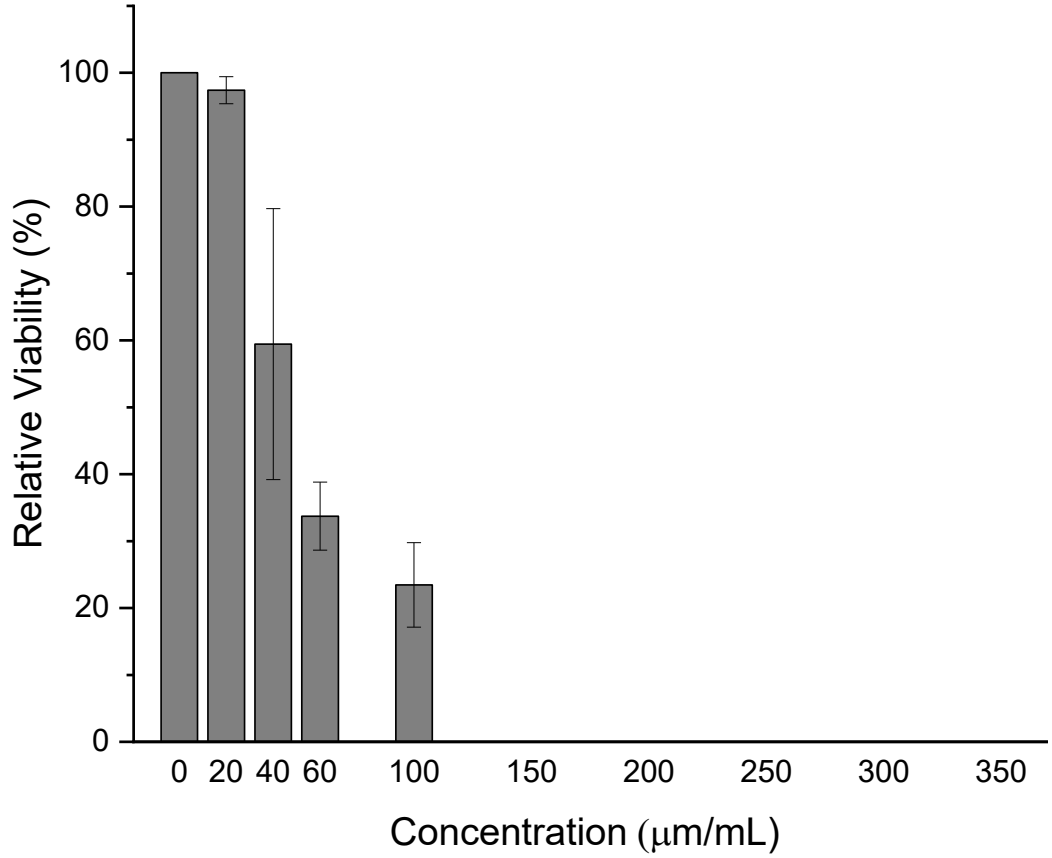


**Figure 5.2: Effects of $H_2O_2$ on HaCaT cell viability determined by the MTT assay. Cells were exposed to different concentrations of $H_2O_2$ for 24 h. Each bar represents mean ± SD (standard deviations).**

To normalize the cell viability data following exposure to irradiated and unirradiated pigment/ink suspensions, a control experiment was performed to account for the potential effects of media dilution (reduction of nutrition). Serial dilutions of cell culture media were prepared using only distilled water in the same ratios as those used for the pigment/ink treatments (Table 5.2). These water-media concentrations served as control treatment solutions and were applied to the cells under identical conditions (24 hours at 37 °C in 5 % $CO_2$). Cell viability measurements (Fig. 5.3) obtained from the controls were used to normalize the viability results of cells treated with the corresponding pigment/ink-media solutions. This normalization step ensured that the observed effects on cell viability were specifically due to the ink components rather than dilution of the culture media.

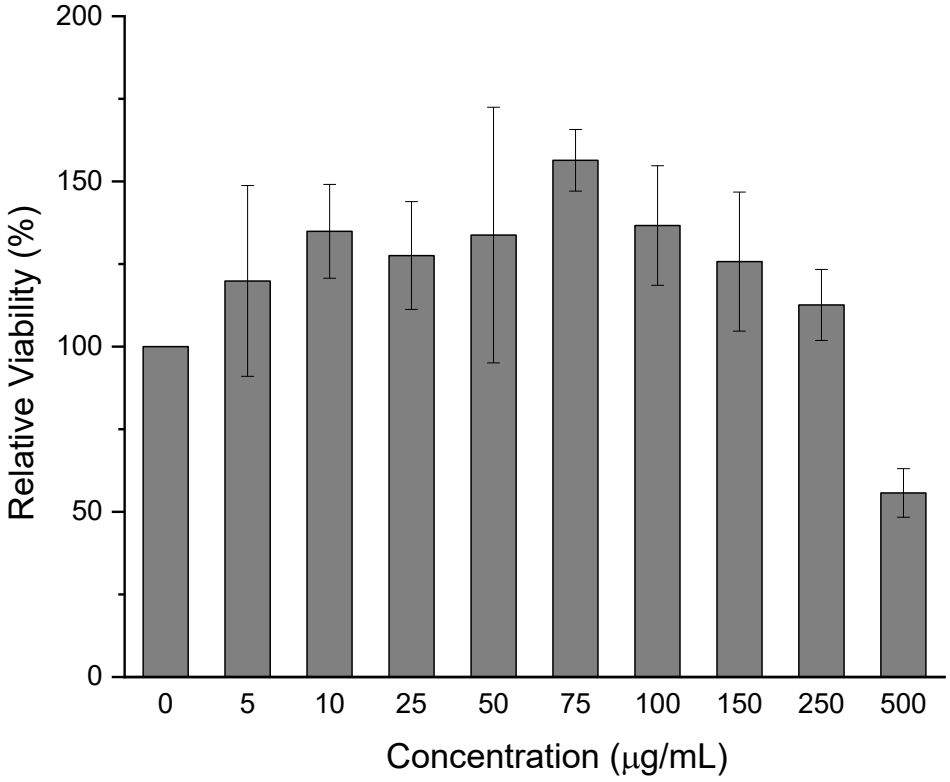


**Figure 5.3: Effects of media dilution with water on HaCaT cell viability determined by the MTT assay. Cells were exposed to various water-diluted media concentrations for 24 h. Each bar represents mean ± SD (standard deviations).**

## Methyl tetrazolium cytotoxicity assay (MTT)

The MTT assay, derived from the tetrazolium salt, relies on a colorimetric method to assess mammalian cell proliferation and viability [37]. It hinges on the capacity of viable cells to metabolise the soluble yellow tetrazolium salt [37, 38]. Metabolic function can be assessed through the colorimetric transformation of thiazolyl blue tetrazolium bromide, resulting in the formation of purple formazan crystals by NAD(P)H-dependent oxidoreductases found within functional mitochondria, following the methodology as previously outlined [8, 39, 40].

Following the treatment, an MTT solution, with a concentration of 0.5 mg/ml, was introduced and allowed to incubate for four hours at 37 °C. Afterward, the MTT solution was removed, and 100 µL of DMSO was added to each well and incubated at 37 °C for 10 min to solubilize the formazan crystals. The absorbance (ODs) was measured on a spectrophotometric plate reader using a reference wavelength of 570 nm. These absorbance values were plotted against known cell concentrations to create the standard curve, which was used to quantify HaCaT cells under a variety of experimental circumstances. A standard curve was used in each experiment to convert OD values to cells per well. The total number of cells were determined by converting the average absorbance values to cells

number using the equation from the standard curve. The percentage of cell viability is calculated using the following equation [41]:

$$\%\text{Viability} = \frac{\text{mean total number of viable cells (treatment)}}{\text{mean total number of viable cells (control)}} \times 100.$$

## Standard curve

In order to create standard curves for HaCaT skin cells, the initial step was placing 200 µL of the cell suspension with a concentration of $4\times10^4$ cells in the first well of a 96-well plate. Further serial dilutions were carried out in order to get a variety of defined cell concentrations. This procedure entailed transferring a 100 µL volume of the cell suspension from one well to the next, ensuring complete mixing at each stage, and repeating the process. As a result, each well was filled with a distinct concentration of cells, which formed the basis for the standard curve. Following seeding, the cells were allowed to adhere for 24 hours under ideal culture conditions, particularly in a suitable medium at 37 °C and 5% $CO_2$. To avoid contamination and maintain cell viability, all processes were carried out in a sterile environment.

## Data analysis

Data were collected from three different trials. Mean results for each experiment condition were compared with the mean rank of unexposed controls. Three-way ANOVA for multiple group comparisons was used (and Tukey's post hoc test). Statistically significant differences compared with untreated cells were defined as $*P \leq 0.05$ $**P \leq 0.01$, and $***P \leq 0.001$.

### 5.2.2 Laser irradiation and characterization of samples

All dispersions were prepared by mixing the pigments or inks with deionized water (1mg/mL) followed by 30 min sonication. A dispersion containing pigment or ink was subjected to laser irradiation at 532 nm (QS Nd:YAG laser, Spectra Physics Quanta-Ray GCR12; 125–140 mJ pulse energy, 10 Hz, 6 ns pulse duration) until transparency was observed (Fig. 5.1), indicating sample fragmentation. For most samples, this was achieved within approximately 15 minutes. This irradiation process aimed to induce degradation of the pigments in dispersion.

Following irradiation, the aqueous suspension containing the laser-generated fragmentswas subjected to extraction using ethyl acetate. To extract laser tattoo fragments, 1 mL of ethyl acetate was added to 1 mL of the irradiated sample. The mixture was gently agitated for a few seconds to ensure thorough mixing, then allowed to settle and separate into two distinct layers. The organic tattoo fragments dissolved in the ethyl acetate layer, facilitating their isolation for further analysis. The ethyl

acetate layer was then collected using a glass syringe and transferred to a clean glass vial for GC analysis. The photodegradation products in aqueous suspension were analysed using UV-Vis spectroscopy .

### 5.2.3 Instrumental analysis

## GC-MS

Gas chromatography mass spectroscopy (GC-MS) analysis was carried out using an Agilent Technologies 7890A GC system with a 5975C inert XL EI/CI MSD Triple Axis Detector and a 7693 sampler. The apparatus was fitted with an Agilent Technologies HP-5MS 5% Phenyl Methyl Silox column (29.4 m x 250 μm x 0.25 μm in size) with a $He_2$ mobile phase at a flow rate of 0.9 ml/min. The injection volume was 1 μL. The oven temperature was set at 40 °C, and remained for 1 min followed by the first ramp with 30 °C/min to 80 °C, the second ramp with 10 °C/min to 260 °C, and the third ramp with 99 °C/min to 320 °C, which was held for additional 4 min. The GC analysis for PY65 differed from the other samples because the oven temperature was set to 70 °C. The ion source and quadrupole temperatures were set at 230 °C and 150 °C, respectively. All m/z values between 40 and 500 were taken in scan mode. The fragment compounds produced upon laser irradiation of pigments, inks, were identified using the NIST database and literature [29, 42, 43].

## UV-Vis

Unirradiated and irradiated samples were transferred into 10 mm optical path length quartz cuvettes. The UV–Vis spectra was measured in the range 300 nm to 800 nm with a Cary 50 and Agilent 60 UV-vis spectrophotometer.

## 5.3 Results and discussion

### 5.3.1 Pigment and ink selection

PY14 and PY65 (Fig. 5.4a and b) were selected as they are both azo pigments, with PY14 representing a di-azo type pigment and PY65 representing a mono-azo type pigment. These pigments are known ingredients in yellow tattoo inks, and they possess a structure consisting of four and two aromatic rings, respectively. Azo pigments are potentially toxic and may decompose into hazardous substances with small particle size [2, 7, 44-46]. A comprehensive review of the literature yielded no reports on the laser irradiation of PY14 and PY65 leading to the formation of hazardous by-products. However, our Py-GCMS analysis reveals that these pigments degrade into harmful compounds, including styrene, aniline, o-toluene isocyanate, o-toluidine, benzene isocyanate, and m-xylidine (Table SI 5.1). Given that laser-induced tattoo pigment degradation is reported to proceed via a thermal mechanism [47, 48], it is plausible that laser irradiation of these pigments could result in the formation of toxic compounds.

The potential toxicity on skin cells from these pigments both before and after irradiation remains unexplored. In addition to these reference pigments, GR and BO tattoo ink were selected for study. These inks were chosen as they are reported to contain PY14 and were readily available to the researchers. Neither of these inks, nor their laser degradation products have been evaluated for their potential toxicity to HaCaT cells.

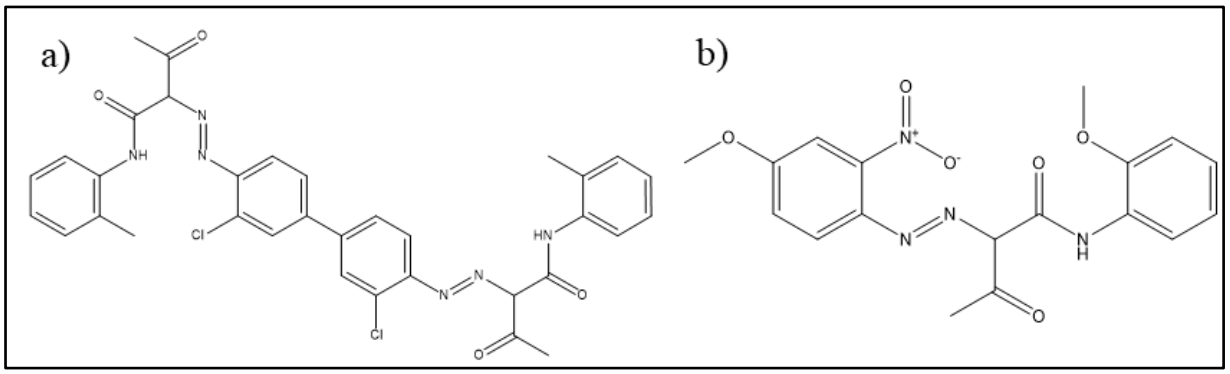


**Figure 5.4: A schematic representation of the chemical structures of a) PY14 and b) PY65.**

### 5.3.2 The toxicity impact of pigments and tattoo inks

This study evaluated the impact of unirradiated and irradiated inks/pigments on mitochondrial metabolic activity, a key indicator of cell survival. Fig. 5.5 shows the MTT results, which demonstrated a dose-dependent decrease in cell viability, indicating cytotoxicity. Specifically, exposure to both PY14 and PY65 (unirradiated) led to a reduction in cell viability to approximately 25-50% at concentration ranges from 10 to 500 µg/mL. Cytotoxicity was increased post-irradiation. Irradiated PY14 further induced a significant decrease in HaCaT cell viability with complete loss of the cell viability (0%) for concentrations larger than 75 µg/mL ($p < 0.001$). Irradiated PY65 exhibited the highest toxicity, reducing cell growth to 0% across all tested doses (50-500 µg/mL) ($p < 0.05$) after 24 hours. Although, PY65 has not been associated to any hazard-related outcomes [33], the MTT results demonstrate that this pigment exhibited the highest level of toxicity among the samples tested. This finding is consistent with a previously reported medical case, where a patient developed an allergic response a few weeks after being tattooed with inks containing PY65. The allergic reaction affected all areas of the tattoo where PY65 was injected [49].

Unirradiated GR and BO inks displayed high levels of cytotoxicity, reducing survival rates to 25-70% at concentrations ranging from 0.01 to 500 µg/mL. Laser irradiation of both inks (GR, BO) significantly increased the cytotoxicity of both inks, with a statistically significant reduction in cell viability to 15-20% ($p < 0.001$) at concentration 100 µg/mL and 0% at concentrations 500 µg/mL.

The observed toxic effects of GR and BO tattoo inks align with previous studies demonstrating that ink injection leads to varying degrees of reduced metabolic activity in reconstructed human skin [8]. Significant reductions were reported for Eternal Light Red ink (89.9% ± 0.8%), Star Brite Colours Light Red ink (88.7% ± 1.9%), Kuro Sumi Glow ink (86.6% ± 0.7%), Intenze Sculpting Black ink (65.0% ± 14.1%), and Intenze Dragon Red ink (56.4% ± 7.6% SEM). Whilst these inks typically contain pigments such as Pigment Red 170 and carbon black (an amorphous carbon-based pigment) which differ structurally and chemically from the diarylide yellow pigments (e.g., PY14 and PY65) examined in this study, Pigment Red 170 is a monoazo pigment (akin to PY65). These studies provide useful insight into the general biological responses induced by different classes of tattoo pigments, and confirm the potential for high cytotoxicity of these tattoo inks, consistent with the results of our spectral analyses [8].

The different toxicity levels of unirradiated and irradiated tattoo inks could be related to changes in particle size. As identified in our previous study [50], DLS analysis of both inks showed that BO inks have a larger particle size than GR inks. Additionally, EDX analysis along with 2018 safety data sheets (SDS) revealed that both inks have the same ingredients [33], except for the presence of $TiO_2$ in BO ink, which could be associated with its larger particle size [50]. Therefore, the larger particle size of BO ink may reduce its toxic effects on cells, whereas the smaller particle size of GR ink might contribute to a stronger toxic impact on HaCaT cells. However, after laser irradiation, both inks exhibited high toxicity, which might be due to a reduction in particle size or the formation of toxic degradation fragments

Notably, all irradiated samples showed statistically significant toxicity at concentrations larger than 50 µg/mL. Despite this, irradiated GR and BO inks exhibited lower toxicity compared to PY14 and PY65, likely due to differences in their chemical composition, which may influence the fragmentation process during laser treatments. Moreover, the variation in toxicity effects on cell survival may also be attributed to the fact that this study used ink suspension consisted of 25 % - 50 % pigments along with additives and preservatives, as stated in the SDS [35] of these, whereas the pigment suspensions consisted of pure pigment powders dispersed in solvent without additional ink formulation components. This increases the likelihood of generating a greater quantity of toxic fragments from pure pigments compared to inks after laser irradiation.

In addition, the inks examined in this study contained chemicals classified as skin or eye irritants, and those with pronounced irritating properties were associated with lower metabolic activity in cells [35]. Prolonged exposure times may further exacerbate cytotoxic effects.

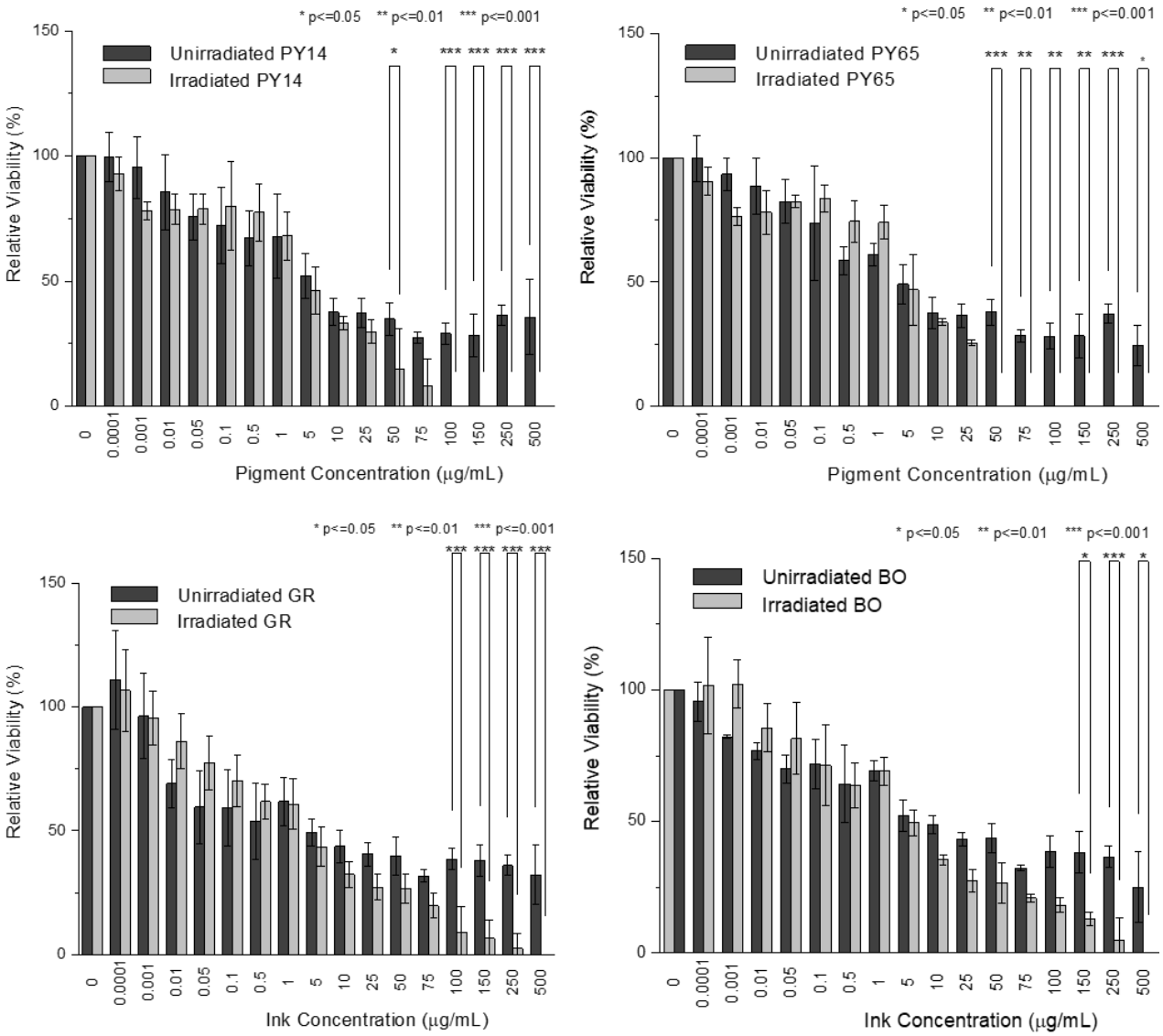


**Figure 5.5: Effects of unirradiated and irradiated PY14, PY6, GR and BO ink on HaCaT cell viability determined by the MTT assay. Cells were exposed to different dilutions of unirradiated and irradiated pigments and inks for 24 h. Each bar represents mean ± SD (standard deviations). Statistically significant differences compared with untreated cells were defined as *P ≤ 0.05 **P ≤ 0.01, and ***P ≤ 0.001.**

### 5.3.3 Characterization of laser tattoo fragments

Optical images of the irradiated samples reveal that the ink/pigment dispersions were transparent after laser exposure (Fig. 5.1). The UV-Vis spectra and the GC chromatogram of pigments and tattoo inks were obtained from the prepared samples pre-and-post laser treatments.

## UV-Vis spectra

The UV-Vis spectra of pigments and tattoo inks were acquired from the dispersions following laser treatments in the 200-800 nm range. The spectra of the post-irradiated samples of PY14, PY65, GR, and BO ink and the reference unirradiated aqueous dispersions are shown in (Fig. 5.6a-d). In water-based sample dispersions, irradiation resulted in a noticeable reduction in absorbance intensity, as

observed in the spectral characteristics. This confirmed the capability of these pigments and inks to absorb 532 nm laser light, hence breaking down the chromophore. The UV-Vis spectral intensity is contingent upon the state of aggregation and rises when the size of aggregates permits the creation of stable colloidal suspensions in the solvent [51]. Additionally, some additives are designed to enhance the stability of pigments in a water-based dispersion. The presence of these additives could increase the turbidity of the dispersion [51] leading to an overall rise in intensity and broadening of the spectrum, as observed in the unirradiated samples.

While considering the distinctions between polychromatic and laser light, it is important to note that laser treatments of pigments and additives (two components of tattoo inks) have the potential to produce soluble species that absorb green visible light. The UV-Vis spectra of irradiated samples suggest down-sizing and/or decomposition of the chromophores after laser treatment as indicated by the decrease in peak intensity.

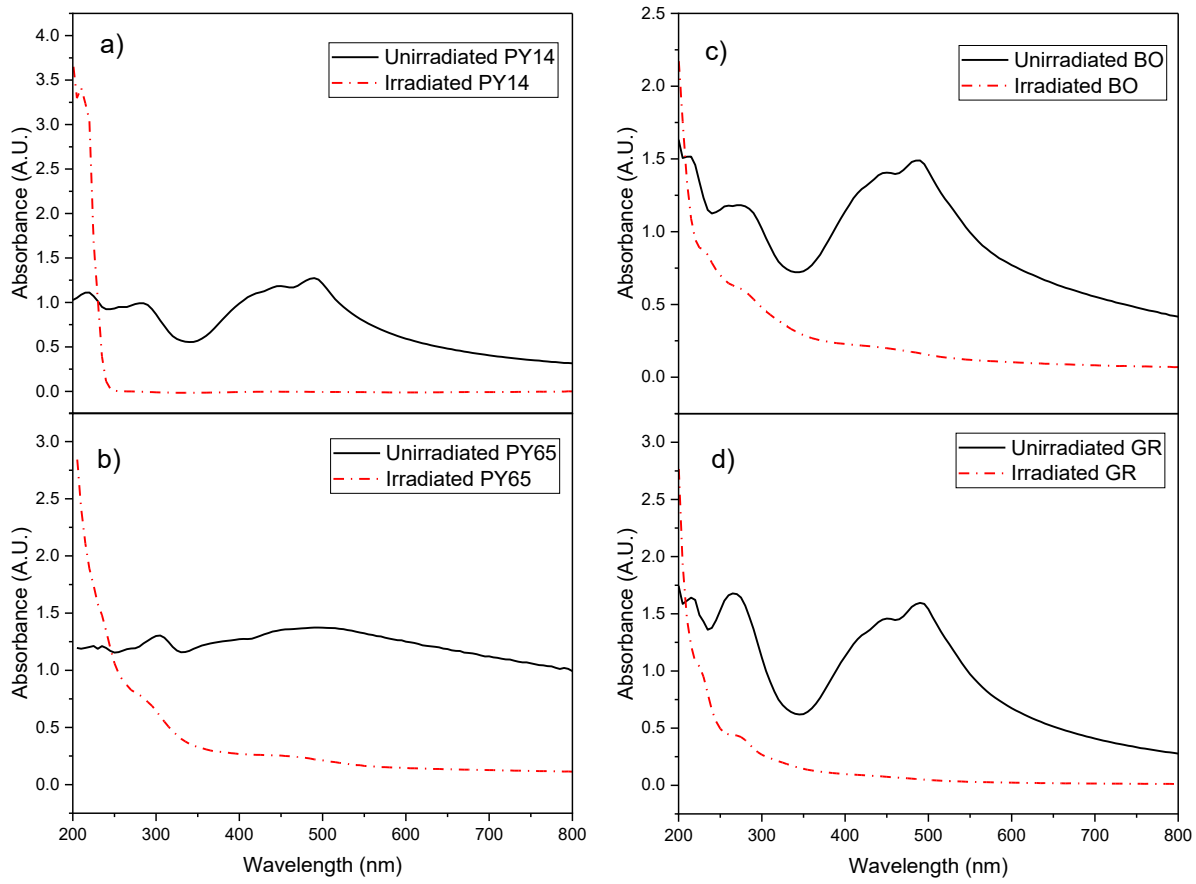


**Figure 5.6: UV-Vis spectra before and after laser irradiation of a) PY14, b) PY65, c) BO, and d) GR inks.**

## GC-MS and Py-GC-MS

Using the GC-MS, soluble fragments from irradiated pigment and ink suspensions were analysed. Fig. 5.7a-d shows the GC-MS chromatograms of irradiated PY14, PY65, GR, and BO inks. As observed in the GC chromatogram of the irradiated sample suspensions, two primary peaks of differing intensities were detected. The GC-MS analysis of the ethyl acetate, unirradiated PY14, PY65, and inks had no peaks (see to supporting information Fig. SI 5.1). This indicates that these peaks correspond to fragments that were produced during the irradiation and breakdown processes that occurred during the laser treatment. The cleavage products from irradiated samples were identified as benzene1-isocyanato-2-methyl (*o*-toluene isocyanate) and 2-methyl-benzenamine (*o*-toluidine) at Rt of 5.7 and 5.8 min, respectively (Table 5.3). Notably, the detected peaks in PY14 were more intense compared to GR and BO inks. This result is expected, given that both inks contain PY14 in addition to various additives that form the suspension medium of the tattoo ink. This discrepancy in peak intensity could be attributed to the presence of these additional components. The GC chromatogram of irradiated PY 65 shows one peak at 7.28 min which correlated to the presence of 2-methoxyphenyl isocyanate.

To confirm the formation of the three fragments detected in the GC chromatograms of PY14 and PY65, Py GC-MS analysis was conducted for these pigments. The findings, as interpreted from the pyrograms of the two pigments, are presented in Fig. SI 5.2 and Table SI 5.1. A comparison between the pyrolysis products and the GC analysis revealed additional fragments, especially in PY65, which exhibited a range of compounds, several of which are known to be hazardous and potentially carcinogenic to humans [35]. Among the additional fragments identified, *o*-anisidine, 2-methoxyphenyl isocyanate, and 5-methoxybenzofurazan have been reported in the literature as hazardous substances [35]. However, these compounds were not detected in our ethyl acetate extracts. Further work would need to be done to determine if these compounds are formed on laser irradiation of PY65.

Based on the GC chromatogram of irradiated BO ink and its constituents, including $TiO_2$, which is known to generate reactive oxygen species (ROS) when exposed to laser irradiation, it was expected that this ink would exhibit significant toxicity impact to cells [52]. However, the MTT assay results revealed that the GR ink exhibited slightly higher toxicity than the BO ink. This discrepancy might be attributed to the specific laser wavelength used in this study (532 nm), which is not well absorbed by $TiO_2$, and the high concentration of $TiO_2$ in the ink, as reported in our previous work [50]. It has been suggested that $TiO_2$ may reflect or fail to absorb this laser wavelength effectively, thereby reducing the likelihood of ink fragmentation and led to the formation of the brown solid during laser irradiation (Fig. SI 5.3) [50].

These findings, together with previous studies involving femtosecond laser treatment of tattoo pigments [29], highlight a broader concern: laser irradiation regardless of the specific laser type or pigment structure can induce the formation of potentially toxic degradation products. For example, prior work showed that PO13 and PY138 resulted in the formation of hexachlorobenzene, aniline, and 3,3'-dichlorobenzidine [29], whilst other work on PG7, showed that chlorinated benzonitriles, benzonitriles, phthalimides, benzaldehydes, and benzene chlorinated byproducts are produced by femtosecond laser [53].

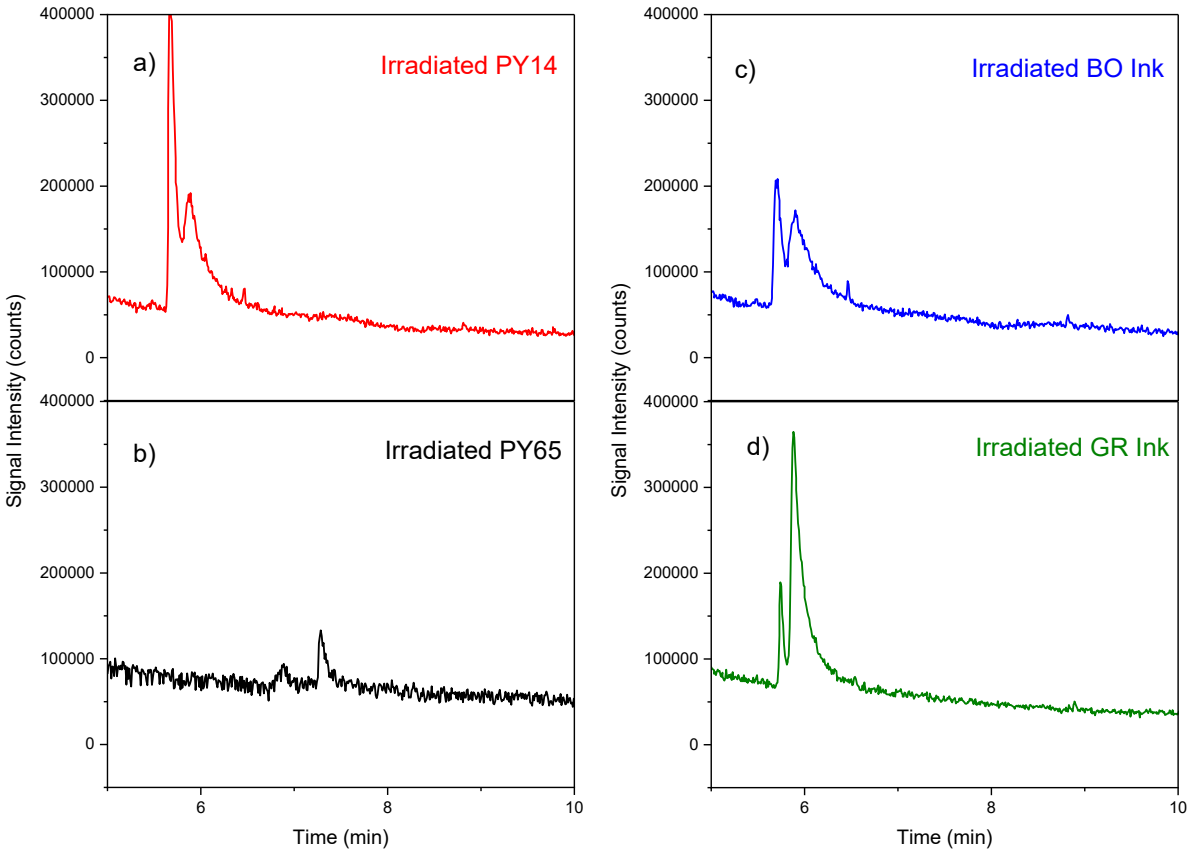


**Figure 5.7: GC-MS taken after irradiation of a) PY14, b) 65, c) BO ink and d) GR ink. The spectra present from 5 to10 minutes, as no detectable peaks were observed prior to this time.**

**Table 5.3: The soluble cleavage products released during laser irradiation of pigments, inks, as well as the retention times, major mass losses and hazard statement.**

| Chemical/ CAS | Rt(min) | Main (m/z) | Present in | Hazard code | Hazard category | Description [35] |
|---|---|---|---|---|---|---|
| ***o*-toluene isocyanate/ 614-68-6** | 5.70 | 133, 104, 91, 78, 51. | PY14, GR, BO | H227 | 4 | Combustible liquid. |
| | | | | H302 | 4 | Harmful if swallowed |
| | | | | H312 | 4 | Harmful in contact with skin. |
| | | | | H315 | 2 | Causes skin irritation. |
| | | | | H319 | 2/2A | Causes serious eye irritation. |
| | | | | H331 | 3 | Toxic if inhaled. |
| | | | | H334 | 1, 1A, 1B | May cause allergy or asthma symptoms or breathing difficulties if inhaled. |
| | | | | H335 | 3 | May cause respiratory irritation |
| ***o*-toluidine/ 95-53-4** | 5.81 | 106, 77, 43, 32. | PY14, GR, BO | H227 | 4 | Combustible liquid. |
| | | | | H301 | 3 | Toxic if swallowed. |
| | | | | H319 | 2/2A | Causes serious eye irritation. |

| | | | | | |
|---|---|---|---|---|---|
| | | | H331 | 3 | Toxic if inhaled. |
| | | | H350 | 1, 1A, 1B | May cause cancer. |
| | | | H400 | 1 | Very toxic to aquatic life. |
| **2-methoxyphenyl isocyanate/ 700-87-8** | 7.28 min | 149,134, 106, 78, 51 PY65 | H302 | 4 | Harmful if swallowed. |
| | | | H312 | 4 | Harmful in contact with skin. |
| | | | H315 | 2 | Causes skin irritation. |
| | | | H317 | 1, 1A, 1B | May cause an allergic skin reaction. |
| | | | H319 | 2/2A | Causes serious eye irritation |
| | | | H332 | | Harmful if inhaled. |
| | | | H334 | 1, 1A, 1B | May cause allergy or asthma symptoms or breathing difficulties if inhaled. |
| | | | H335 | 3 | May cause respiratory irritation. |

| | | | |
|---|---|---|---|
| | H351 | 2 | Suspected of causing cancer |

### 5.3.4 The toxicity impact of laser-generated fragments

Our GC-MS analyses identified the formation of *o*-toluidine, *o*-toluidine isocyanate and 2-methoxyphenyl isocyanate. These chemicals are likely to be causing toxicity seen in the HaCaT cells. Moreover, o-toluidine is a carcinogenic compound that has been identified as a by-product of laser degradation from red pigments [46].

The cytotoxic effects of *o*-toluene isocyanate, *o*-toluidine and 2-methoxyphenyl isocyanate on cell viability were evaluated using the MTT assay (Fig. 5.8a-c). To assess their impact, a series of concentrations of these compounds (0.0001-500 µg/mL) were prepared. The results indicate that at lower concentrations (0.0001-0.1 µg/mL), *o*-toluene isocyanate did not significantly impact cell growth, suggesting that cells can tolerate low levels of exposure to this compound. However, as the concentration increased (0.5-10 µg/mL), cell viability declined to 60-20%, indicating a dose-dependent cytotoxic effect. Complete loss of cell viability (0 %) was observed at concentrations above 10 µg/mL, confirming the compound's toxicity at higher doses.

In contrast, *o*-toluidine exhibited cytotoxic effects at very low concentrations. Cell viability was reduced to approximately 75 % within the range of 0.0001-0.01 µg/mL, highlighting its higher toxicity compared to *o*-toluene isocyanate at low doses. A further decline in cell viability was observed at concentrations between 0.05-1 µg/mL, reaching 20-10 %, while complete inhibition of cell growth was noted at 5 µg/mL and higher. This suggests that *o*-toluidine is more potent in affecting cell viability at lower concentrations than *o*-toluene isocyanate.

Laser irradiation of PY5 resulted in the identification of 2-methoxyphenyl isocyanate as a major degradation fragment, which demonstrated a distinct cytotoxicity profile on HaCaT skin cells in comparison to the fragments produced from irradiated PY14. Exposure to lower quantities of 2-methoxyphenyl isocyanate (0.0001-0.01 µg/mL) reduced cell proliferation by roughly 65 %. As concentrations increased (0.5-10 µg/mL), cell viability decreased by approximately 50-40 %. Notably, concentrations over 25 µg/mL resulted in full cell viability loss (0 %).

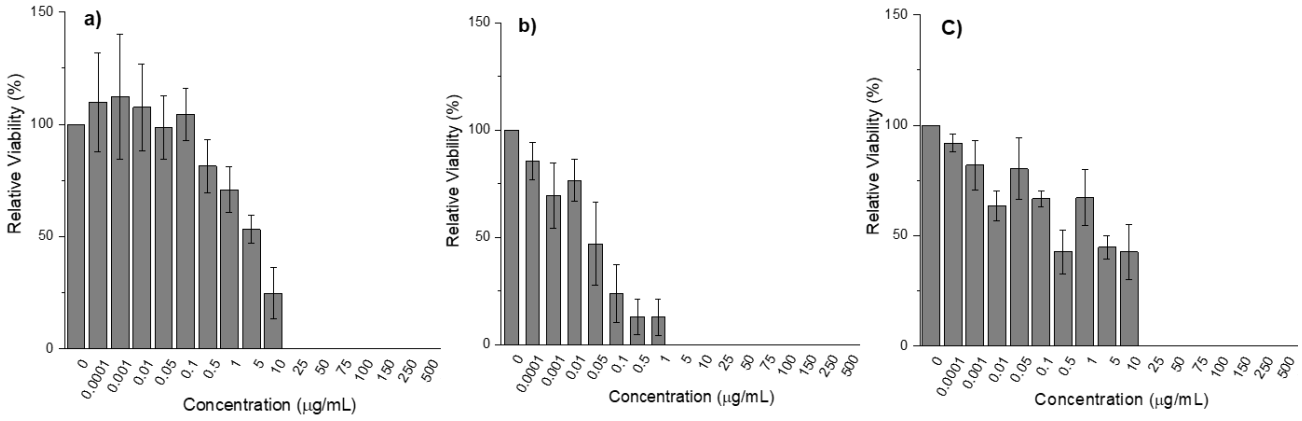


**Figure 5.8: Effects of a) *o*-toluene isocyanate, b) *o*-toluidine, and c) 2-methoxyphenyl isocyanate on HaCaT cell viability determined by the MTT assay. Cells were exposed to different dilutions of this chemical for 24 h. Each bar represents mean ± SD.**

## 5.4 Conclusion

In conclusion, this study highlights significant health concerns associated with the use of tattoo inks and the breakdown products generated during laser tattoo removal. Our findings demonstrate a marked reduction in the survival rate of human skin cell lines (HaCaT) when exposed to irradiated inks compared to non-irradiated ones, indicating the potential for adverse effects on human health. The process of irradiation was shown to produce hazardous chemicals, including *o*-toluidine, *o*-toluene isocyanate, and 2-methoxyphenyl isocyanate, all of which negatively impact the viability of HaCaT cells. These harmful fragments, released during laser treatments, may not only damage the skin locally but also pose systemic risks by entering the body and affecting other tissues. To safeguard patient health, it is essential to implement comprehensive toxicity assessments and further research the characteristics of laser-induced tattoo ink degradation products. Additionally, limiting the amount of ink removed during each session and spacing laser treatments at regular intervals would allow the body time to process and eliminate these hazardous substances, thereby reducing potential health risks.

## 5.5 Acknowledgements

B.A. extends deepest gratitude to the following organizations for their invaluable support and contributions to this research: King Faisal University, Ministry of Education, Saudi Arabia, for their generous support and encouragement throughout the duration of this study. We acknowledge the facilities and technical assistance of Medical Biotechnology, College of Medicine and Public Health,

Flinders University. Finally, the authors acknowledge Benjamin Boyle (Flinders University) for his invaluable assistance with the Py-GC-MS analysis.

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# 6 CHAPTER 6: CONCLUSION AND FUTURE WORKS

## 6.1 Conclusion

This thesis has explored the challenges surrounding the safety and behaviour of tattoo inks and the impact of laser treatments on their degradation. In Chapter 2, an evaluation of commercially available tattoo inks revealed substantial discrepancies in labelling, with numerous products containing unlisted pigments and elements. These findings underscore a need for stringent regulatory measures to ensure consumer safety and transparency within the tattoo industry. The potential health risks associated with these undeclared components highlight the importance of implementing robust monitoring systems and enforcement of labelling standards.

Chapter 3 explored the photodegradation of tattoo pigments, focusing on the role of $TiO_2$ in modifying the degradation pathways of yellow pigments during laser irradiation at 532 nm. The study demonstrated that $TiO_2$ significantly influences the behaviour of pigments by forming complex aggregates, which in turn reduce the production of volatile fragments. These insights provide an understanding of the chemical interactions occurring during laser tattoo removal, shedding light on both the challenges and safety concerns associated with the process.

Chapter 4 involved the examination of laser treatment effects on darker skin tones, where melanin pigments complicate the tattoo removal process. This research highlights the role played by melanin pigments in absorbing laser light, which reduces yellow pigment and ink degradation during treatment processes. Melanin's presence influences how lasers interact with these substances by decreasing volatile fragments while promoting conglomerate formation and increasing particle size. This underscores the need for personalized treatment approaches to address the unique requirements of individuals with darker skin to ensure more effective outcomes.

Chapter 5 assessed the cytotoxic effects of tattoo ink degradation products, revealing that both unirradiated and irradiated inks exhibit toxicity. While unirradiated pigments reduced skin cell viability by approximately 50%, irradiated products displayed greater toxicity to the cells. These results bring to light the potential health hazards associated with both the application and laser removal of tattoos, emphasizing the importance of understanding the long-term implications of these practices.

In conclusion, this thesis highlights the multifaceted issues related to tattoo inks and their removal, emphasizing the need for regulatory oversight, public education, and advancements in laser technology. By addressing the gaps in knowledge regarding pigment composition, degradation

behaviour, and health risks, this study provides a foundation for future research and the development of safer practices. These findings contribute to the ongoing effort to protect public health while supporting innovation and informed decision-making in the tattoo and laser removal industries.

## 6.2 Future Directions Based on Preliminary Method Development

### 6.2.1 Advanced characterization of laser degradation products

The nature and behaviour of degradation products formed by laser treatment needs to be further explored using advanced analytical techniques such as mass spectrometry, spectroscopy, and imaging techniques. Initial work on this is described below. In Chapters 3 and 4, all studied samples were irradiated in their dried form and analysed using GC-MS to examine volatile degradation products. To investigate non-volatile laser degradation products, LC-MS should be used. The following outlines the initial method development for this LC-MS analysis, representing work that has been initiated and remains in progress.

## Sample preparation and laser irradiation set up:

PY74, PY14, and PY65 were prepared and irradiated using the same laser setup described in Chapter 3 with a varied number of pulses. Ethyl acetate (1 mL) was added to a GC glass vial containing irradiated pigment and vigorously shaken for one minute. 200 µL of the extracted sample was diluted with ethyl acetate for LC-MS analysis.

## LC-MS instrument analysis:

Thermo Scientific Vanquish UHPLC equipped with an autosampler, UV-Vis detector, and an ISQ EC mass spectrometer was used to analyse the unirradiated and irradiated samples.

Various solvent gradients were trialled using water in combination with one of the following: methanol, acetonitrile (ACN), dilute aqueous formic acid, or tetrahydrofuran (THF), in an attempt to improve separation. Similarly, different columns (C18 and C3), solvent extraction methods (ethyl acetate, methanol, isopropanol), and gradient conditions were tested to further improve separation. The results of this method development are discussed in sections below.

The optimal LC method was performed using an LC-MS system equipped with a ZORBAX 300SB-C3 column (4.6 × 250 mm, 5 µm). The column was operated at a flow rate of 0.6 mL/min with a two-phase solvent system: water (A) and acetonitrile (ACN, B). The best solvent gradient began with 70% A, held for 4 minutes, then linearly decreased to 10% A over 20 minutes. This composition was maintained for 6 minutes, followed by a return to 90% A over 6 minutes. Post-run conditioning was performed using 30% water and 70% ACN, held for 5 minutes. The sample injection volume was 2

µL. The ISQ EC mass spectrometer operated with an ionisation energy of 70 eV and was set to detect positive ions in the range of 10 to 1000 amu. UV-Vis detection was carried out in the range of 190–680 nm

## Preliminary results

Initial LC-MS work was conducted using a Phenomenex Kinetex column (150 mm × 4.6 mm, 2.6 µm), operated at a flow rate of 0.6 mL/min with a two-phase solvent system: water (A) and UPLC-grade methanol (B). The initial gradient started with 95% A, held for 3 minutes, then linearly decreased to 55% A over 10 minutes, followed by a further decrease to 5% A over the next 10 minutes. This was maintained for 2 minutes before returning to 95% A over 2 minutes. Post-run conditioning was performed with 30% water and 70% methanol, held for 5 minutes. The ISQ EC mass spectrometer operated with an ionisation energy of 70 eV and was set to detect positive ions in the range of 10 to 1000 amu. UV-Vis detection was carried out over a wavelength range of 190–680 nm.

The LC-MS chromatogram of irradiated PY74 showed that increasing the number of laser pulses led to its degradation. This was evidenced by the appearance of several new peaks with higher retention times (Fig. 6.1). Notably, peaks at 15.05 and 15.4 minutes were observed in the irradiated samples, whereas the unirradiated PY74 displayed a primary peak at 13.9 minutes. The intensity of the 13.9-minute peak decreased significantly with an increasing number of laser pulses, while a new peak at 14.5 minutes appeared, suggesting the formation of degradation products.

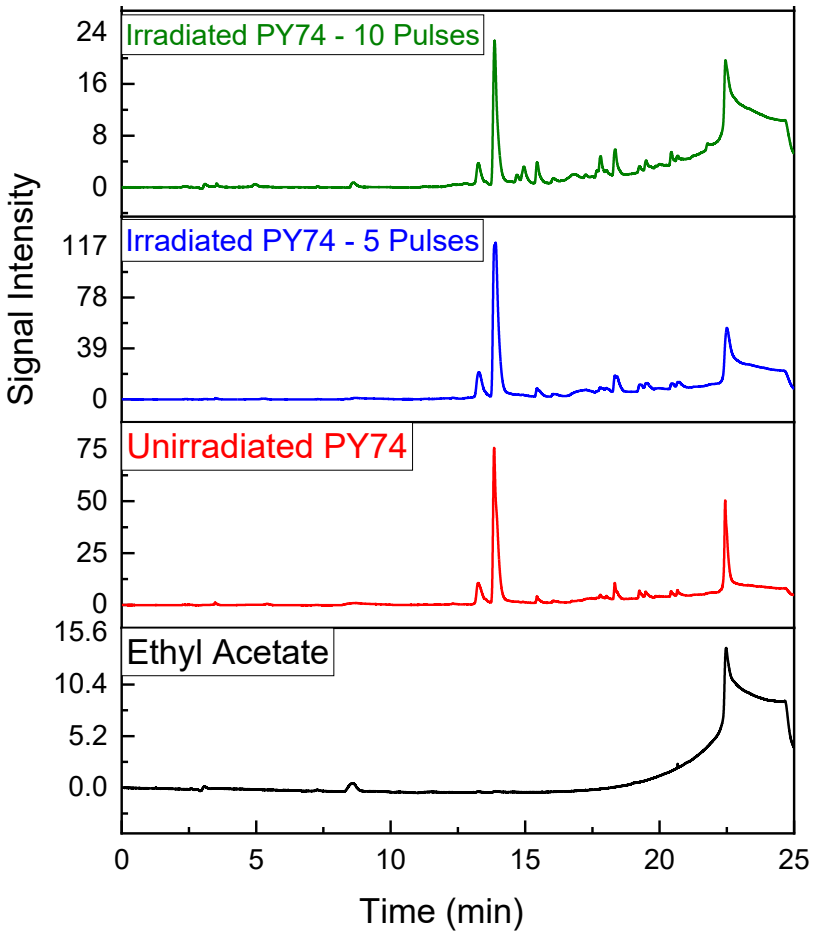



**Figure 6.1:LC-MS chromatogram of unirradiated and irradiated PY74 with different number of pulses.**

Based on the previous observation, PY74 was further irradiated with 20 pulses (Fig. 6.2), as previous studies typically used a pulse duration of 20 ns [1-4]. As shown in Fig. 6.2, some fragmentation peaks were detected with increasing the pulse duration to 20 pulses. When LC-MS analysis was performed for PY74, the signal intensity of most of the analytes was low (peaks at 12.3 min and 14.9 min). This indicated that the analytes protonated poorly in the water-methanol mobile phase conditions. Therefore, individual analyses were performed with the MS in electrospray positive ionisation mode to optimise LC-MS parameters.

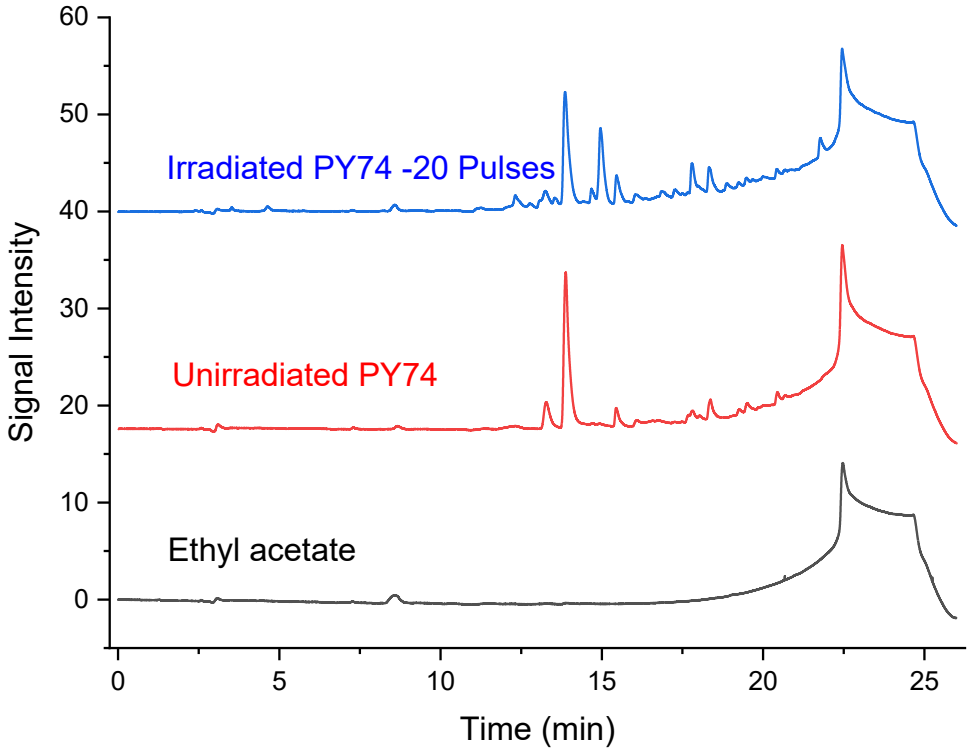



**Figure 6.2: LC-MS chromatogram of unirradiated and irradiated PY74 with 20 pulses laser radiation using water: methanol mobile phase conditions.**

### Addition of the formic acid

Previous research found that the pH of mobile phase solvents affects the MS detection of aromatic amines noticeably. As a result, mobile phase additives such as formic acid are necessary to increase ionisation and fragmentation signal intensity [5]. Adding 0.1% or 0.2% of formic acid to the solvent system was examined to enhance the molecules' peak shape. It has been commonly utilised as a modifier in the mobile phase. This is owing to formic acid's comparatively low capacity to suppress ion detection during light MS analysis employing electrospray ionisation (ESI) of samples, which is why formic acid was chosen as a mobile phase modifier.

Therefore, the first step in optimising the LC-MS analysis was the addition of formic acid to water in an attempt to increase the signal strength. Results from this experiment showed that the addition of 0.2% formic acid in both organic and aqueous mobile phases provided adequate retention and peak characteristics (shape and intensity) (Fig. 6.3).

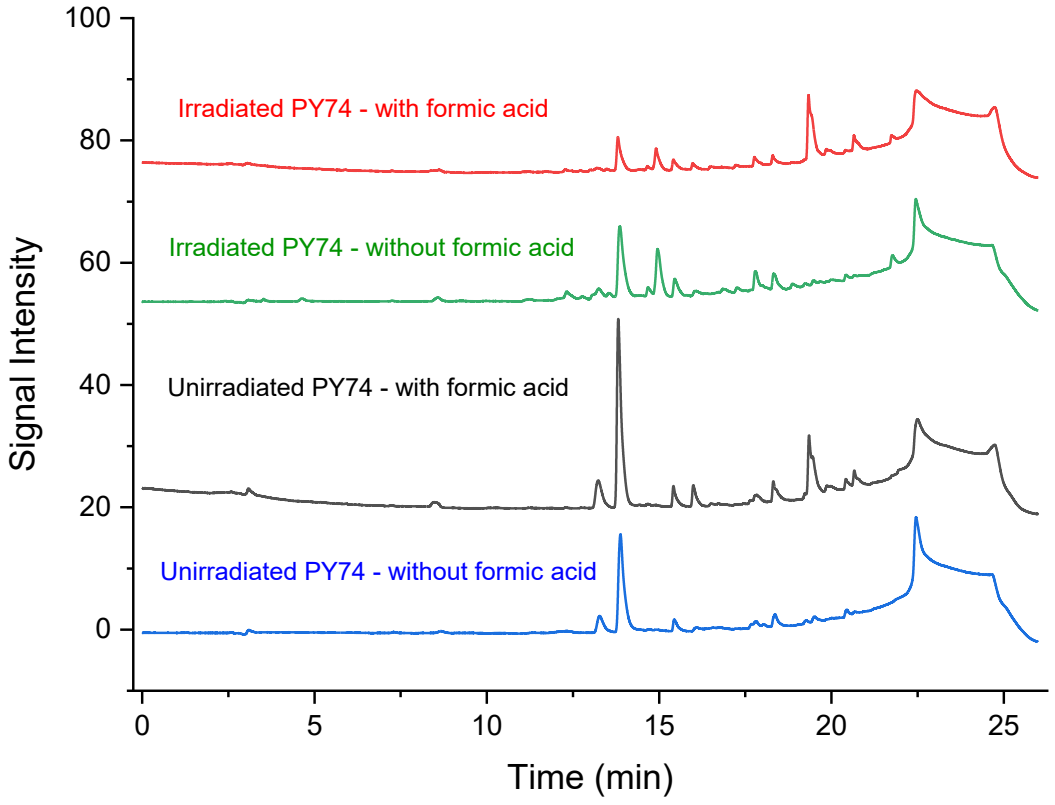



**Figure 6.3: LC-MS chromatogram of unirradiated and irradiated PY74 using water: methanol mobile phase conditions & 0.2% formic acid.**

### Extraction solvents

To improve the detection of laser-induced degradation products, alternative sample preparation solvents were explored. Signal intensity remained suboptimal despite initial adjustments (the addition of formic acid). The MS peak intensity of the PY74 fragmentation remained relatively low when compared to unirradiated PY74. Consequently, the second step was to use organic solvents such as methanol and isopropanol and test for their ability to dissolve different types of yellow pigments and their degradation products.

PY14 has been irradiated and analysed using the same laser condition and LC-MS method that was used to irradiate and analyse PY74, with the only variable being the organic solvent used. Fig. 6.4 depicts the LC-MS chromatogram of irradiated PY14 that dissolved in two different solvents. The resolution of peaks corresponding to degradation products was significantly improved when isopropanol was used, compared to ethyl acetate and methanol. Additionally, the intensity of photodegradation product peaks increased when samples were dissolved in isopropanol.

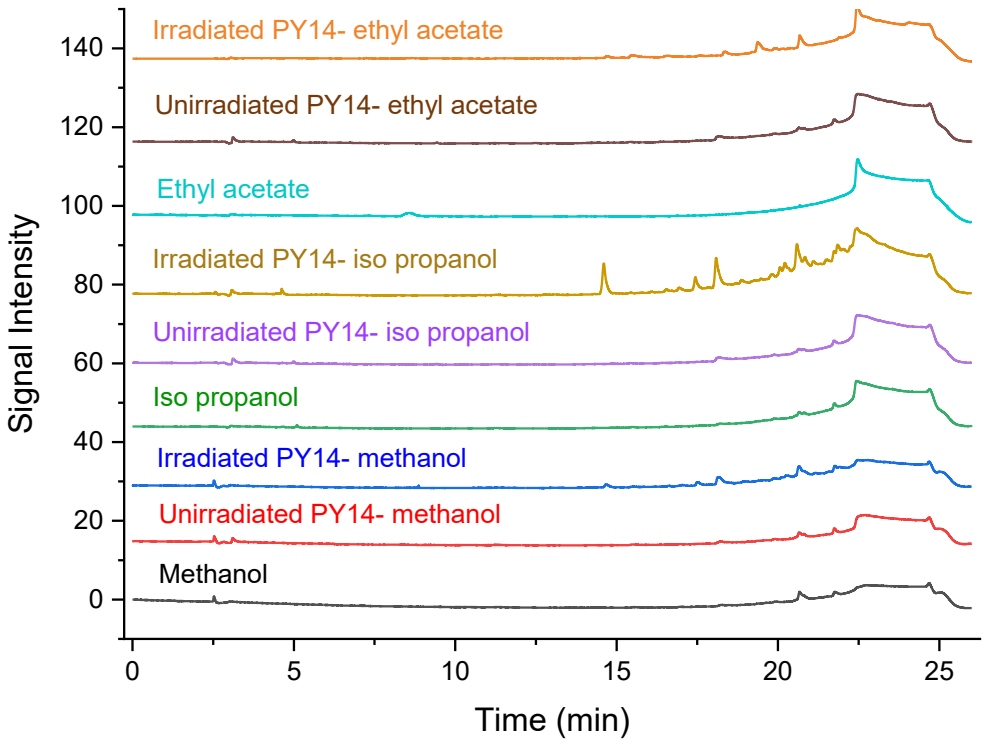



**Figure 6.4: LC-MS chromatogram of unirradiated and irradiated PY14 dissolved in different organic solvents (methanol, isopropanol, and ethyl acetate).**

Furthermore, PY65 was exposed to the same laser conditions as PY74 and PY14. Interestingly, the LC-MS chromatogram of irradiated PY65 shows high-intensity peaks that are associated with the degradation products when the sample was dissolved in isopropanol (Fig. 6.5).

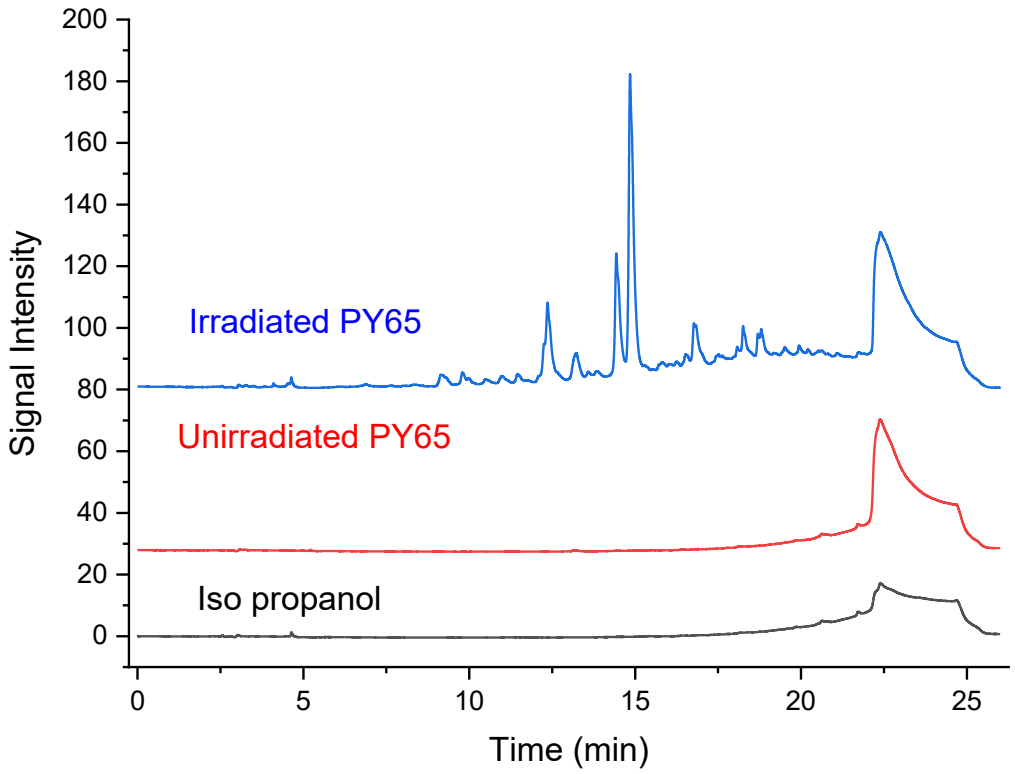



**Figure 6.5: LC-MS chromatogram of unirradiated and irradiated PY65 dissolved in the isopropanol.**

**Gradient conditions**

Building on the improved detection achieved by optimizing solvents, further refinement of the LC-MS method was necessary to enhance separation of degradation products. One unexpected observation with separation of the breakdown products is that the switch (in the elution) between stationary phase and mobile phase at 22 minutes (Fig. 6.5) resulted in an intense peak, which means there may be some degradation products that co-eluted later. Therefore, LC-MS analysis was performed with a new gradient elution method.

The new gradient condition of water-methanol was 95% of water, which was decreased to 1% gradually over 20 min and then maintained at 1% of water for 6 min. After that, the initial conditions were restored from 26 to 32 min and kept for 2 min, allowing the system to equilibrate (Table 6.1). The total run time was 35 min, and the curve was kept at 5 (linear). The new gradient had a noticeable effect on eluting the photodegradation products early. This was confirmed by detecting peaks between 10-20 minutes in the LC-MS chromatogram of irradiated PY65 (Fig. 6.6).

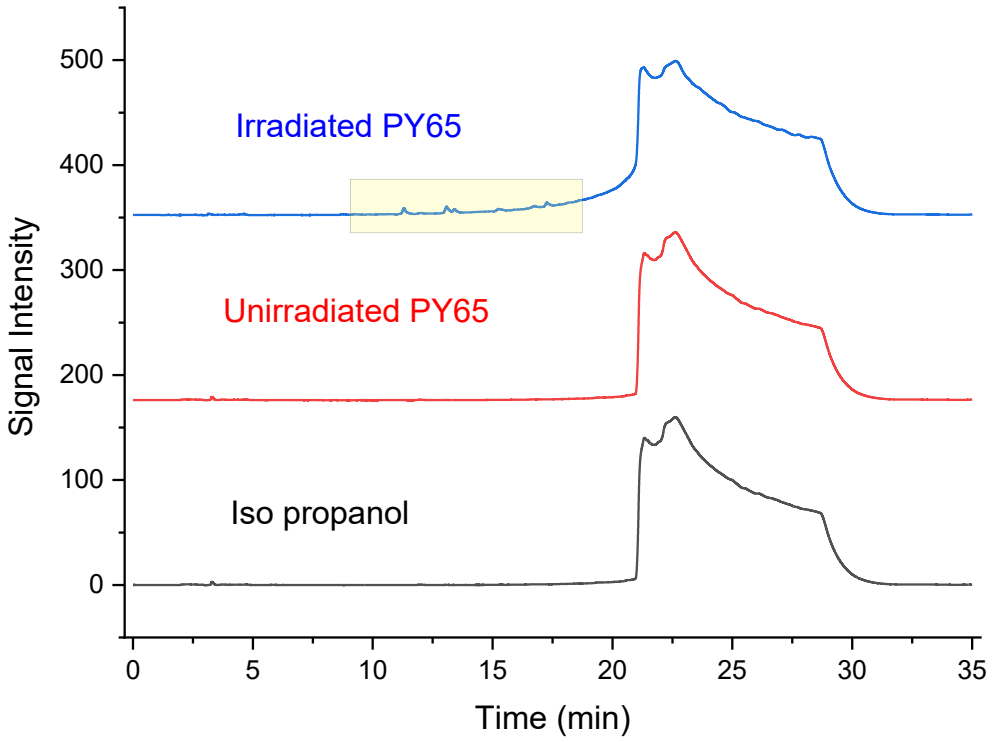



**Figure 6.6: LC-MS chromatogram of unirradiated and irradiated PY65 using a new gradient elution.**

**Curves**

Following the implementation of the new gradient method to improve separation, additional optimisation focused on improving peak intensity and elution efficiency for irradiated PY65. The intensity of the peaks detected in the LC chromatogram of irradiated PY65 (Fig. 6.6) remained very low. Therefore, the previously described gradient was retained, but the curve setting was adjusted from 5 to 3 and 7 (Table 6.1). Results from these adjustments showed that, in all cases, the degradation products were still strongly retained (Figs. 6.7 and 6.8). Changing the curve to 7 was not effective in eluting the products, whereas curve 3 eluted them at approximately 17 minutes.

**Table 6.1: Gradient condition used in the analysis of irradiated PY65 by LC-MS.**

| Time/min | Water (%) | Methanol (%) | Curve 5 (linear) | Curve 7 (concave) | Curve 3 (convex) |
|---|---|---|---|---|---|
| **0** | 95 | 5 | 5 | 5 | 5 |
| **20** | 1 | 90 | 5 | 7 | 3 |
| **26** | 1 | 90 | 5 | 7 | 3 |

| | | | | | |
|---|---|---|---|---|---|
| **32** | 95 | 5 | 5 | 5 | 5 |
| **35** | 95 | 5 | 5 | 5 | 5 |

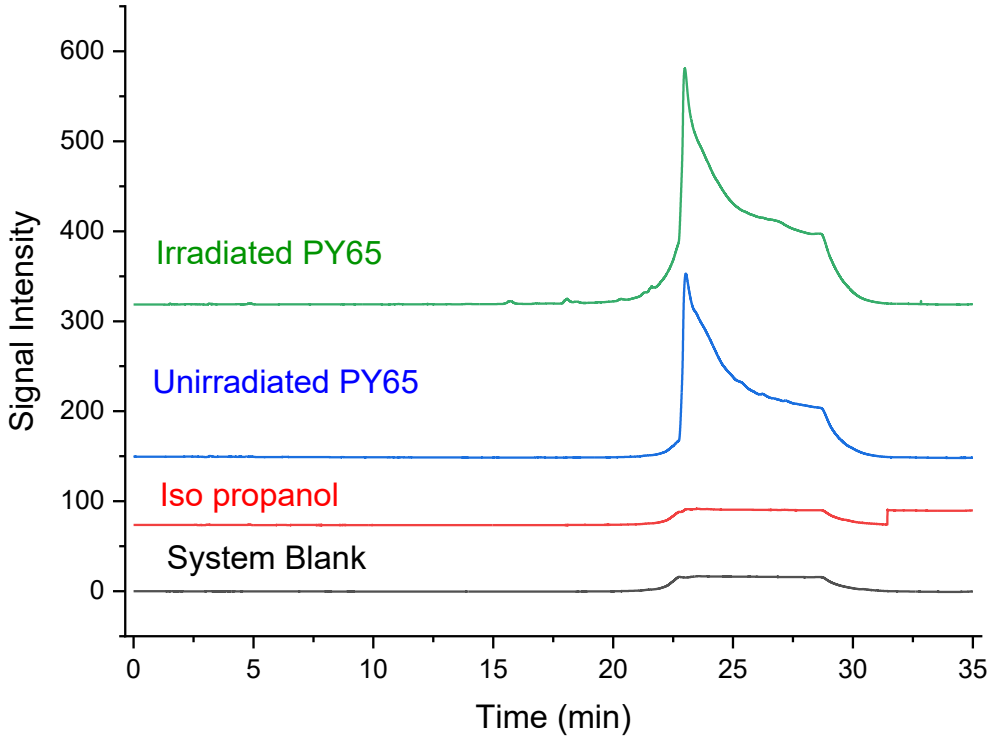



**Figure 6.7: LC-MS chromatogram of unirradiated and irradiated PY65 using a new gradient elution (curve 7).**

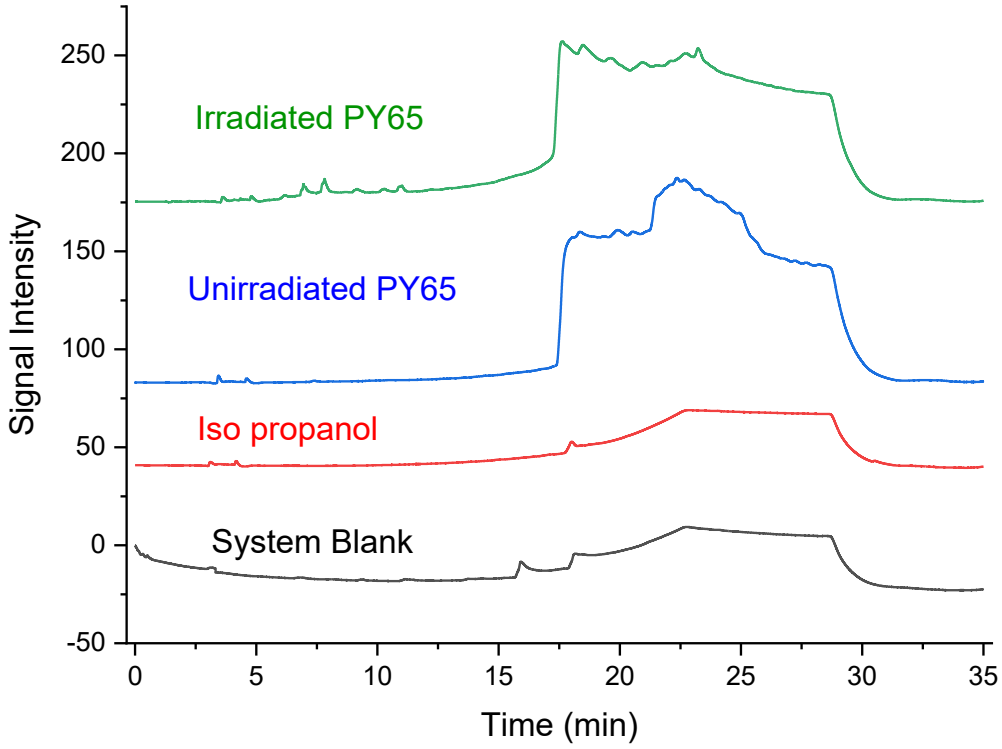



**Figure 6.8: LC-MS chromatogram of unirradiated and irradiated PY65 using a new gradient elution (curve 3).**

**Mobile phase concentration:**

After adjusting the gradient curve to improve elution time, further optimisation was necessary to address the poor peak intensity and retention issues observed in earlier trials.

Even when low intensity peaks corresponding to early-eluting products were detected, as shown in Fig. 6.8, a high concentration of organic solvent was required to elute them from the column. Therefore, the initial mobile phase concentration was modified. The organic solvent content (methanol) in the LC-MS parameters was incrementally increased to 25% and 50% (Figs. 6.9 and 6.10). The degradation products began to elute at 20 minutes and 17 minutes when the concentration was raised to 25% and 50%, respectively. However, these increased concentrations did not effectively elute the products, as peak intensity and shape remained suboptimal. As a result, a different mobile phase solvent was explored.

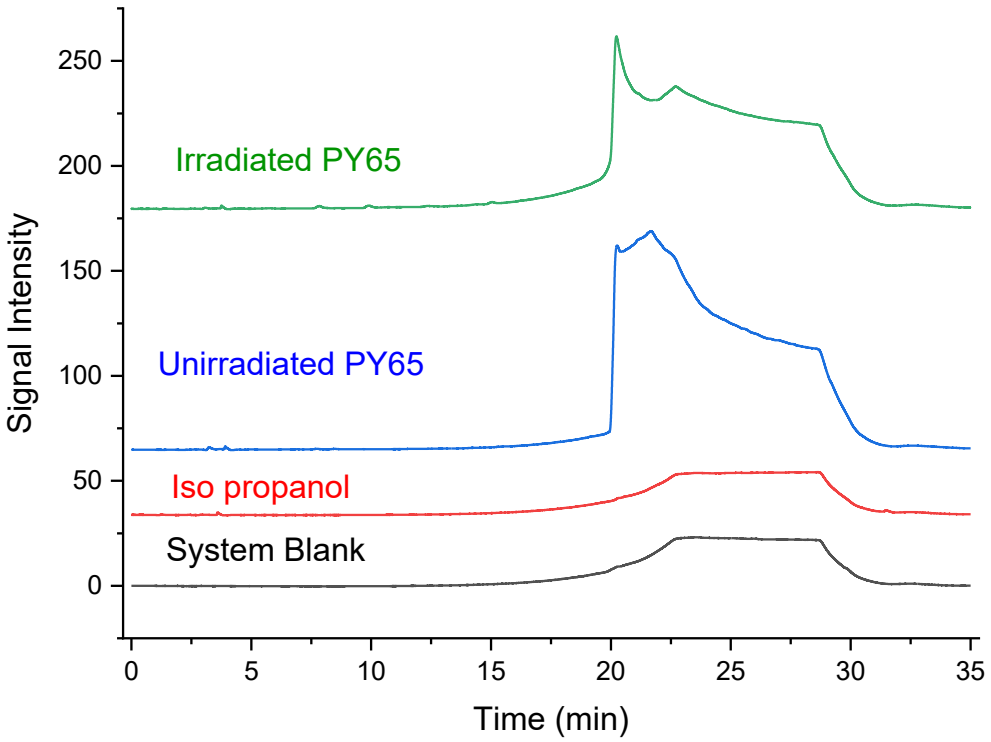



**Figure 6.9: LC-MS of unirradiated and irradiated PY65 using a new gradient elution and changing the mobile phase concentration was increased from 5 to 25%.**

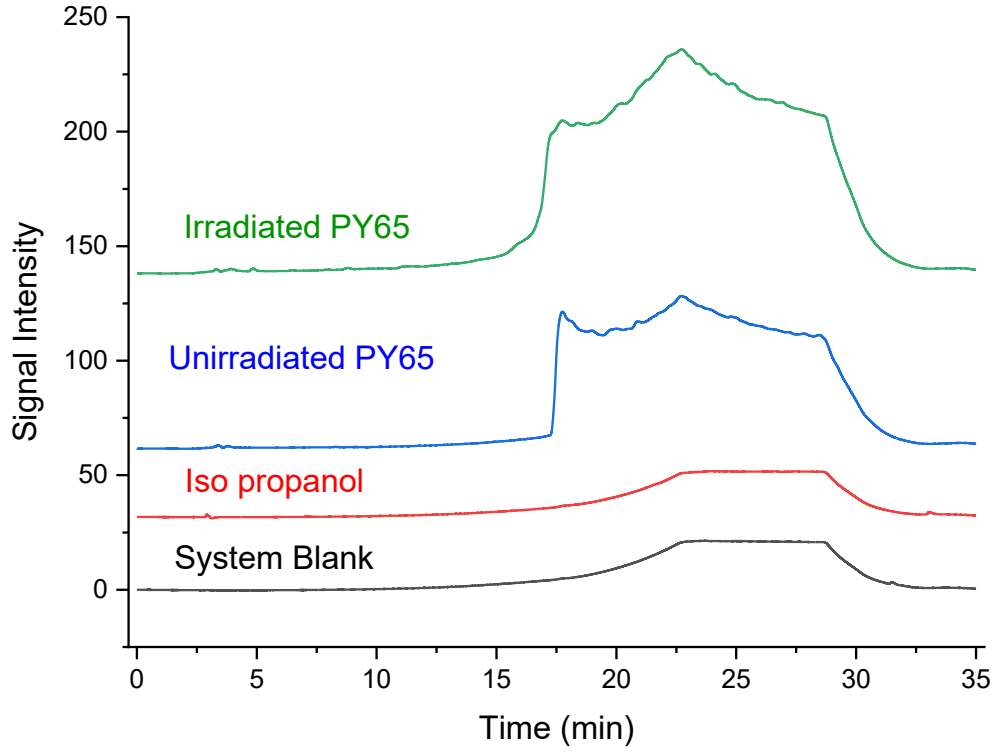



**Figure 6.10: LC-MS chromatogram of unirradiated and irradiated PY65 using a new gradient elution and changing the mobile phase concentration was increased from 5 to 50%.**

**Mobile phase type**

Following the limited success of increasing methanol concentrations in the mobile phase, alternative organic solvents were evaluated to further enhance product elution and peak shape. Organic solvents such as ACN and THF were tested as mobile phase additives to assess their efficiency in eluting degradation products from a C18 column under different gradient conditions (Table 6.2). Selection of these solvents was based on their polarity indices, (5.8 for ACN, and 4.0 for THF), which are higher or lower relative to methanol. The use of ACN resulted in a significant improvement, with degradation products eluting earlier at 13 minutes and displaying more optimal peak shapes (Figs. 6.11, 6.12, and 6.13). Furthermore, using 25% THF led to product elution in before 5 minutes. However, increasing the THF concentration to 50% caused irregular peak shapes and shifts in retention time, likely due to column compatibility issues.

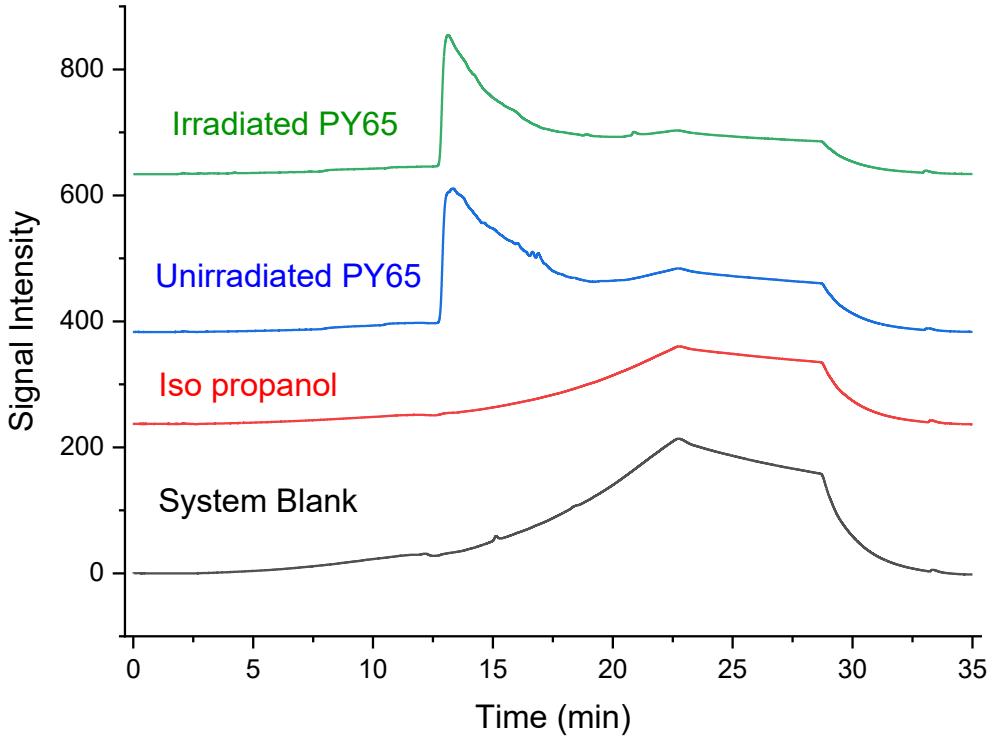



**Figure 6.11: LC-MS chromatogram of unirradiated and irradiated PY65 using Acetonitrile.**

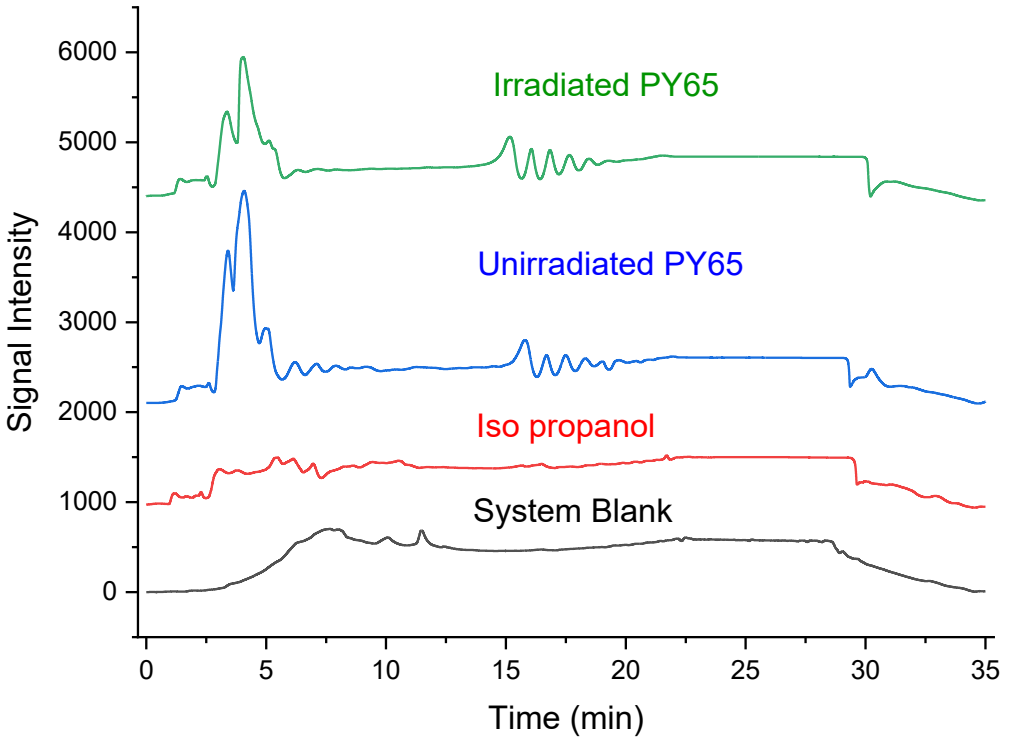



**Figure 6.12: LC-MS chromatogram of unirradiated and irradiated PY65 using THF 25%.**

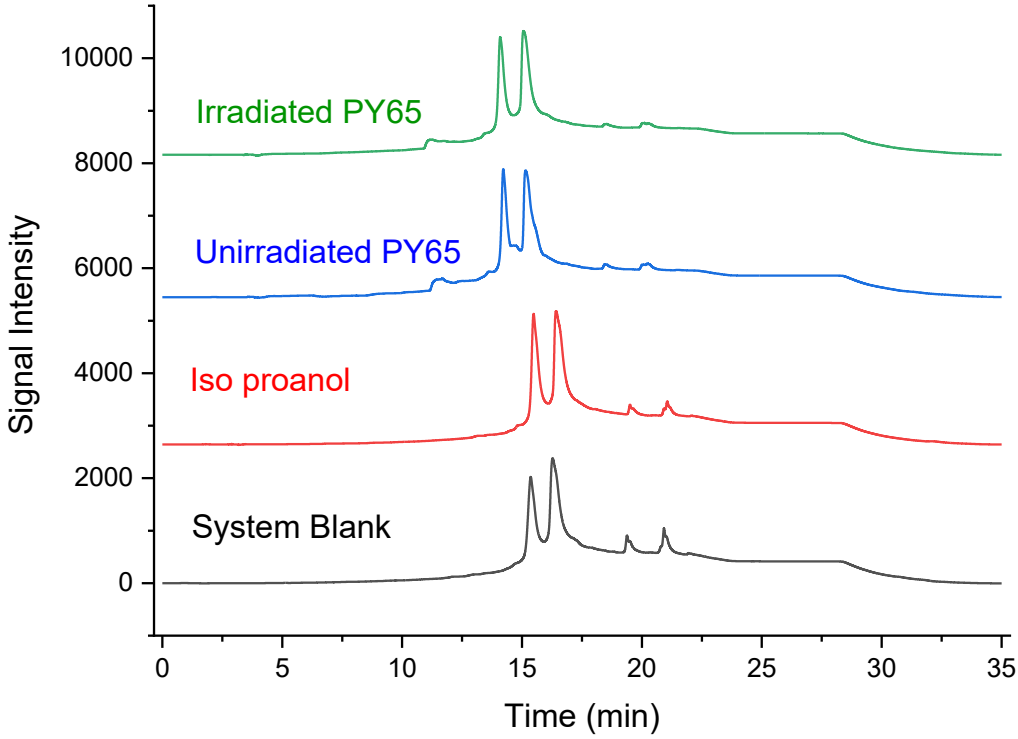



**Figure 6.13: LC-MS chromatogram of unirradiated and irradiated PY65 using THF 50%.**

**Table 6.2: Gradient conditions used to optimize the LC-MS analysis of irradiated pigments. The mobile phase consisted acetonitrile or tetrahydrofuran, with water containing 0.2% formic acid.**

| Time/min | CAN (%) | THF (%) | THF (%) | Curve |
|---|---|---|---|---|
| **0** | 50 | 25 | 50 | 5 |
| **20** | 99 | 75 | 99 | 5 |
| **26** | 99 | 75 | 99 | 5 |
| **32** | 50 | 25 | 50 | 5 |
| **35** | 50 | 25 | 50 | 5 |

## Column type

Due to irregular peak shapes and retention shifts observed with the C18 column, likely caused by column degradation, a new column with a C3 stationary phase was trialled to improve separation of laser fragmentation products.

A new column with a C3 stationary phase was trialled with different concentration of acetonitrile and water mobile phase to improve laser fragmentation separation (Table 6.3). The LC-MS was outfitted with a ZORBAX 300SB-C3 4.6 × 250 mm, 5 µm LC column and ran at 0.6 mL/min with a two-phase solvent gradient of water and ACN. This gave improved results when compared with the C18 column.

**Table 6.3: LC-MS condition using ACN as a solvent and C3 column.**

| Time/min | ACN (%) | ACN (%) | ACN (%) |
|---|---|---|---|
| **0** | 10 | 20 | 30 |
| **20** | 90 | 90 | 90 |
| **26** | 90 | 90 | 90 |
| **32** | 10 | 10 | 10 |

The results from these attempts indicate that using ACN is the best solvent that could elute the breakdown products before the switch between the stationary phases (Fig. 6.14). Therefore, the final LC-MS method that could be used in future studies is described below.

The optimal LC method was performed using an LC-MS system equipped with a ZORBAX 300SB-C3 column (4.6 × 250 mm, 5 µm). The column was operated at a flow rate of 0.6 mL/min with a two-phase solvent system: water (A) and acetonitrile (ACN, B). The best solvent gradient began with 70% A, held for 4 minutes, then linearly decreased to 10% A over 20 minutes. This composition was maintained for 6 minutes, followed by a return to 90% A over 6 minutes. Post-run conditioning was performed using 30% water and 70% ACN, held for 5 minutes. The sample injection volume was 2 µL. The ISQ EC mass spectrometer operated with an ionisation energy of 70 eV and was set to detect positive ions in the range of 10 to 1000 amu. UV-Vis detection was carried out in the range of 190–680 nm

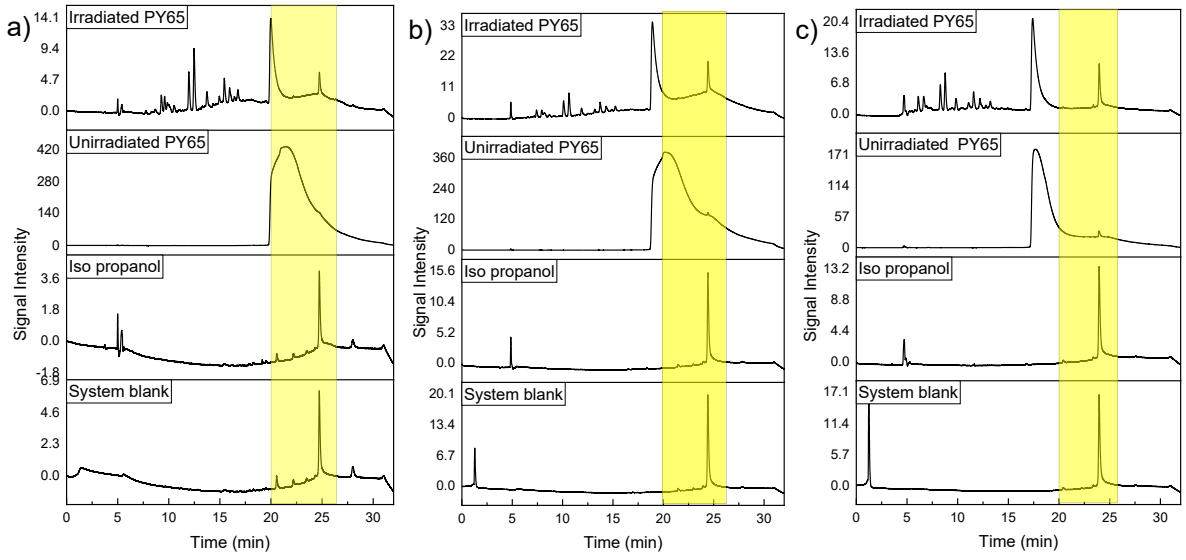



**Figure 6.14: LC-MS chromatogram of unirradiated and irradiated PY65 using a C3 column with different concentration of ACN: a) 10%, b) 20%, and c) 30%.**

### 6.2.2 Extended photodegradation studies on other pigments and ink colours

Future research can broaden the scope to examine the photodegradation of a wider range of pigments and inks, including other colours frequently used in tattoos. This would provide a more comprehensive understanding of pigment behaviour and breakdown under laser treatment.

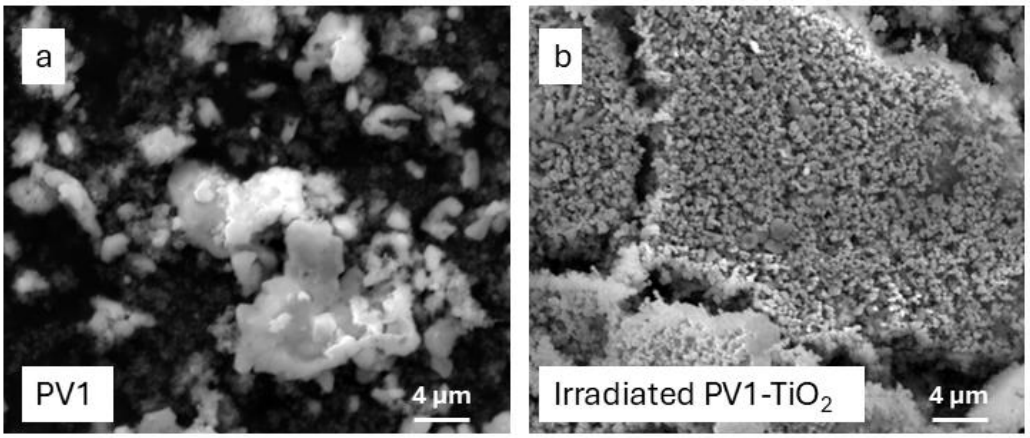
Research presented in Chapter 2, 3, 4, and 5 primarily focused on yellow tattoo inks, a popular but challenging colour to remove. The reference pigments were selected based on their simple chemical structures containing elements such as H, C, O, N, and Cl. However, other pigments, like PG7, PG36, PG50, PB15, and PV32, contain additional atoms such as Cu, Br, S, and more Cl. These differences may alter their interactions with laser light in the presence of $TiO_2$ or melanin pigment. Future work should investigate pigments such as PV1, PB15, PG36, PG7, and PO13 mixed with $TiO_2$ using the laser irradiation method described in Chapter 3. GC-MS, DLS, and SEM imaging should be conducted on these mixtures before and after laser irradiation.

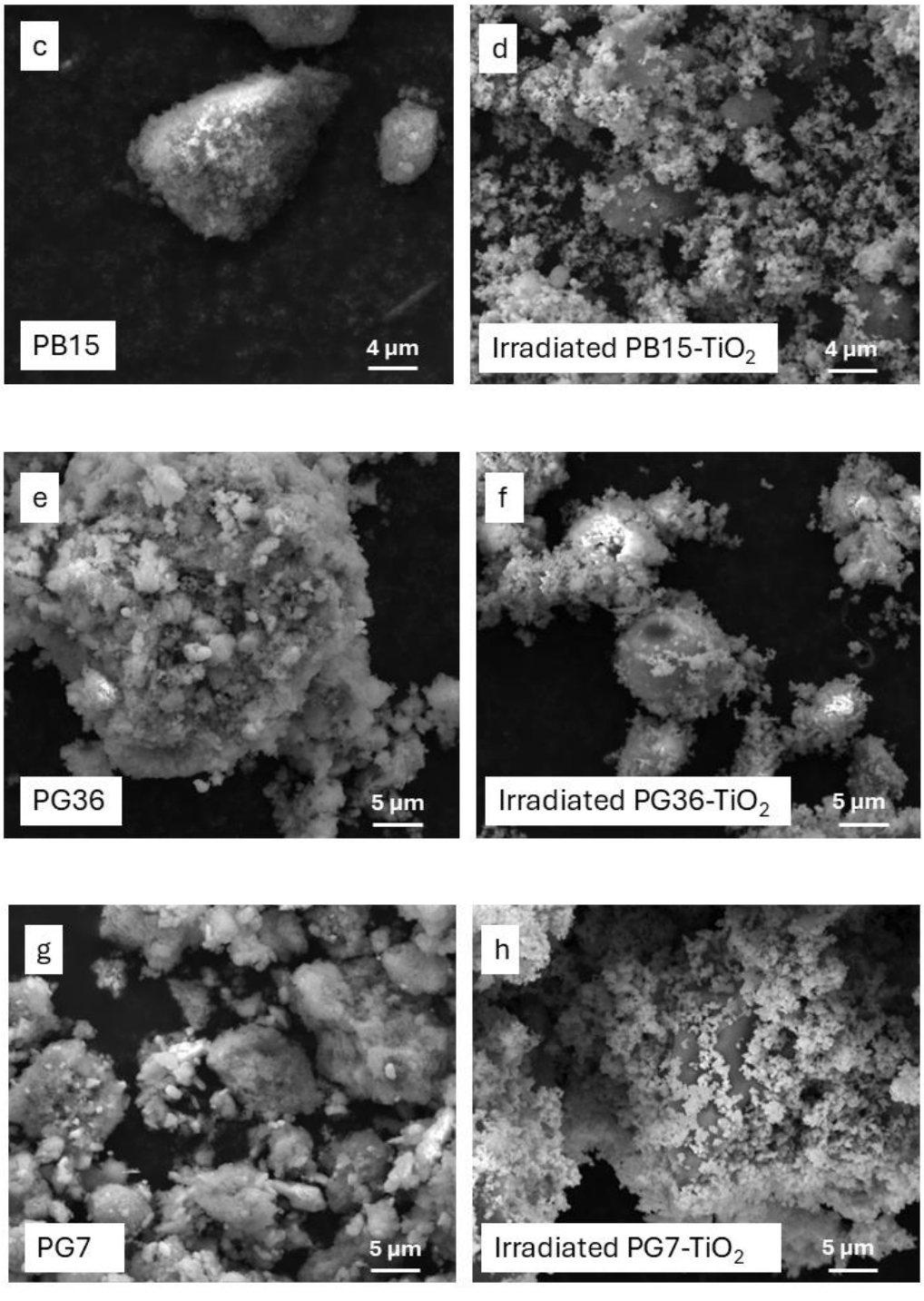
## Sample preparation and laser experiment setup

Preliminary work was undertaken for PV1, PB15, PG36, PG7, and PO13 mixed with $TiO_2$ where the pigments were irradiated using the same conditions as outlined in Chapter 3(Section 3.2.2). The resulting pigment morphologies examined by SEM. The SEM analysis was conducted as described in Chapter 3 (Section 3.3.2).

## Preliminary results

Preliminary SEM analysis, presented below, suggests that different pigments exhibit various morphologies and distinct $TiO_2$ aggregation behaviours after laser treatment (Fig. 6.15). Therefore, it is expected that $TiO_2$'s influence on volatile fragments and particle size following laser irradiation may differ from that observed with yellow pigments




c
PB15
4 µm
d
Irradiated PB15-TiO2
4 µm
e
PG36
5 µm
f
Irradiated PG36-TiO2
5 µm
g
PG7
5 µm
h
Irradiated PG7-TiO2
5 µm

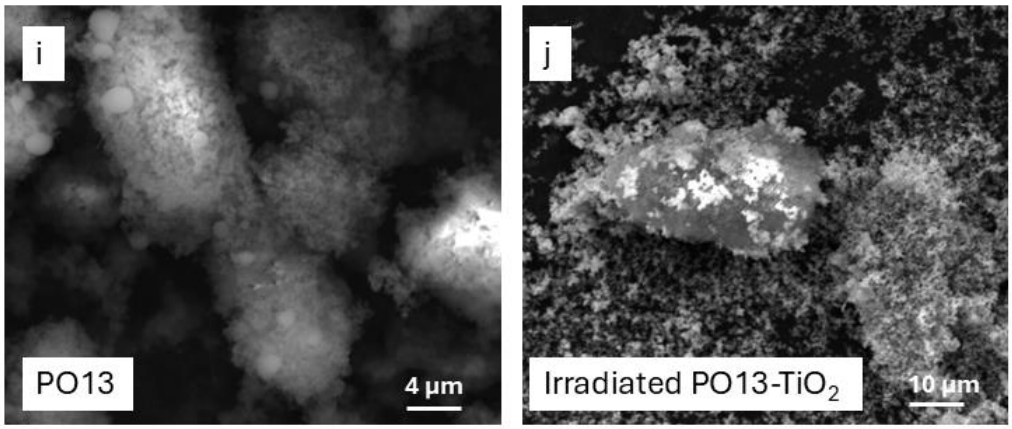



**Figure 6.15: SEM Images of unirradiated PV1, PB15, PG36, PG7, PO13, and irradiated mixture of these pigments with $TiO_2$.**

### 6.2.3 Influence of picosecond pulse duration on the photodegradation of yellow pigments

Tattoos are becoming more widespread in society. According to reports, up to a quarter of the Australian and US populations have at least one tattoo. Although tattoos have grown in popularity in recent years, it's estimated that about 17% of people with tattoos are considering having them removed, with roughly one in five expressing a desire to have their tattoos removed [6, 7]. The most effective method for removing tattoos is Q-switched lasers; however, the mechanism underlying tattoo ink fragmentation remains relatively unknown. As a result, there is little agreement on the best laser settings for first and future therapy sessions. According to a recent study, eight or more sessions are frequently required for a desirable outcome. Greater knowledge of the mechanisms of pigment reduction is required to optimise laser parameter selection, increase the effectiveness of each treatment session, and reduce the total number of treatment sessions required [7, 8].

Conventional laser techniques utilised for tattoo removal have pulse lengths within the nanosecond range [9, 10], with more recent technology exploiting picosecond lasers as most tattoo chromophores possess a thermal relaxation time of less than 10 nanoseconds ($10^{-9}$) [11]. Picosecond-domain lasers have been gradually gaining popularity because of their shorter pulse durations, which heat the targeted chromophores more quickly and improve tattoo removal [9, 12]. Picosecond laser technology was first developed for commercial usage in order to enhance tattoo clearing by more effectively breaking nanometre-sized tattoo ink particles accumulated within macrophages and fibroblasts than nanosecond domain lasers [13].

When compared to nanosecond lasers (NSLs), picosecond-domain lasers (PSLs) target chromophores by irradiating the tattooed skin at greater peak power and extremely short pulse duration[14, 15]. Laser therapy with picosecond pulse duration allows more efficient energy transfer to target chromophores of smaller size than nanosecond-domain laser treatment. Tattoo fragmentation is caused by photoacoustic impacts as described in Chapter 1 rather than photothermal effects with shorter picosecond pulse durations [16]. In terms of side effects after laser irradiation, such as tattoo darkening, applying a picosecond laser creates less heating, preventing additional darkening and reducing heat-related adverse effects. Moreover, PSLs may provide better mechanical breakdown of pigment particles (breaking up particles into smaller fragments that are more easily phagocytosed)[17]. In addition to improving the effectiveness of treatment, picosecond technology allows for lower fluences to be given during treatment, which presumably reduces the chance of undesirable side effects [18, 19].

Picosecond lasers have not only improved tattoo clearing efficiency [19] but frequency-doubled Nd:YAG at 532 nm picosecond lasers have been demonstrated to be particularly successful in the

removal of yellow ink [9]. Yellow tattoo inks have historically been challenging to remove using lasers because their absorption peaks (440 nm, 470 nm, and 485 nm) do not match the current wavelengths that are available in QS laser systems. Therefore, it is important to investigate the impact of PSLs on yellow tattoo inks.

Alabdulrazzaq *et al.* (2015) used a 1064/532 nm PSL on 6 yellow-pigmented tattoos [20]. All obtained 75% or higher clearance, with two-thirds approaching it in one to five sessions. In 16.5 % of instances, hypopigmentation was seen, whereas 50 % had bullae or blisters [20]. Alabdulrazzaq's achievements in the removal of yellow ink are encouraging for more dependable treatment of this challenging pigment [20]. However, greater sample size investigations should be undertaken in the future in order to verify these findings [9].

A picosecond frequency-doubled 1064/532 nm Nd:YAG was evaluated on 31 tattoos with various colours by Bernstein *et al.* (2015) [21]. An average clearance of 79 % was noted following an average of 6.5 sessions. The pigments that were most effectively removed were black, red, and yellow. Furthermore, 16% of patients had hyperpigmentation, while 9.7% had hypopigmentation [21, 22] .

Short pulses, in the picosecond range, are characteristic of picosecond lasers. Theoretically, PSLs should perform better than NSLs in terms of the effectiveness of tattoo removal, given that the thermal relaxation period of tattoo ink particles is often shorter than 10 ns [22, 23] .

## Picosecond laser versus Nanosecond laser

In a split-lesion technique, Pinto *et al.* [24] contrasted the QS NSLs with the PSLs on 30 black tattoos. After two sessions, there was no statistically significant difference in the two types of techniques. There was no substantial distinction in cases of hyperpigmentation and hypopigmentation. However, during the QS nanosecond laser therapy, individuals experienced noticeably higher discomfort [24].

The study by Ross *et al.* used 16 tattoos with various colours over the course of four sessions to compare a 35-PS and a 10-NS Nd:YAG [14] On a scale of 0 to 10, clearance was scored; a score of 10 indicated 90% or higher removal. The mean score for a picosecond laser was 6.72, whereas the mean for a nanosecond laser was 3.16. The NSLs group showed no hyperpigmentation, whereas the PSLs half showed 6.25%. PSL resulted in no visible scarring, compared with NSL, where scarring was visible [14, 22].

Lorgeou *et al*.[25] used a randomised split-lesion technique to compare two PSLs with one NSLs. In comparison to nanosecond laser, picosecond laser showed a statistically significant improvement in blue-black tattoos. Both showed similarities between hyperpigmentation and hypopigmentation[25].

In Chapter 3, yellow tattoo inks, reference pigments and mixtures of yellow pigments and $TiO_2$ was irradiated using nanosecond pulse duration. The result shows partial breaking down of these samples. It is proposed that future research investigate the effect of various laser parameters such as pulse durations (e.g., picosecond lasers) on the photodegradation of samples used in Chapter 3. This will offer insights into the optimization of laser protocols for safer and more efficient tattoo removal.

## Sample preparation and laser experiment setup

Tattoo inks were pipetted onto microscope slides and dried in open air at ambient temperature for 48 h prior to irradiation and/or characterization. Dried inks were scraped from the slide and placed in a GC vial prior to laser treatment. The pigment-$TiO_2$ samples were made by mixing PY14 in separate GC glass vials with $TiO_2$ (rutile form) with a 50:50 % ratio by mass.

Dried tattoo inks and a mixture of PY14 with $TiO_2$ were irradiated using a QS Nd:YAG laser at 532 nm. Each sample was exposed to 3140 laser pulses. The laser pulses were 157 mJ/pulse over an area of 2 mm diameter (i.e., a fluence 0.1 J/cm$^2$). The photodegradation products were analysed using a headspace GC-MS to identify volatile fragments. In addition, SEM and DLS were used to study the changes in particle shape and size following laser irradiation.

## Preliminary results

It is important to note that the laser irradiation conditions used in this study were not optimized to match those detailed in Chapters 3 and 4, due to limited access to a picosecond laser at the University of Adelaide. Given this constraint, we anticipate that having the option to increase the laser fluence could enhance the fragmentation of pigments and inks. Future work should focus on optimizing these laser parameters to improve the efficiency and safety of tattoo removal processes.

The pigment and ink samples were seen to change colour from yellow (pre-exposure) to black in one small area (post-irradiation) (Fig. 6.16). Samples were characterised before and after irradiation using GC-MS, SEM, and DLS to determine alterations in sample chemistry, composition, morphology, and particle size. Results from these analyses are discussed below.

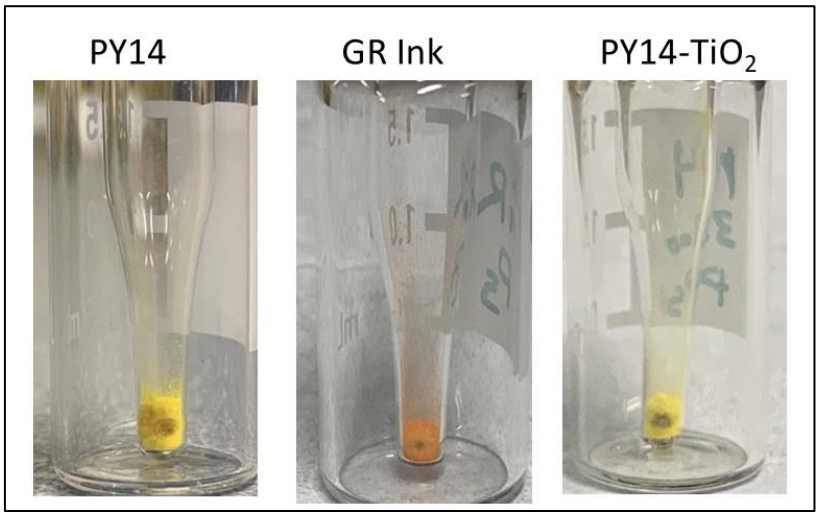



**Figure 6.16: Photographs of pigments and inks pre- and post-irradiation.**

## GC-MS

Headspace GC-MS was used to detect volatile breakdown products produced by the laser irradiation of PY14 and GR, as shown in Fig. 6.17. The GC chromatogram of the irradiated samples shows a peak corresponding to chloroform residues from the blank system. Two low-intensity peaks were observed in the GC chromatogram of the irradiated GR and BO inks, which were associated with the presence of methyl methacrylate at 1.7 min. Overall, the GC-MS analysis indicates that the laser conditions were less effective in breaking down the pigments and particles.

It has been suggested that the photoacoustic mechanism of picosecond lasers can break down tattoo ink particles. If this is the case, we would not expect to detect new volatile fragments, while changes in morphology and particle size would be observed. Therefore, SEM and DLS analyses were conducted to examine this hypothesis.

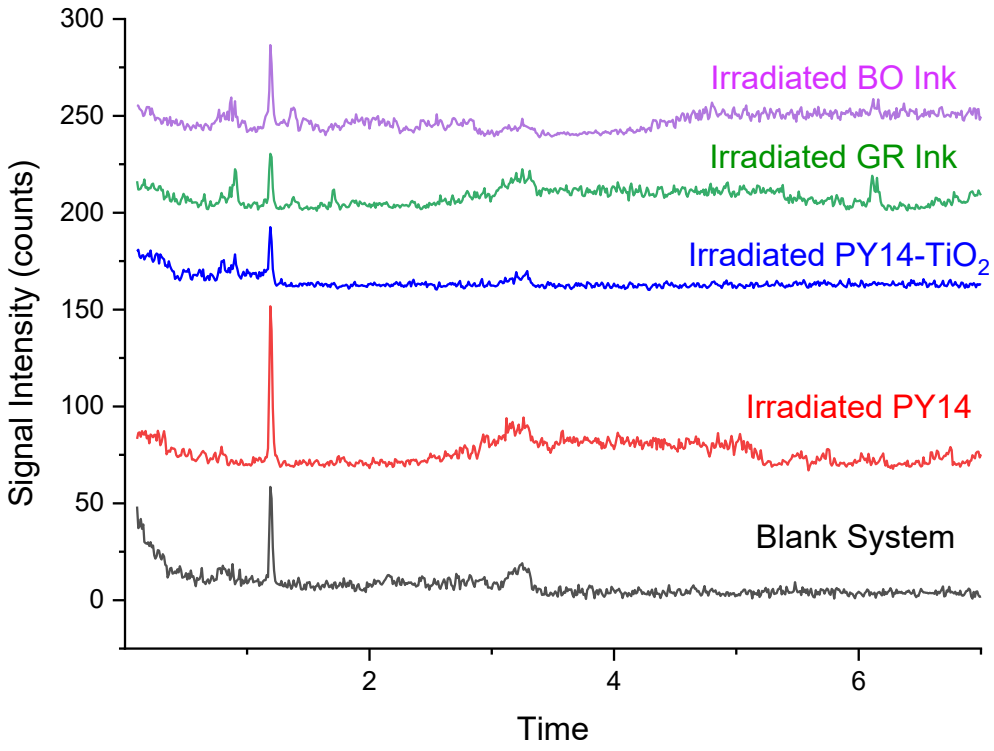
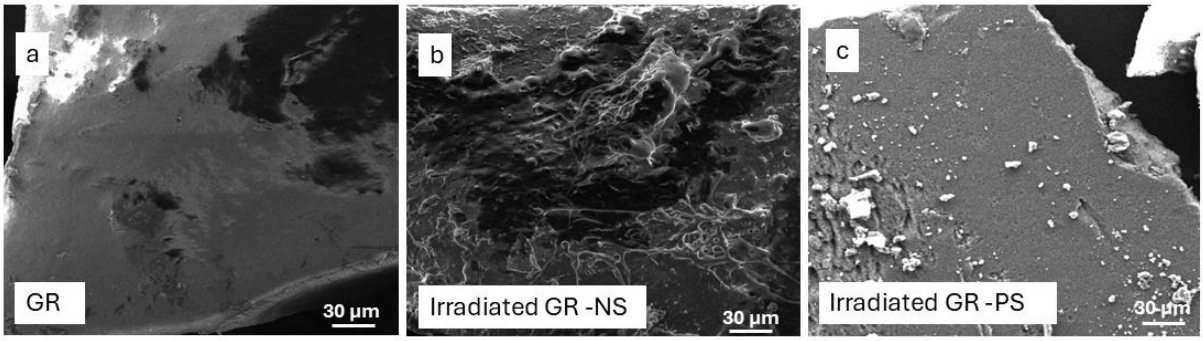



**Figure 6.17: GC-MS chromatogram of irradiated PY14, PY14-$TiO_2$, GR, and BO tattoo inks with Picosecond laser.**

## SEM

The SEM images and DLS data of unirradiated and irradiated GR and PY14 are displayed in Fig. 6.18 and 6.19. As evident from these images and diagrams , the morphology and the particle size of both PY14 and GR ink did not change upon laser irradiation. These results align with the GC-MS data, which confirmed that the laser irradiation method, employing low power energy and low fluence, was insufficient to heat the sample beyond its thermal relaxation time (TRT).



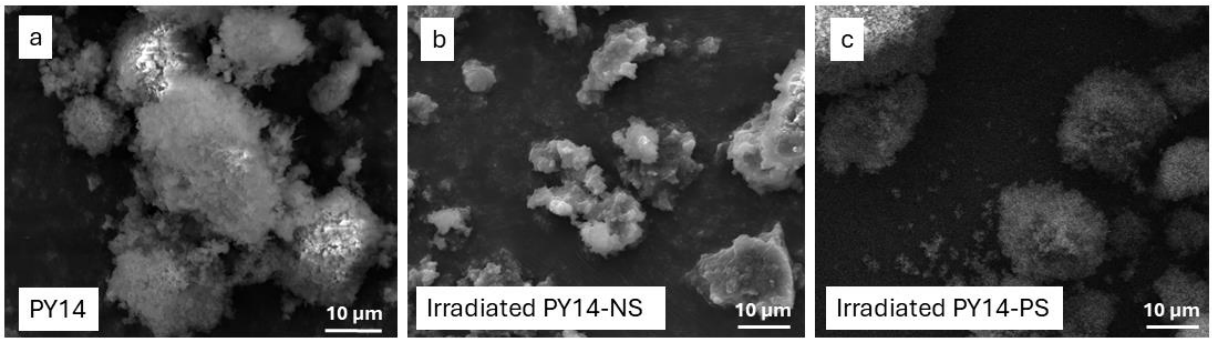



**Figure 6.18: SEM images of unirradiated and irradiated GR and PY14 ink with Nano and Picosecond laser.**

## DLS

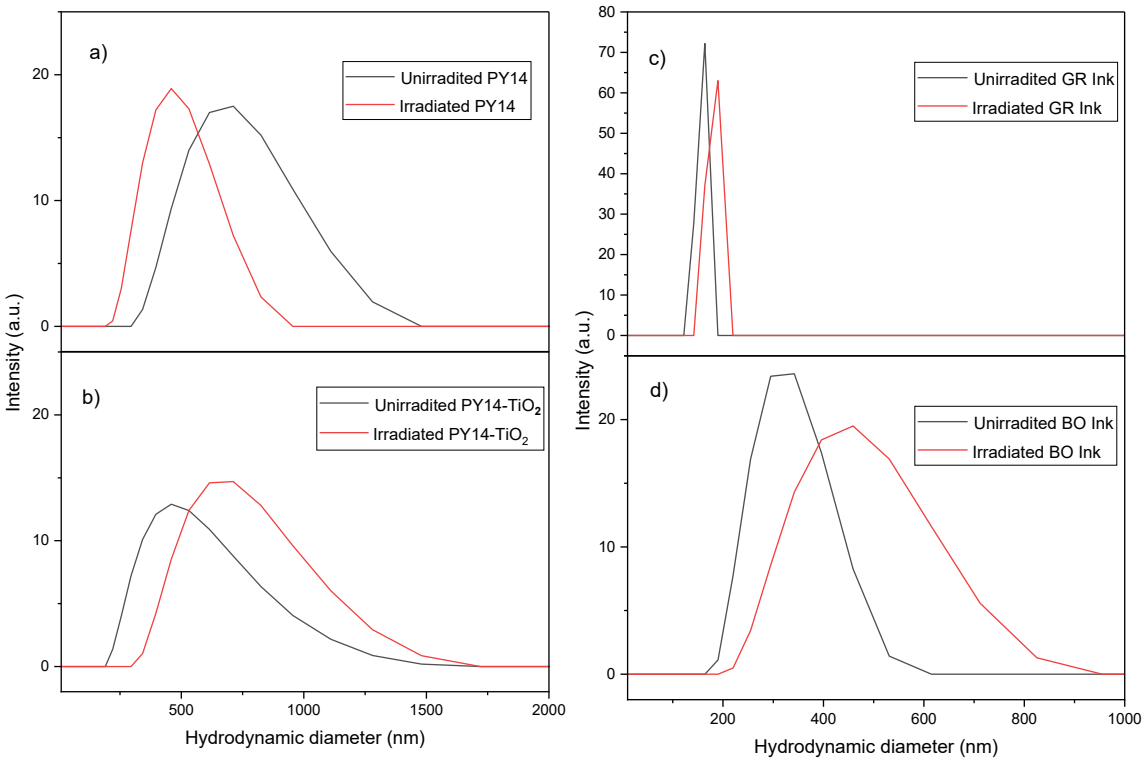



**Figure 6.19: DLS analysis of unirradiated and irradiated GR, BO, PY14, and PY14-$TiO_2$ with Picosecond laser.**

In conclusion, the results obtained from the GC-MS, SEM, and DLS analyses indicate that there was no significant change in the samples before and after laser irradiation under the applied conditions. This lack of observable change in the shape and size may be attributed to the high sample quantity and the low laser power and fluence used. As a result, the next step involves developing a new method for sample preparation to enhance the sensitivity and effectiveness of the analysis.

### Method development for sample preparation

The sample was prepared by dispersed the pigment in methanol and placed in the insert GC glass vial and left for 24h to evaporate the solution and obtain thin film of pigments and inks (see images below

Fig. 6.20). This was done to decrease the surface area. Laser condition of the nanosecond was modified to match what is available in the picosecond laser (20 pulses 532 nm Fluence 0.6 J/cm$^2$).

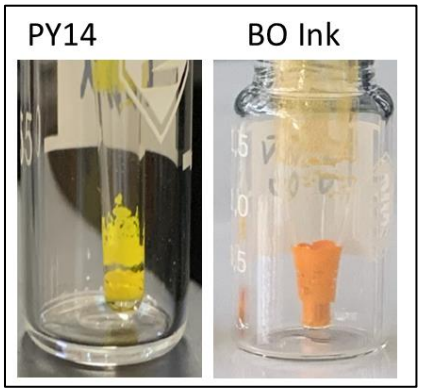



**Figure 6.20: Images of thin film of unirradiated PY14 and BO ink.**

## GC-MS

Headspace GC-MS analysis was performed on the irradiated thin films of PY14 and BO ink to detect any volatile fragments. The resulting GC chromatogram of the irradiated sample is presented in Fig. 6.21. The data indicate that the pigment and ink thin films were not affected by the low fluence laser irradiation, as evidenced by the absence of any detectable peaks. This suggests the need of increasing the power energy in the picosecond laser in the future work.

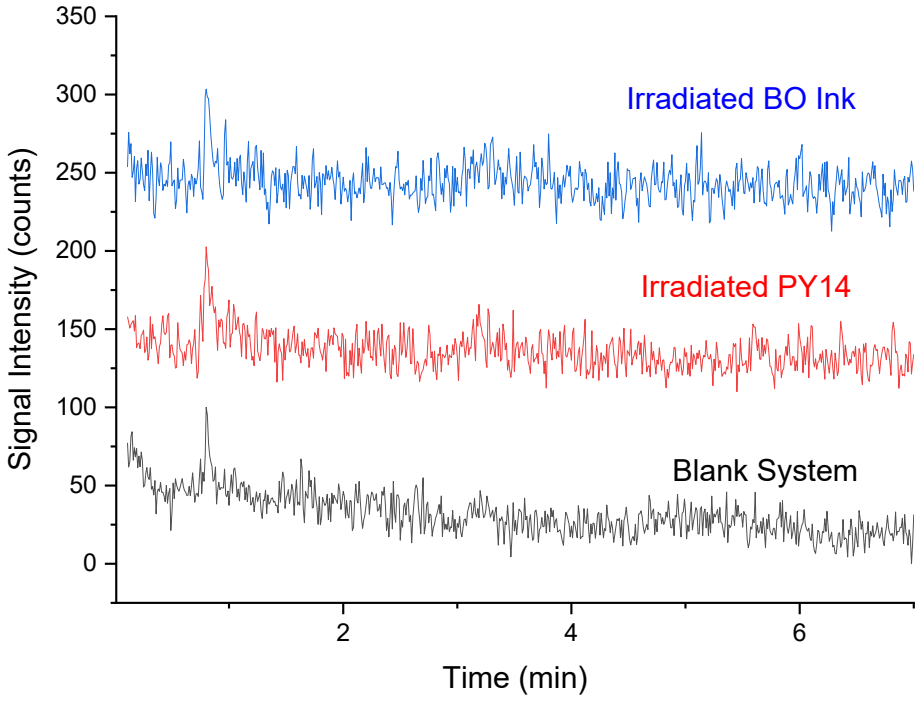



**Figure 6.21: GC-MS chromatogram of irradiated PY14 and BO ink using low power energy of nanosecond laser.**

### 6.2.4 Proposed Future Research Initiatives

## Long-term toxicity and biocompatibility studies

It is proposed that future studies conduct further *in vitro* and *in vivo* studies to assess longer term impact of laser-induced pigment and ink degradation products on skin cells and tissues. It is important to understand chronic toxicity or inflammatory responses would be vital for patient safety.

This could be achieved using the Sartorius Incucyte® system, which enables observation and quantification of cell behaviour via real-time, quantitative live-cell analysis. The Incucyte® Live-Cell Analysis System was developed to monitor cellular alterations *in situ* within the incubator. The system acquires high-resolution fluorescence and bright field photos while documenting data in real time over extended periods, including hours, days, or weeks. This technology allows users to view, and measure biological alterations [26]. This method allows continuous monitoring of cells during preparation, culture, and assays.

This research (Chapter 5) tested the effect of reference pigment and suspension of tattoo inks on HaCaT skin cells. MTT assays which is a traditional endpoint assay, showed different levels of toxicity based on the chemical structure and concentrations of the pigments. Future studies using Sartorius Incucyte® system would be able to observe and quantify cell behaviour [27, 28] .

## Investigate the toxicity impact of additional additives and carriers in tattoo inks

Tattoo inks often contain other additives, such as dispersants, preservatives, and carriers (such as alcohols, glycols, or water-based carriers), that could influence laser interactions. Further research should investigate how these additives might alter pigment degradation or generate new by-products upon laser irradiation.

## Author contribution statement:

Batool Aljubran carried out the experimental work using nanosecond and picosecond lasers, and the data analysis for (LC-MS, SEM, GC-MS, and DLS).

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**AI tools, including Quill Bot AI, were used to help check grammar and editing in this chapter.**

# 7 CHAPTER 7: APPENDICES

## 7.1 Appendix 1: (Supplementary information- Chapter 2)

### Decoding Tattoo Inks: A Multi-Technique Analysis Reveals Discrepancies in Ingredient Composition and Elemental Content When Compared to Label Claims

**Table SI 2.1: Infrared peaks of pigments and tattoo inks(dried (D) and extracted (E). The label (x) indicate the presence of absorption peaks from the sample.**

| Functional group | Wavenumber ($cm^{-1}$) | PY14 | PY65 | PO13 | LY | | GY | GY | GR | GR | BO | BO |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | D | E | D | E | D | E | D | E |
| **C-O** | 1026 | - | x | - | - | - | - | - | - | - | - | - |
| **O=N=O** | 1400-1420 | - | x | - | - | - | - | - | - | - | - | - |
| **C-Cl** | 800 - 714 | x | | x | x | x | x | x | x | x | x | x |
| **N-H** | 3300-3500 | x | - | - | x | x | x | x | x | x | x | x |
| **N-C=O** | 1670 | x | x | x | x | x | x | x | x | x | x | x |
| **C-H C=N** | 2000–4000 | x | x | x | x | x | x | x | x | x | x | x |
| **C=N** | 1400-1600 | - | - | x | - | - | - | - | - | - | - | - |

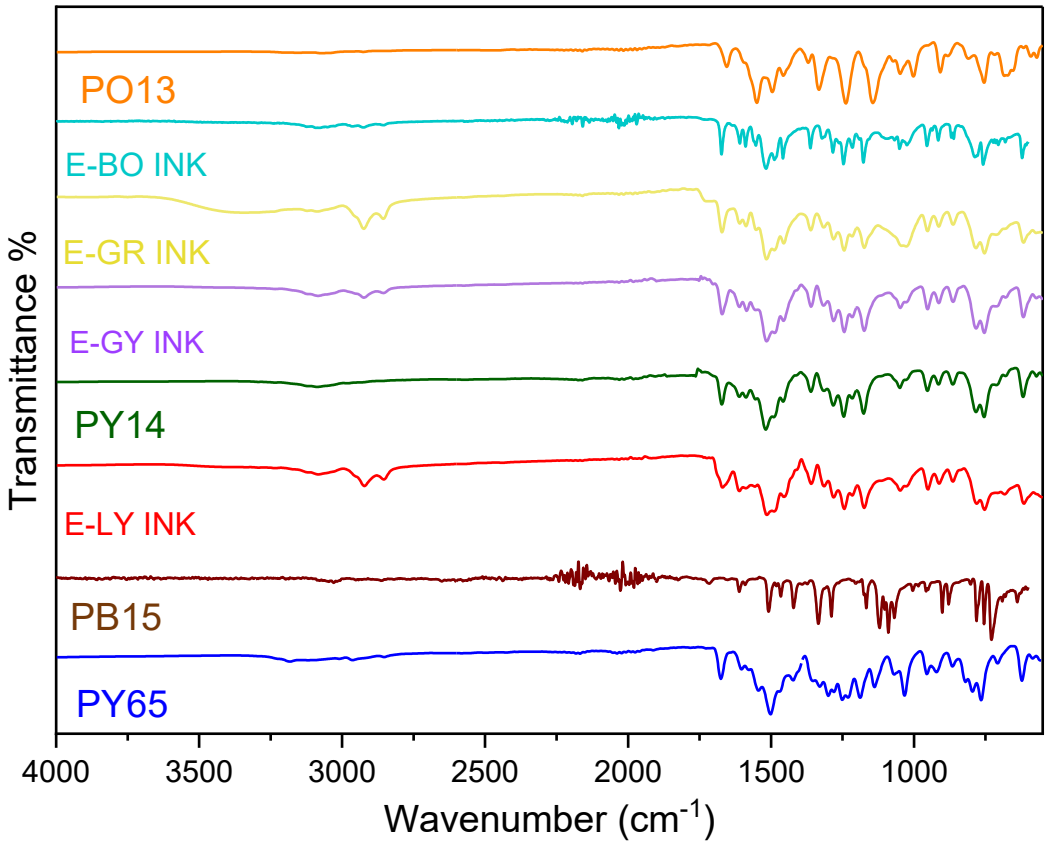



**Figure SI 2.1: The full range of the FT-IR spectrum (4000 $cm^{-1}$ to 550 $cm^{-1}$) of pigments and inks obtained using the Perkin Elmer Spectrum 100 FTIR. IR spectra comparison of inks and pigments reveals the presence of PY14 instead of PY65 in the LY ink. GY, GR, and BO inks did not have PO13.**

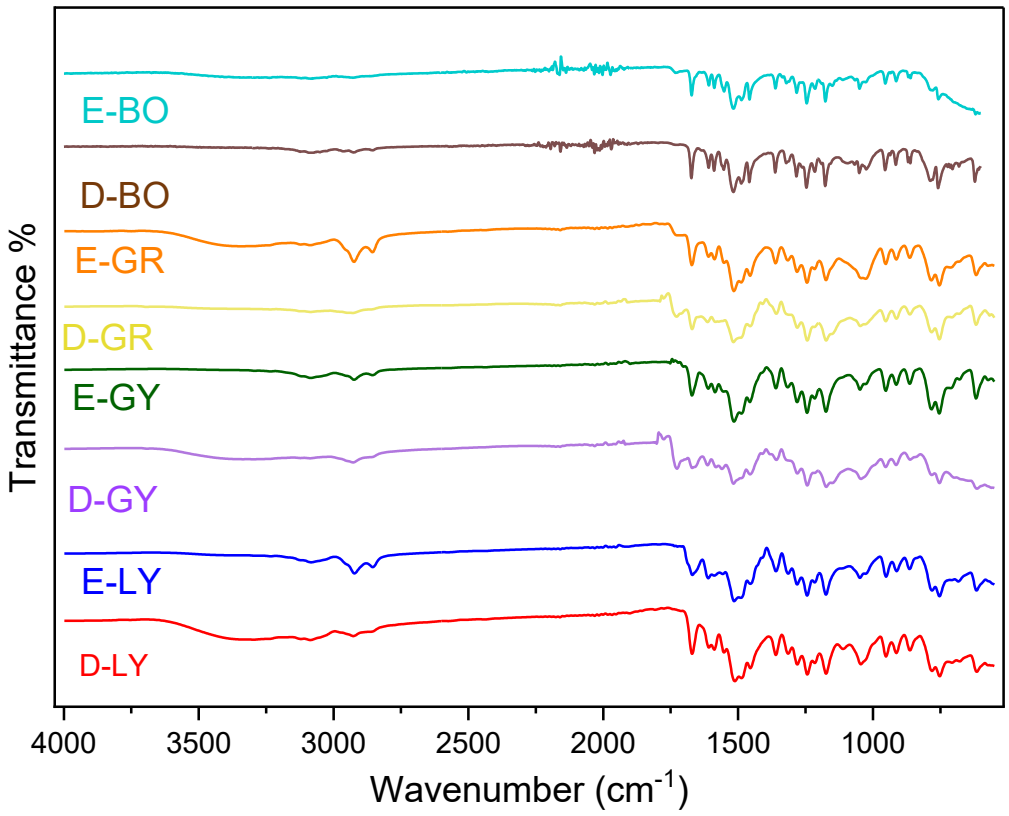



**Figure SI 2.2: FTIR spectra from the dried inks (D-LY, D-GY, D-GR and D-BO) were consistent with those of the extracted inks.**

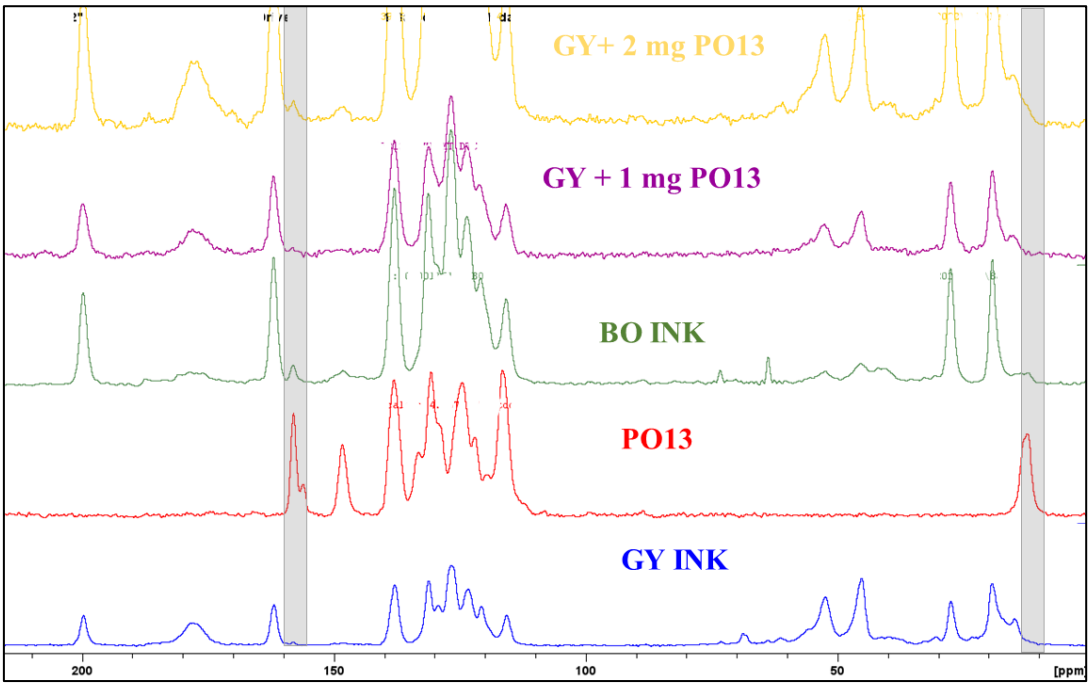



**Figure SI 2.3: $^{13}C$ NMR spectrum of BO, GY, PO13, and mixture of GY-PO13 to identify the limits of detection of the NMR instrument.**

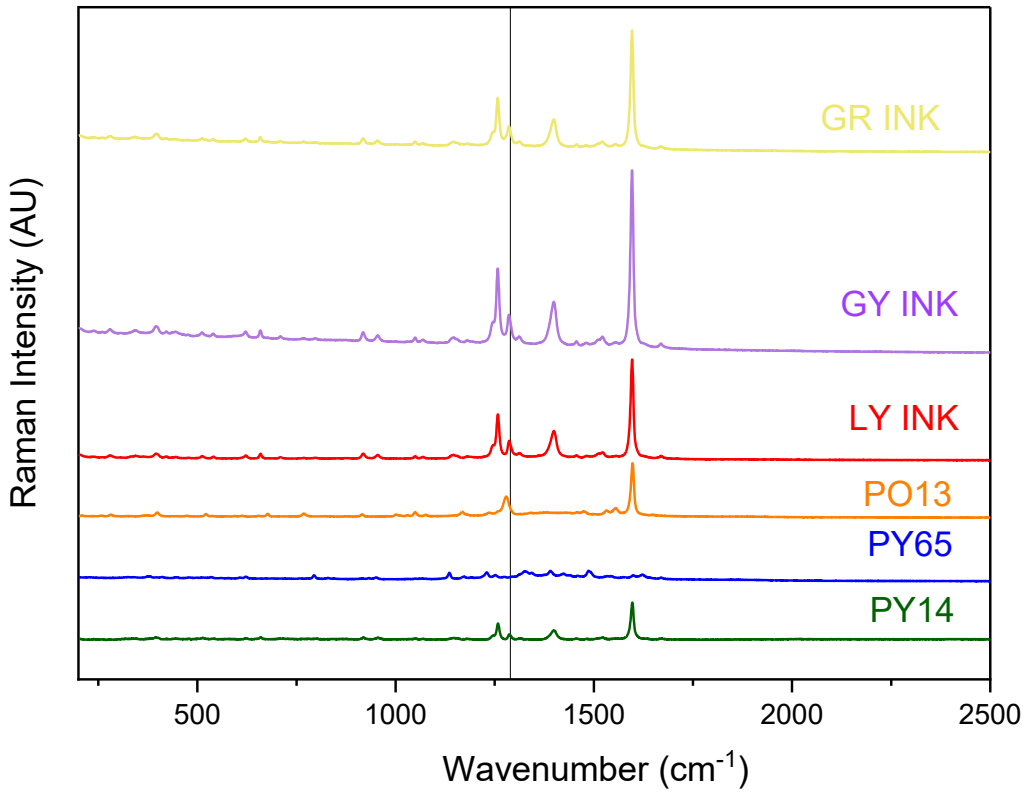



**Figure SI 2.4: Baseline-corrected Raman spectra of LY, GY, GR inks and PY14, PY65, PO13. The Raman data was collected at 786 and 532 nm. The analysis was carried out at 10 and 25% filters from 200 $cm^{-1}$ to 3000 $cm^{-1}$ wavenumbers. For each sample, 12 scans of 20-second pulses were recorded.**

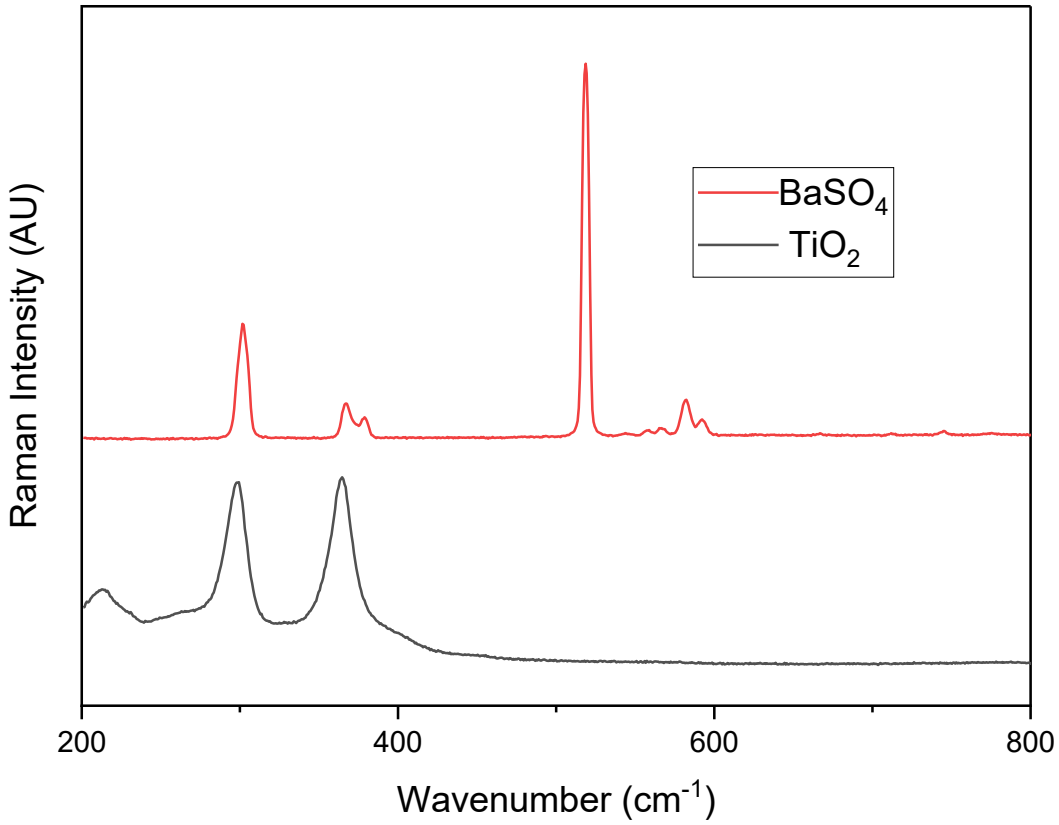



**Figure SI 2.5: Baseline-corrected Raman spectra of $TiO_2$ and $BaSO_4$.**

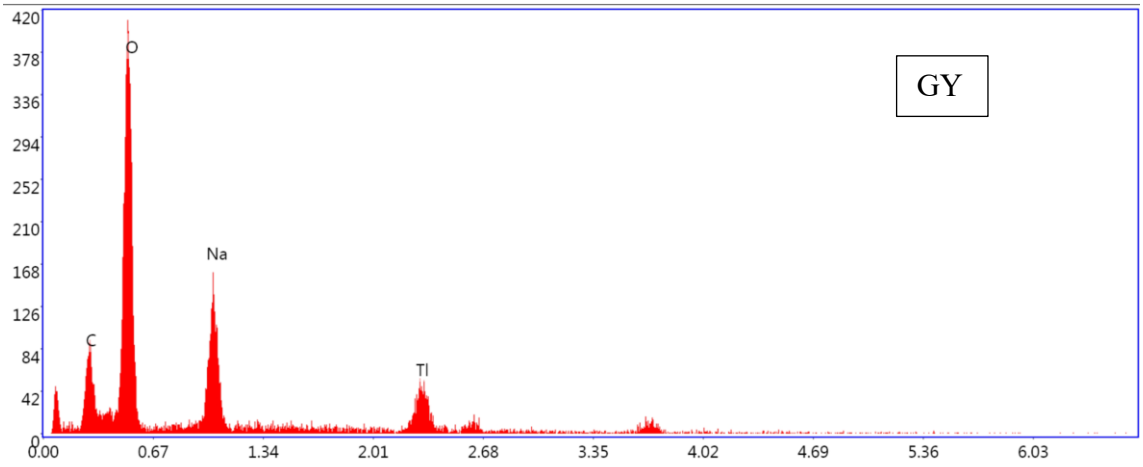



**Figure SI 2.6: Element composition analysis of GY ink using EDX.**

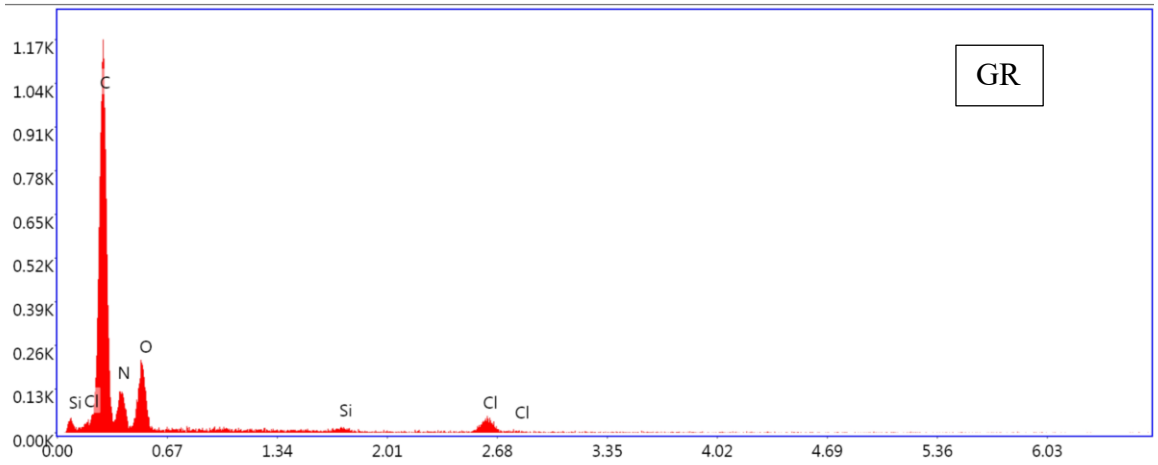



**Figure SI 2.7: Element composition analysis of GR ink using EDX.**

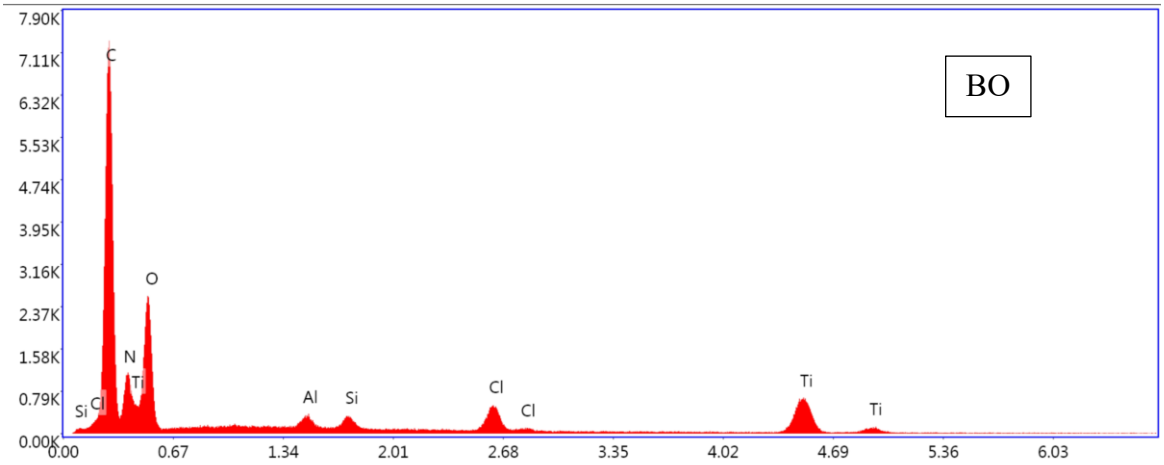



**Figure SI 2.8: Element composition analysis of BO ink using EDX.**

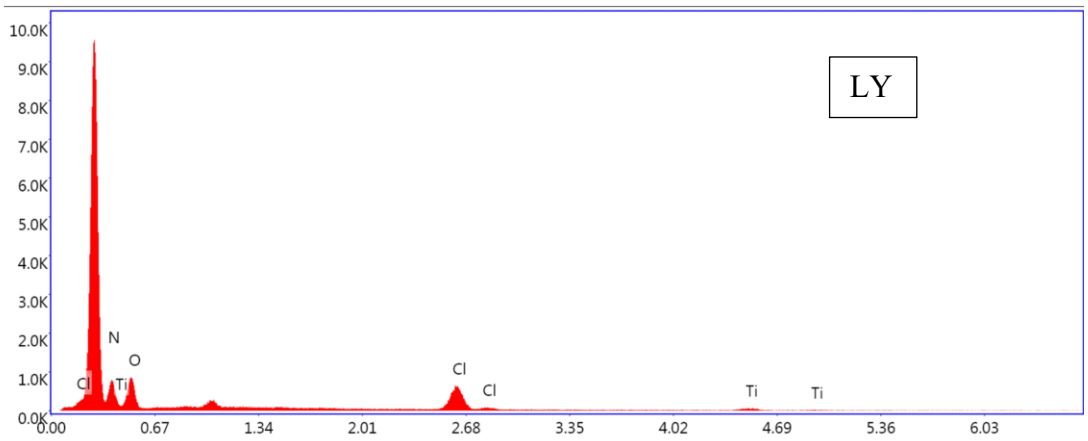



**Figure SI 2.9: Element composition analysis of LY ink using EDX.**

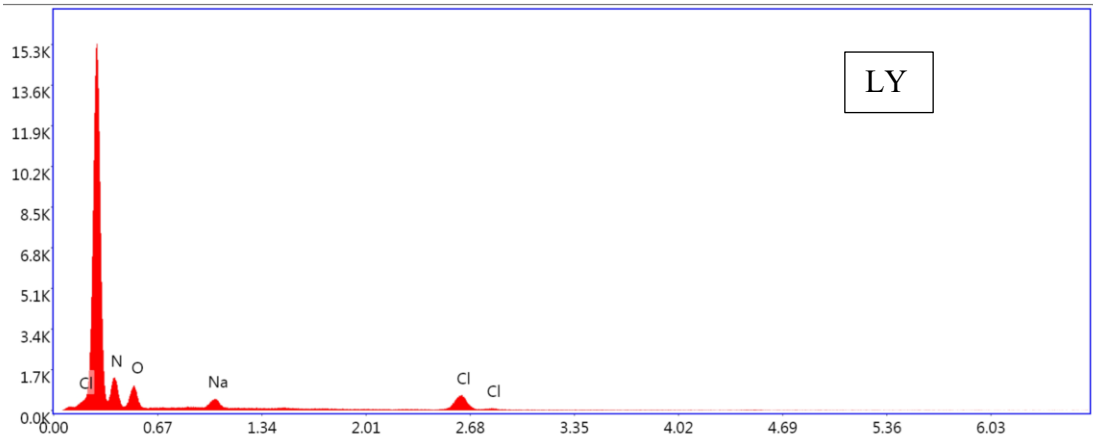



**Figure SI 2.10: Element composition analysis of LY ink using EDX.**

## 7.2 Appendix 2: (Supplementary information- Chapter 3)

### Challenges in Laser Tattoo Removal: The Impact of Titanium Dioxide on Photodegradation of Yellow Inks

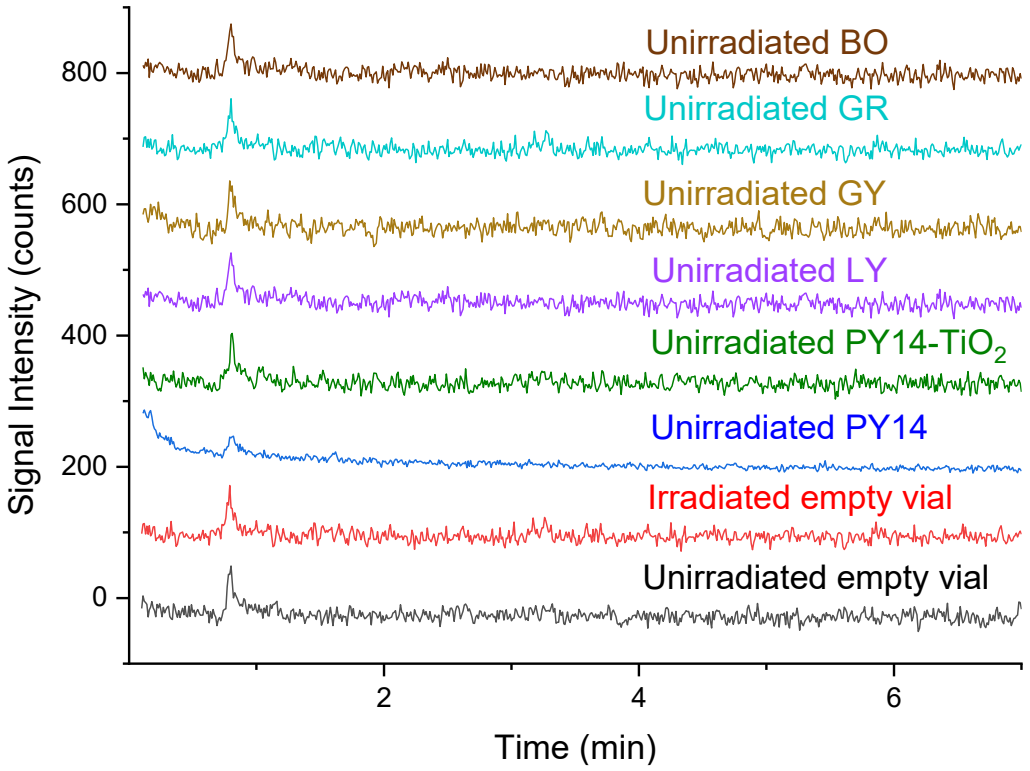



**Figure SI 3.1: GC-MS data of unirradiated and irradiated empty vial, pigments, and inks.**

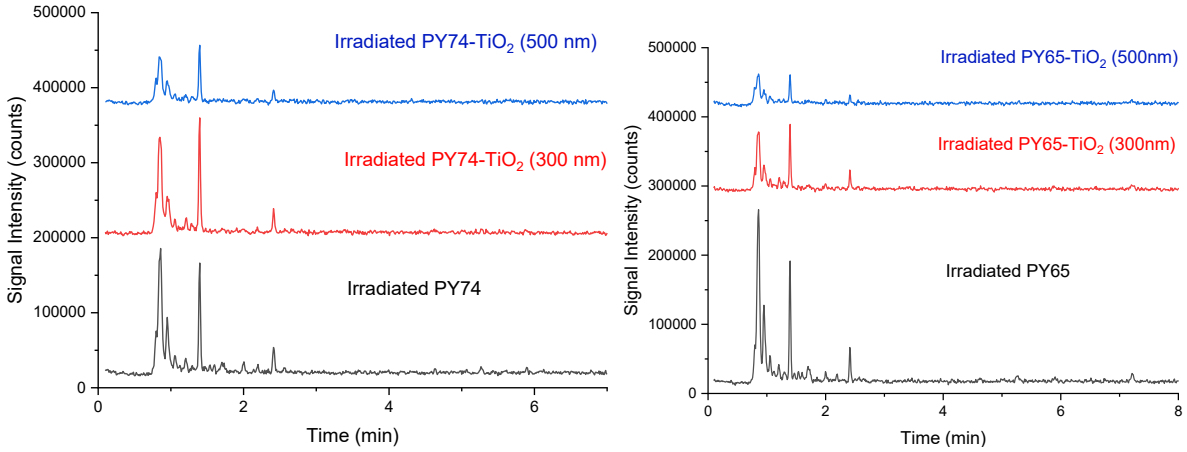



**Figure SI 3.2: GC-MS spectra of irradiated PY74, and PY65 with and without $TiO_2$.**

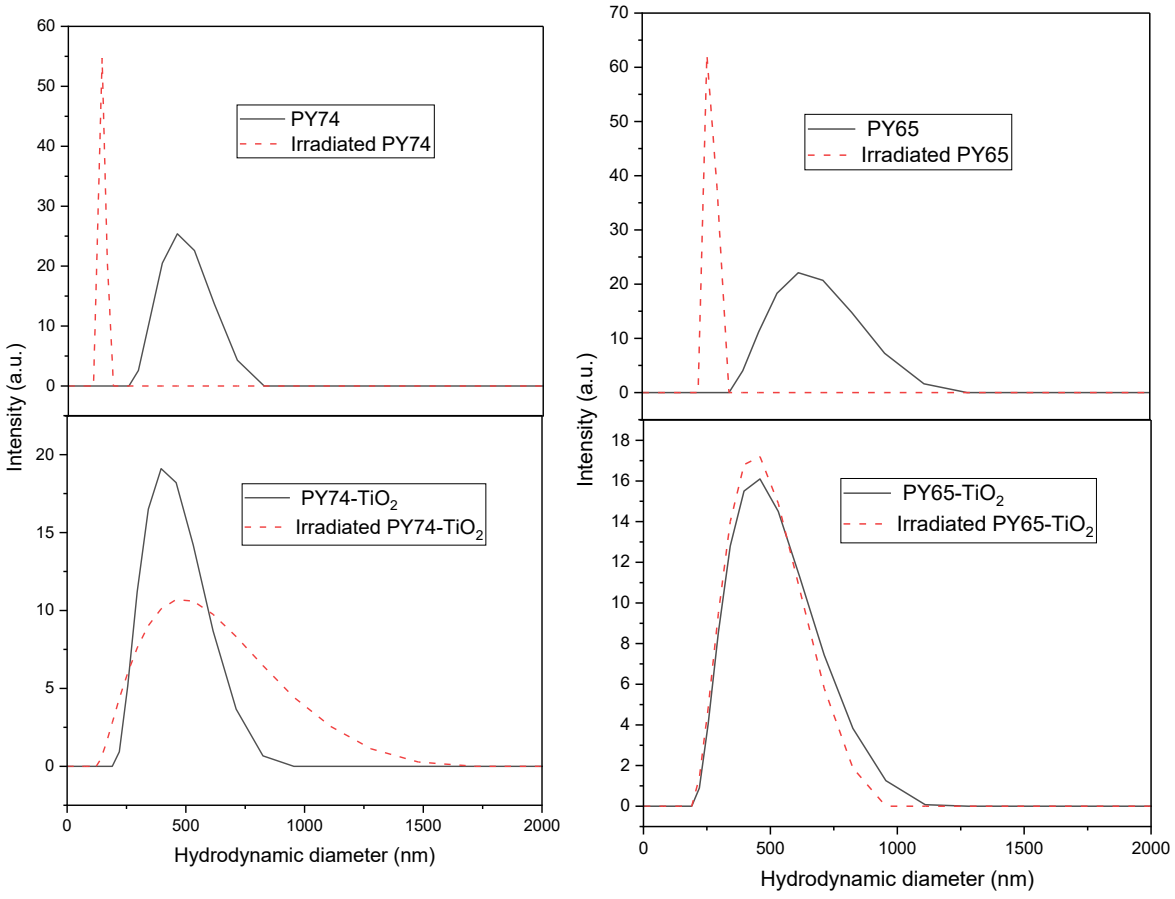
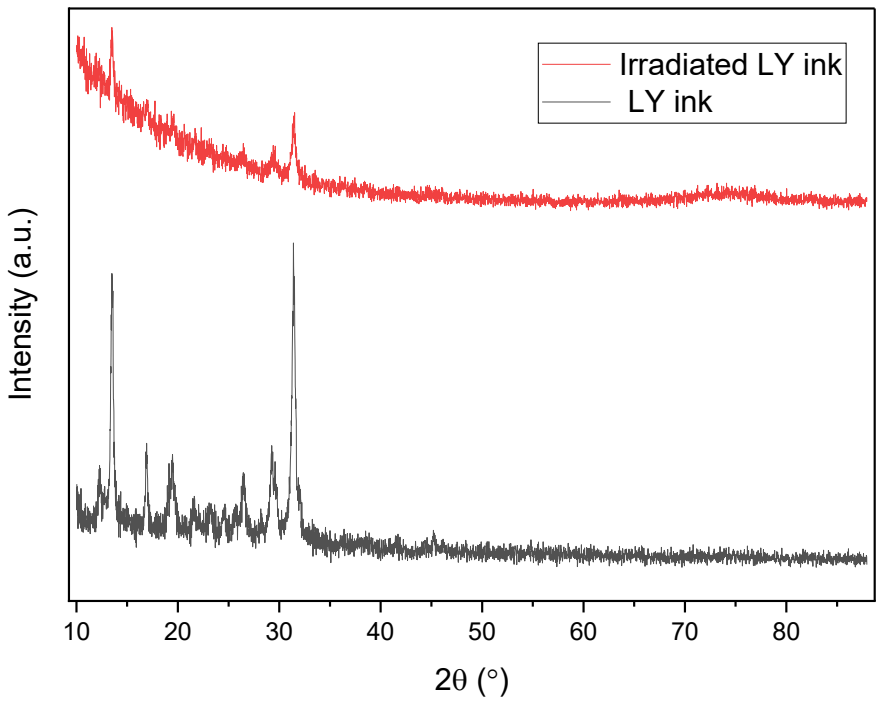



**Figure SI 3.3: DLS measurement of unirradiated pigments and irradiated PY74 and PY65 with and without $TiO_2$. Data represents the average value and standard deviation over three repeated. Comparison of the particle size of irradiated pigments with and without $TiO_2$, showing the particle size of irradiated pure pigments is smaller than irradiated PY-$TiO_2$.**

**Table SI 3.1: DLS data of unirradiated and irradiated pigments and inks.**

| **Samples** | **Particle size (nm)** | |
|---|---|---|
| | Before laser | After laser |

| | | |
|---|---|---|
| **$TiO_2$** | 300 | 473±7 |
| **PY14** | 705±39 | 301±20 |
| **PY14-$TiO_2$** | 531±20 | 461±29 |
| **PY74** | 452±46 | 165±21 |
| **PY74-$TiO_2$** | 396±11 | 334±14 |
| **PY65** | 669±24 | 272 ±16 |
| **PY65-$TiO_2$** | 459±17 | 455±13 |
| **LY** | 153±16 | 194±49 |
| **GY** | 149±20 | 492±43 |
| **GR** | 156±47 | 353±19 |
| **BO** | 329±20 | 353±27 |



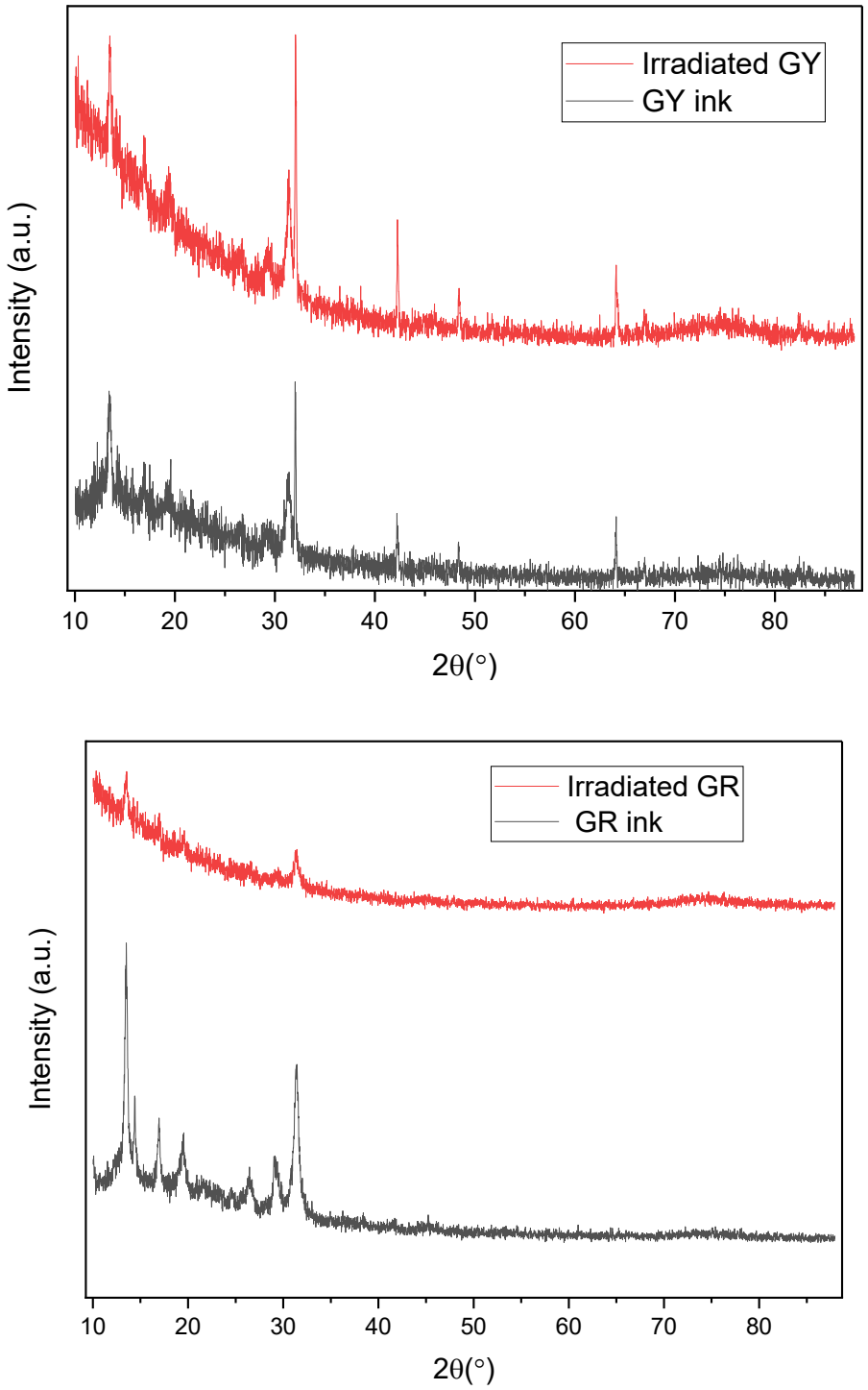



**Figure SI 3.4: XRD analysis of unirradiated and irradiated LY, GY, and GR inks. GY ink surface analysis showed high intensity peaks of $TiO_2$ after laser irradiation.**

## Laser Irradiation Method Development

A sample was prepared by making a thin film from dispersed PY14 in methanol and then it was exposed to 20 pulses of a 532 nm laser at one spot. Two lenses were utilised to focus the laser, which had a spot size of 2 mm. The laser pulses were 157 mJ/pulse over an area of 2 mm diameter (i.e., a

fluence of 5 J/cm$^2$). This laser parameter was selected based on a method that was discovered in the Lenehan's lab and matched to the condition of the clinic tattoo laser removal [1].

**Initial GC-MS method used**

Headspace gas chromatography mass spectroscopy (GC-MS) analysis was carried out using an Agilent Technologies 7890A GC system with a 5975C inert XL EI/CI MSD Triple Axis Detector and a 7693 sampler. The apparatus was fitted with an Agilent Technologies HP-5MS 5% Phenyl Methyl Siloxane column (29.4 m x 250 µm x 0.25 µm) with a $He_2$ mobile phase at a flow rate of 1 mL/min. The headspace injection volume was 5 µL. The GC was operated in isocratic mode with an oven temperature of 40 °C and an inlet temperature of 200 °C throughout the analysis. The MS ion source and quadrupole temperatures were set at 230 °C and 150 °C, respectively. All m/z values between 40 and 500 were taken in scan mode.

The observable results showed no changes in colour, and the amount of degraded product could not be collected for LC-MS analysis. Furthermore, there were no peaks associated with a volatile product in the GC-MS chromatogram (Fig. SI 3.5). There were two peaks detected at 1.7 and 2.2 minutes, which were from the blank system and corresponded to the presence of the chloroform.

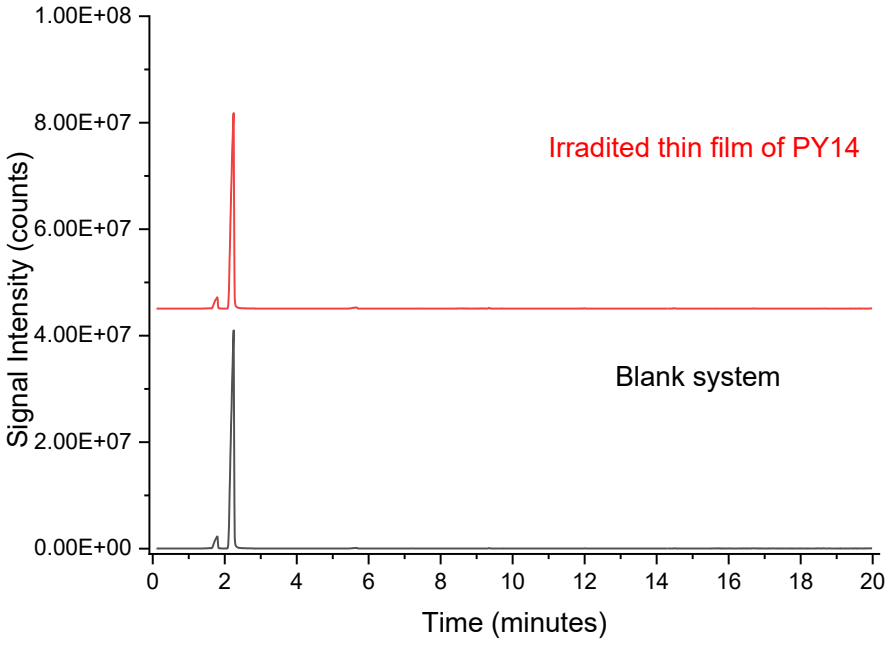



**Figure SI 3.5: GC-MS analysis of irradiated thin film of PY14**

The second method for the laser experiment was done by adding PY74 (1-2 mg) into GC glass vials fitted with reduced volume inserts. A negative control vial was set as an irradiated empty vial for GC-MS analysis. PY74 was exposed a 532 nm laser at one spot with different number of pulses ranged

from 2 pulses to 20 pulses. It is noticeable that irradiating PY74 with 20 pulses caused the colour to shift to green (Fig. SI 3.6).

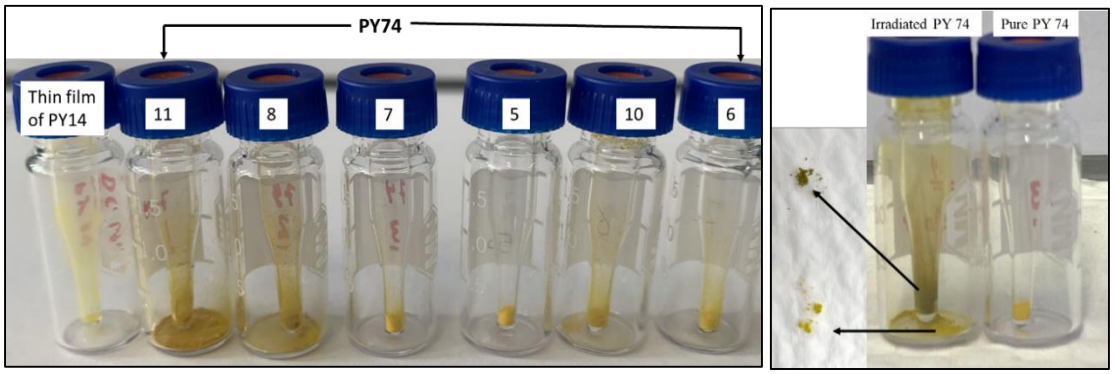



**Figure SI 3.6: : (Lift) Irradiated thin film of PY14 and PY74 with different number of pulses, (Right) irradiated PY74 with 20 pulses using 532 nm laser radiation.**

Samples were subjected to headspace GCMS analysis after laser exposure directly. Initial results were unclear with some samples having a peak has a low signal to noise ratio and other samples have many peaks originated from blank systems (Fig. SI 3.7). Furthermore, this peak could not be replicated. This could be because the concentration of degradation products, if they were formed, was below the GC-MS detection limit based on the method that have been used. Also, the volatile degradation products could not stable for a few minutes after laser irradiation. Furthermore, the presence of high-intensity peaks at roughly 2-3 minutes, due to the rinsing of the needle with chloroform prevents the appearance of fragmentation peaks.

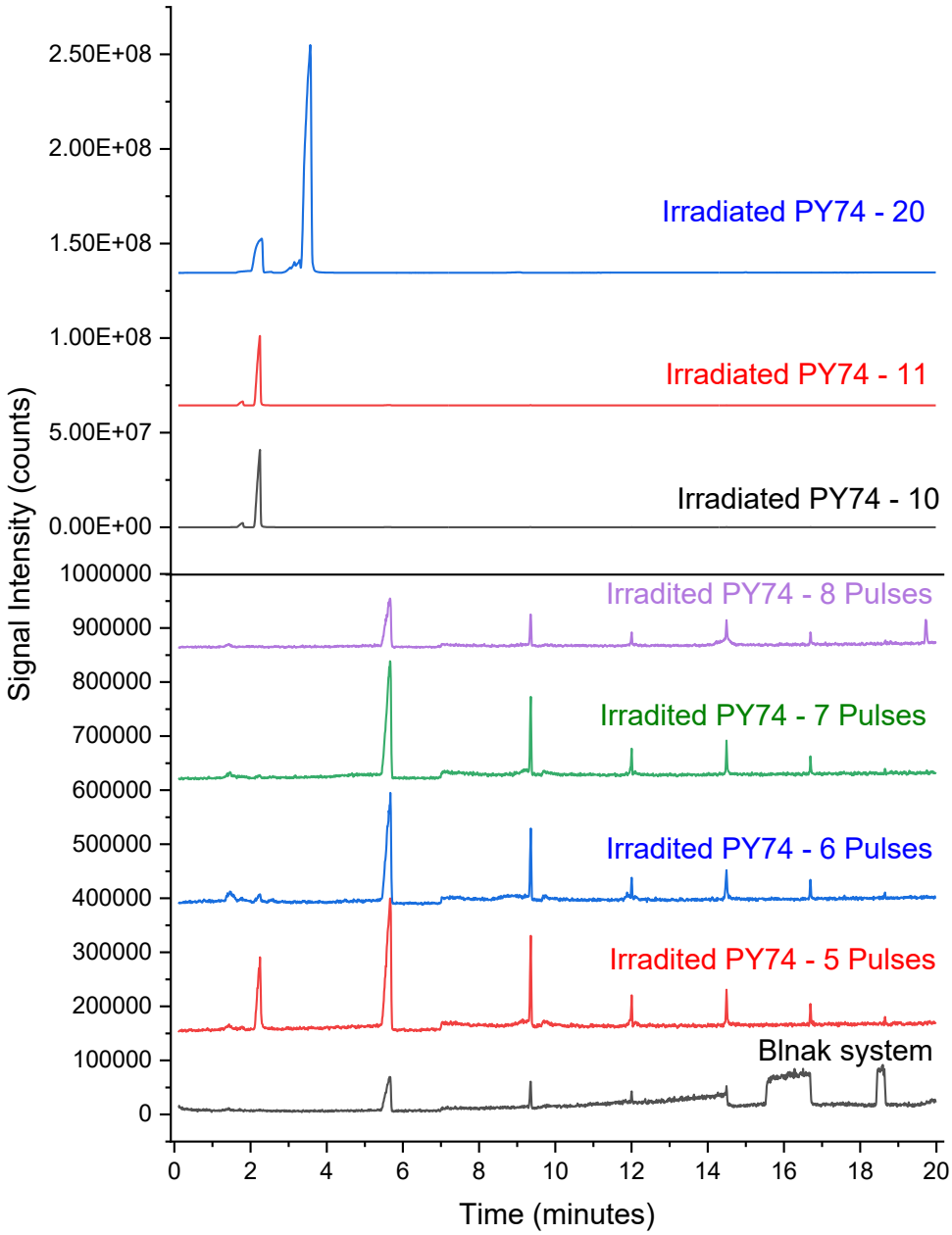
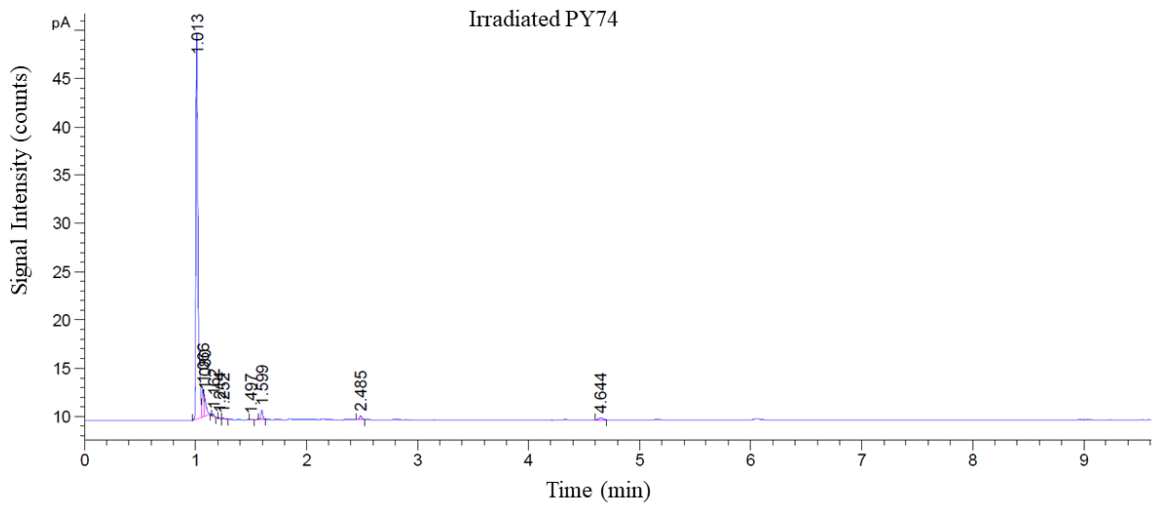
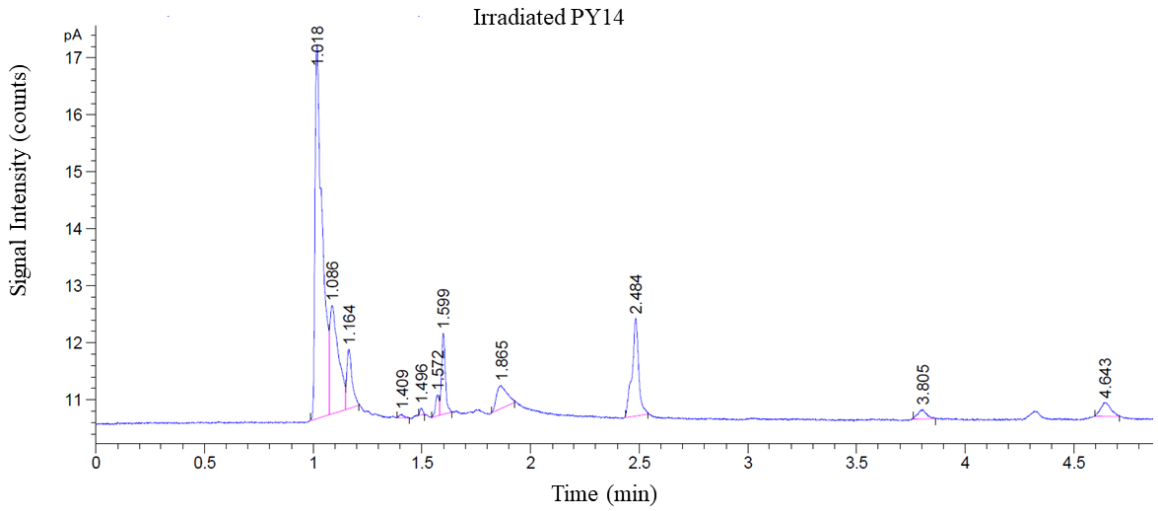
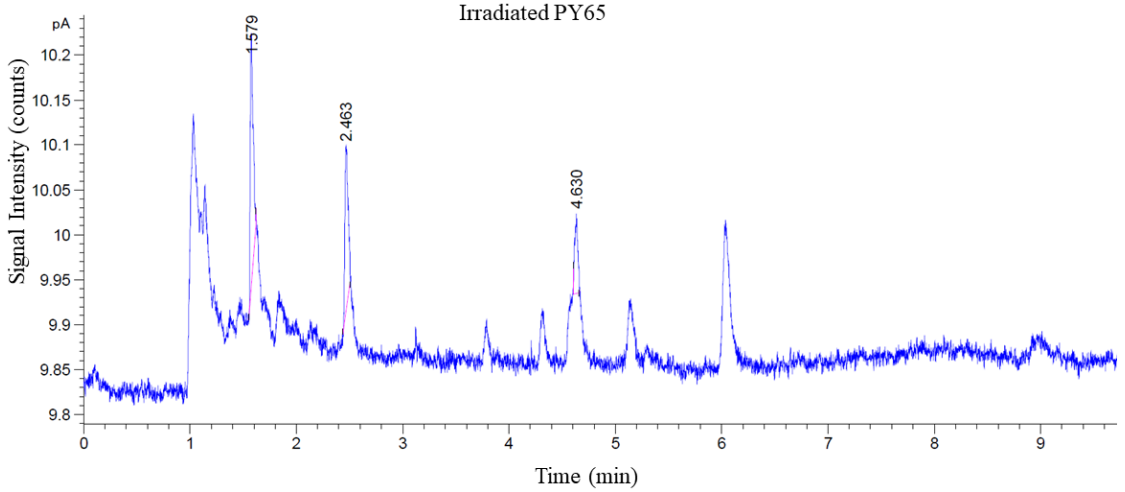


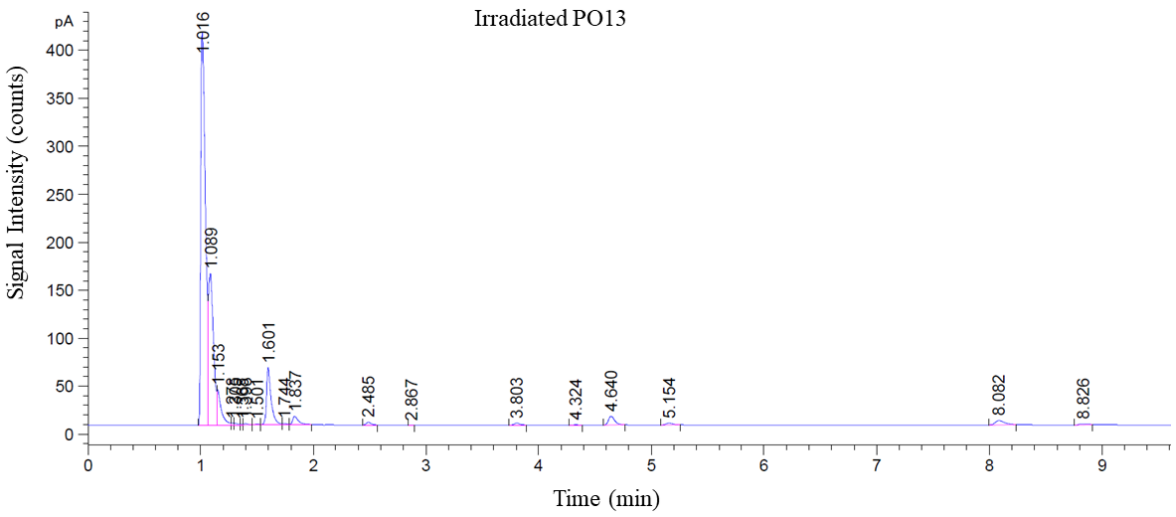

**Figure SI 3.7: GC-MS analysis of irradiated PY74 with different number of pulses.**

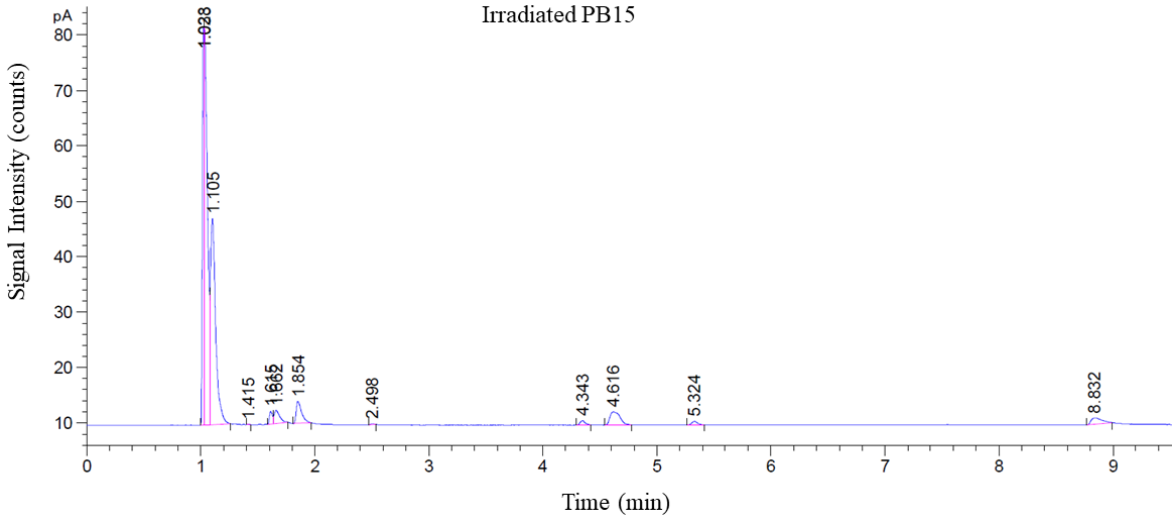
Follow up experiments using headspace GC-FID for analysing irradiated pigments (PY74, PY14, PY65, PO13, PB15) and inks (GY, GR, LY), showed many peaks which can be associated to the fragmentations of pigments (Fig. SI 3.8).

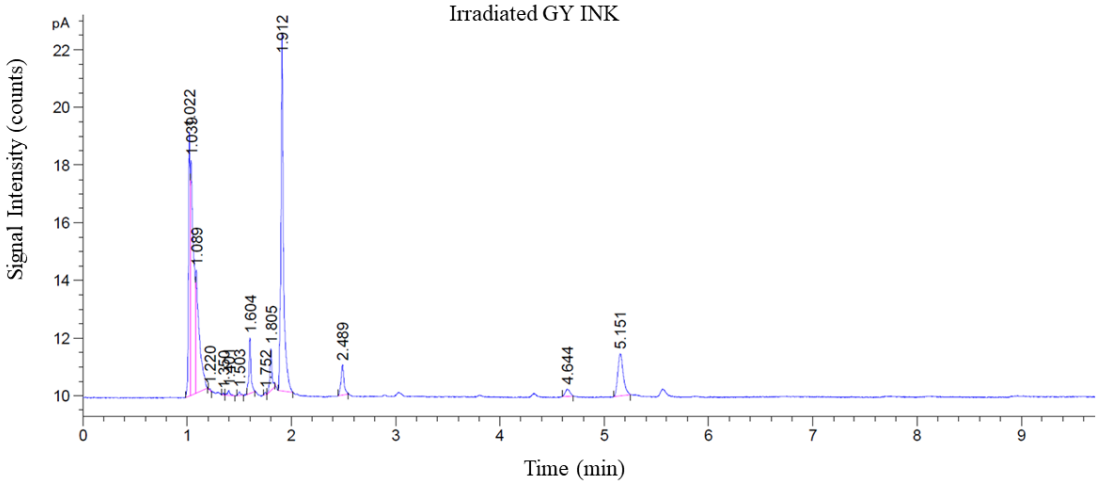

Irradiated PY74
pA
Signal Intensity (counts)
1.013
1.497
1.599
2.485
4.644
Time (min)


Irradiated PY14
pA
Signal Intensity (counts)
1.018
1.086
1.164
1.409
1.496
1.572
1.599
1.865
2.484
3.805
4.643
Time (min)


Irradiated PY65
pA
Signal Intensity (counts)
1.579
2.463
4.630
Time (min)


Irradiated PO13
pA
Signal Intensity (counts)
1.016
1.089
1.153
1.601
1.744
1.837
2.485
2.867
3.803
4.324
4.640
5.154
8.082
8.826
Time (min)


Irradiated PB15
pA
Signal Intensity (counts)
1.105
1.415
1.854
2.498
4.343
4.616
5.324
8.832
Time (min)


Irradiated GY INK
pA
Signal Intensity (counts)
1.022
1.089
1.604
1.805
1.912
2.489
4.644
5.151
Time (min)

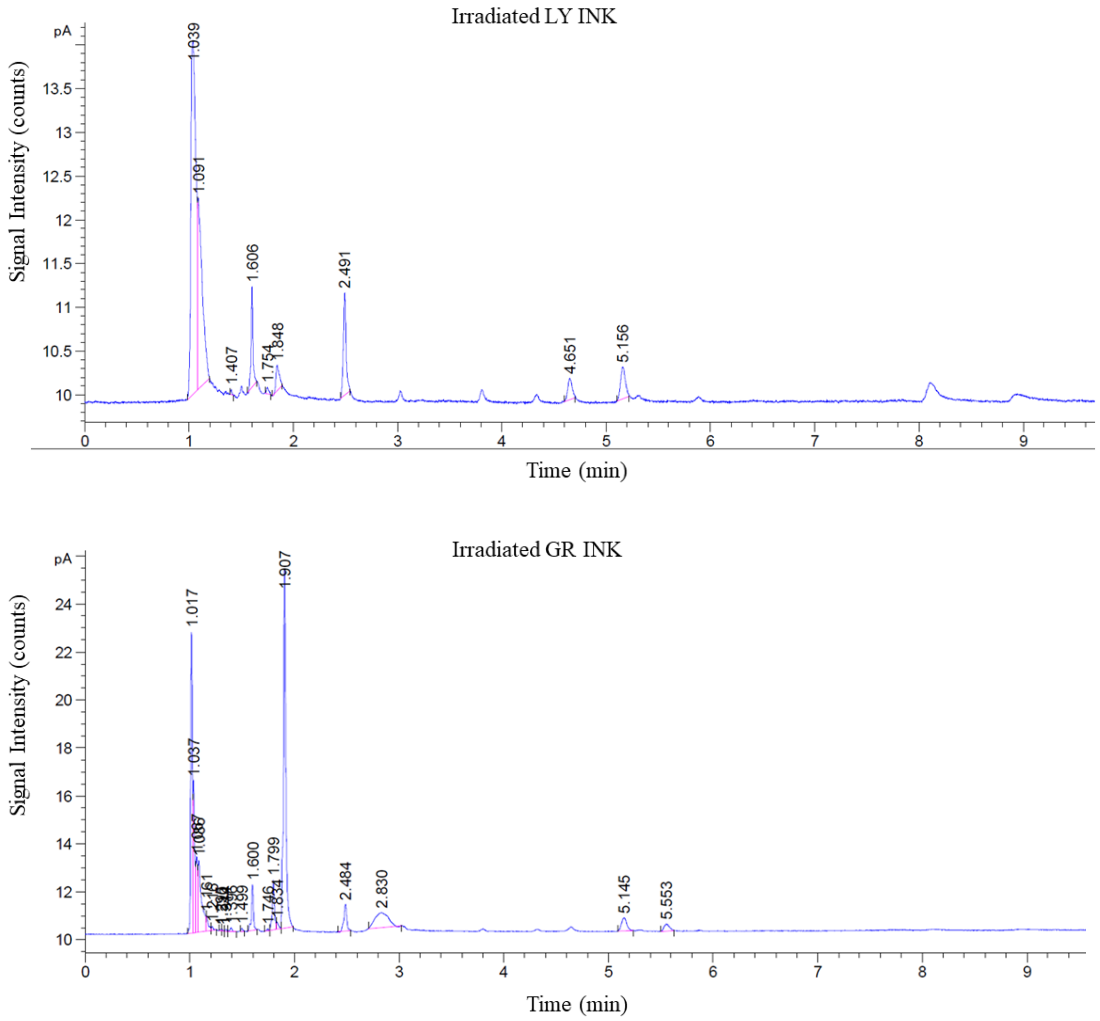



**Figure SI 3.8: GC-FID analysis of irradiated pigments (PY74, PY14, PY65, PO13, PB15) and inks (GY, GR, LY)**

This suggest that the chloroform solvent and delay time on the GCMS may be obscuring peaks. In addition, the inlet temperature was reduced to180 °C to minimise further degradation of compounds in the headspace. On the basis of these results the GC-MS method was revised, and the results now clearly showed high intensity peaks related to the volatile fragments (Fig. SI 3.9). The revised GC-MS method is presented in Chapter 3 (Section 3.2.3).

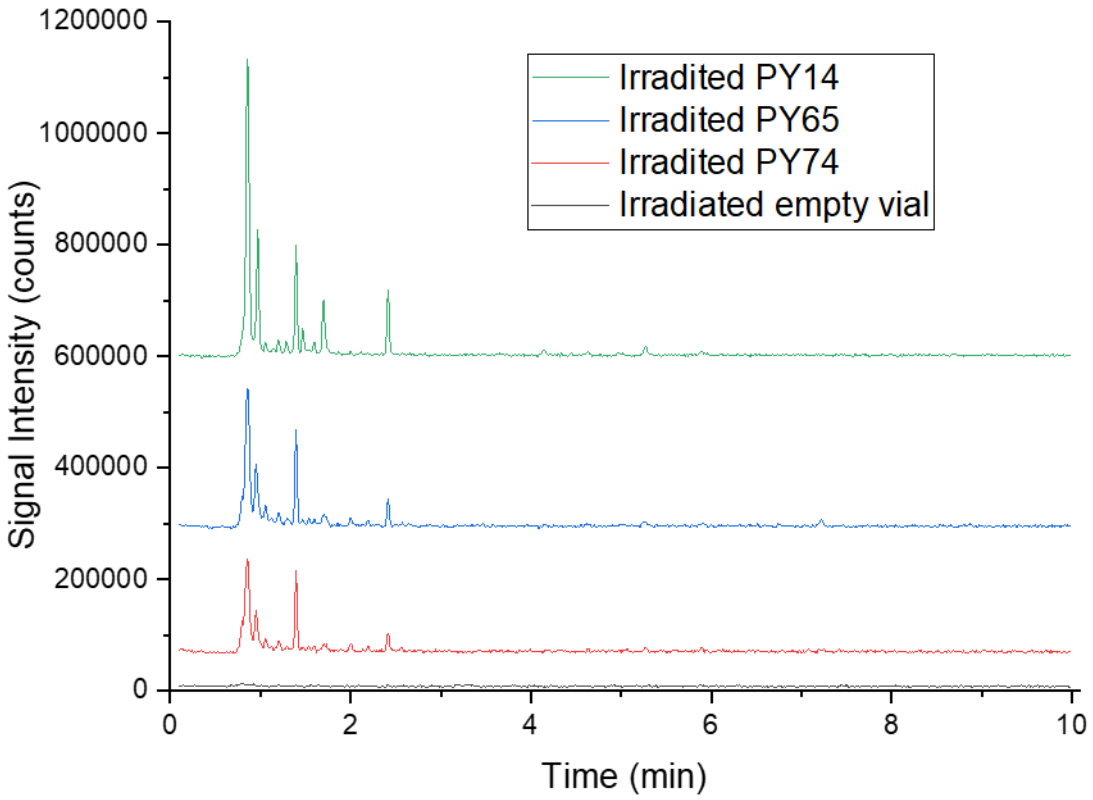



**Figure SI 3.9: New GC-MS analysis method of irradiated PY14, PY74, and PY65**

In light of the availability of pure chemical compounds, we conducted headspace GC-MS analyses on benzene, toluene, and styrene (Fig. SI 3.10). This analytical step was undertaken to assess the retention times (Rt) of these pristine organic solvents and juxtapose them with the Rt values of volatile fragments identified subsequent to laser irradiation. The chromatographic representation of the gaseous phase of these solvents revealed their elution at 1.3, 2.3, and 5.6 minutes, respectively. This outcome serves to validate the GC-MS investigation of the volatile fragments generated following laser irradiation of pigments and inks.

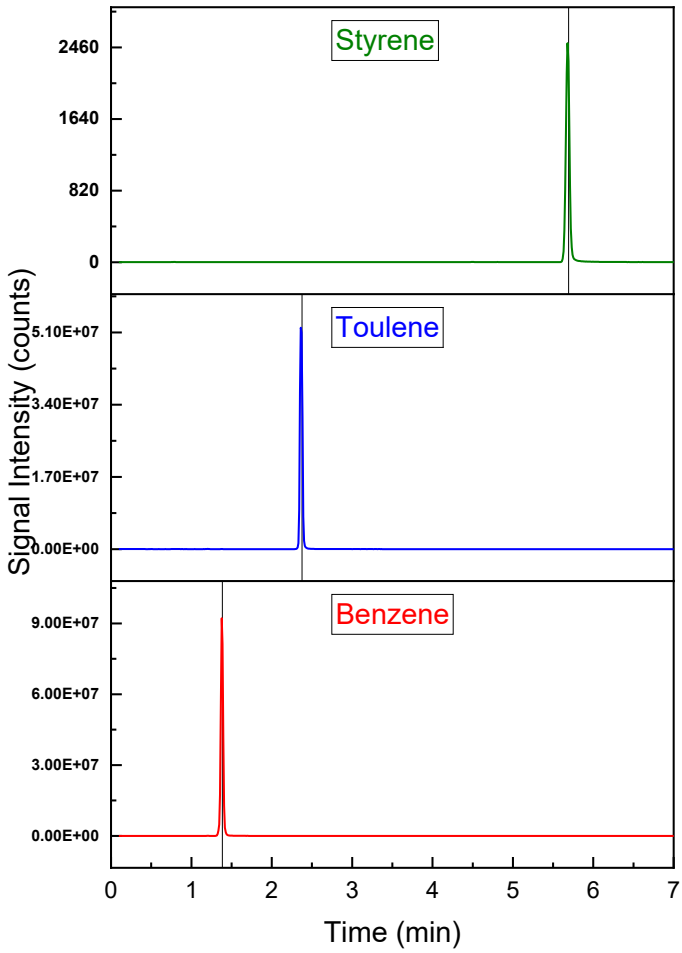



**Figure SI 3.10: GC-MS data of benzene, toluene, and styrene**

## References

1. Yates, B.; Que, S. K. T.; D'Souza, L.; Suchecki, J.; Finch, J. J., Laser treatment of periocular skin conditions. *Clinics in Dermatology* **2015,** *33* (2), 197-206.

## 7.3 Appendix 3: (Supplementary information- Chapter 4)

### Analytical Investigation of Melanin's Impact on the Laser Fragmentation and Morphology of Yellow Tattoo Pigments

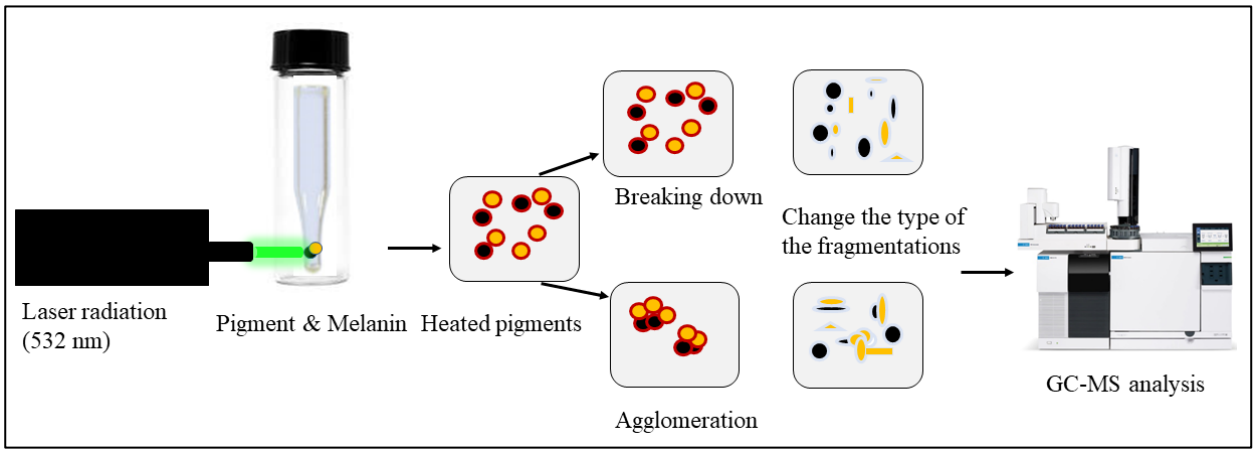



**Figure SI 4.1: General methodology of the project (schematic experimental setup).**

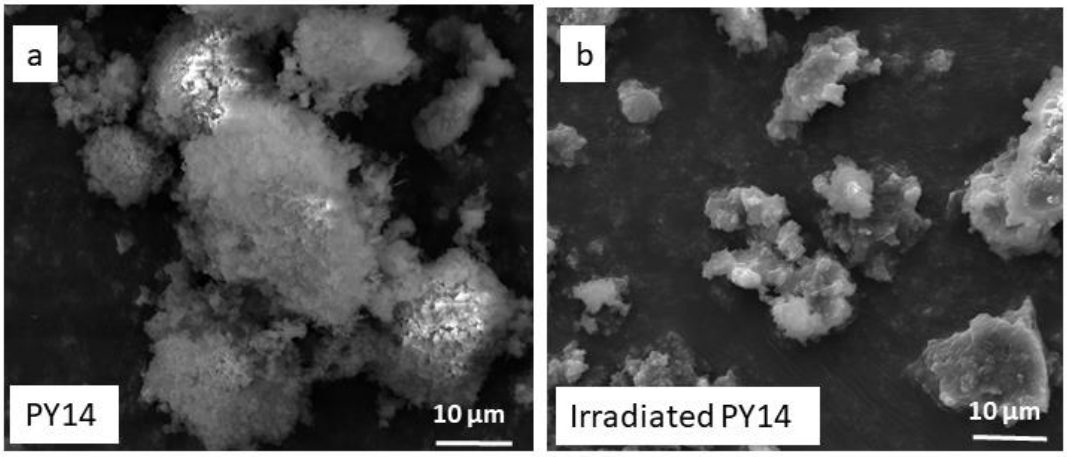



**Figure SI 4.2: SEM images of irradiated PY14 with high fluence.**

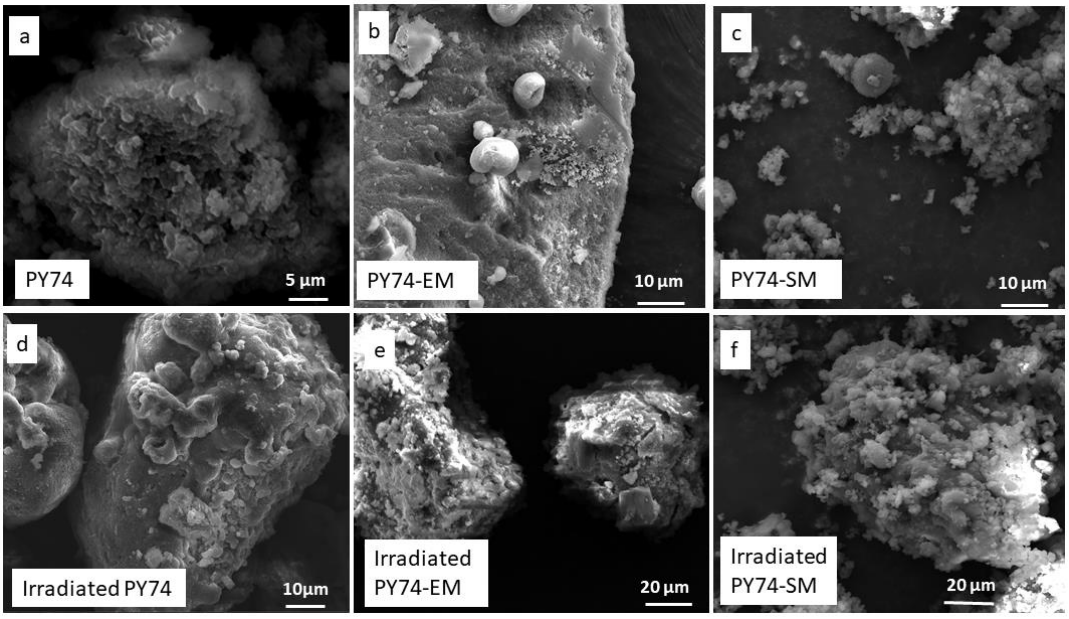



**Figure SI 4.3: SEM images of unirradiated and irradiated PY74, PY74-EM, PY74-SM.**

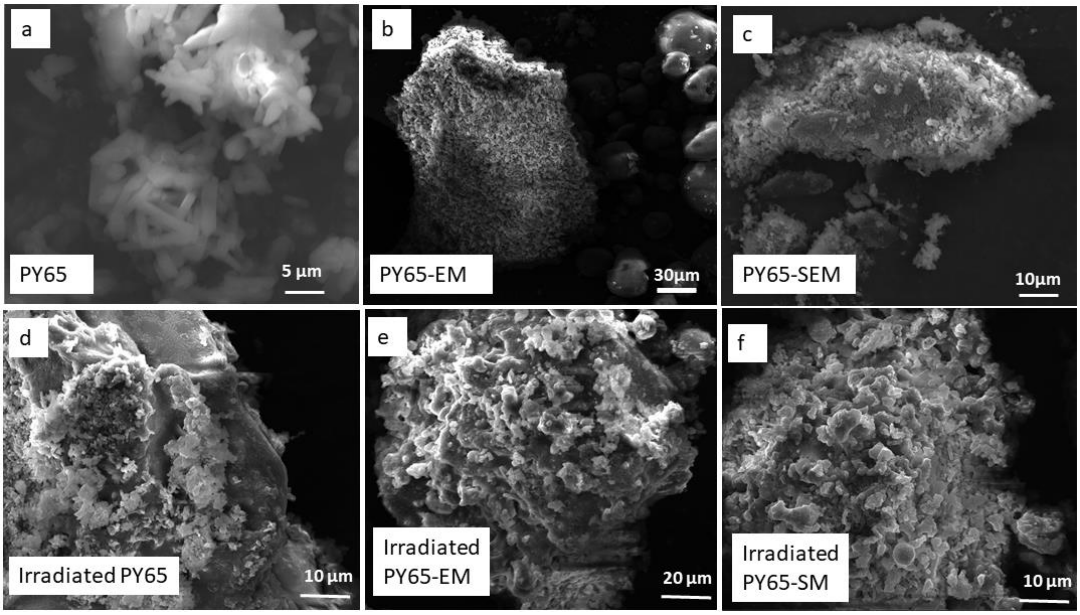



**Figure SI 4.4: SEM images of unirradiated and irradiated PY65, PY65-EM, PY65-SM.**

The aggregation characteristics, furthermore, varied amongst combinations. For example, irradiated PY65-EM clearly showed the effect of laser beam as the fibre shape conglomerated with melanin particles. By comparing the structures of irradiated PY65 and PY65-EM, it demonstrated the crucial role of melanin in absorbing laser energy and inhibiting the melting of the PY65's fibres as observed in the SEM image of irradiated PY65.

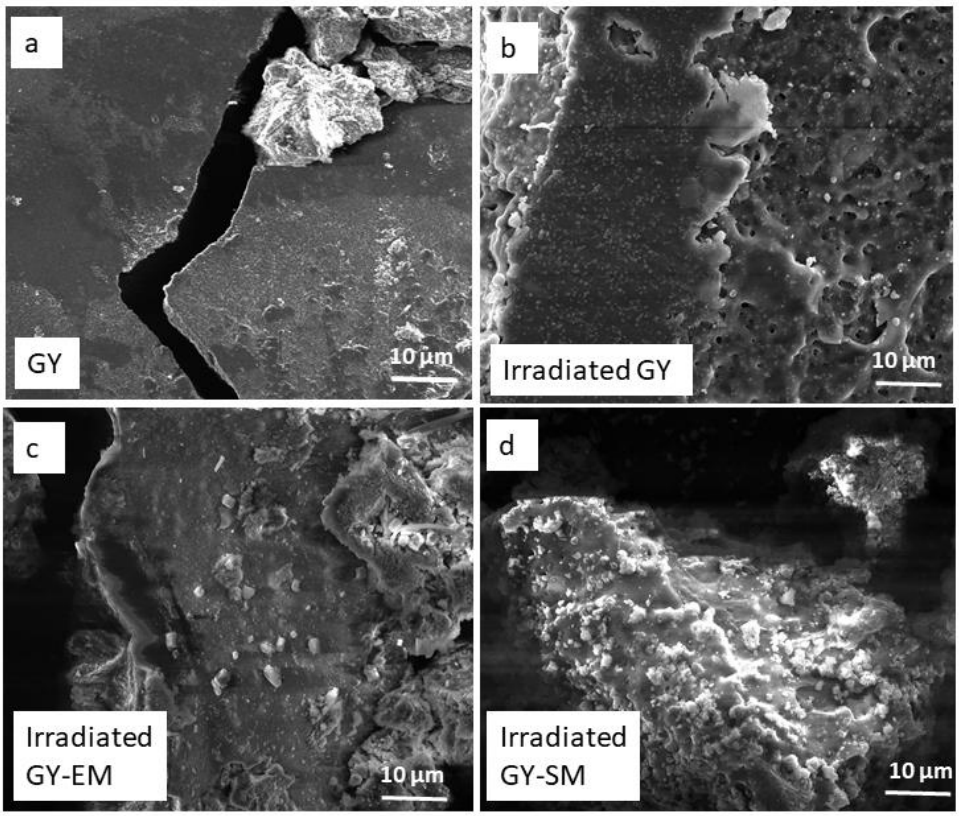



**Figure SI 4.5 : SEM images of unirradiated and irradiated GY, GY-EM, GY-SM.**

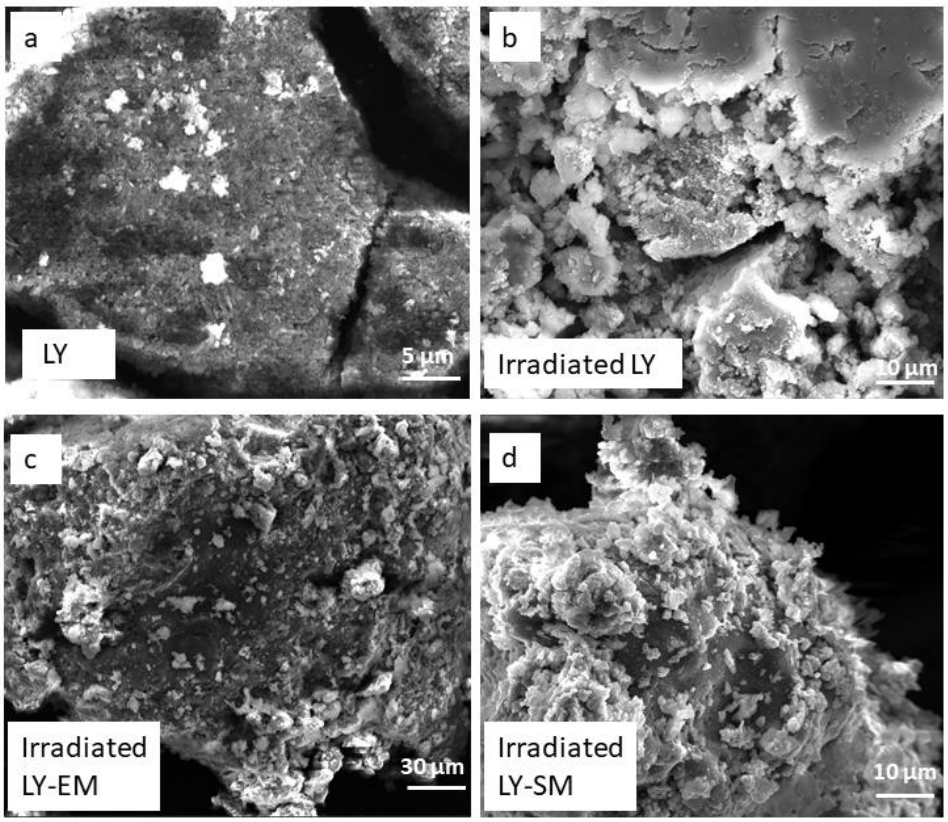



**Figure SI 4.6 : SEM images of unirradiated and irradiated LY, LY-EM, LY-SM.**

Some parts of the inks' surfaces were not fully covered by melanin; hence laser beam was piercing that surface and make holes as seen in LY-EM. This finding was further demonstrated in Fig. SI6, which revealed thin fibres structure following laser irradiation of GY-EM and ink's surface does not appear to be melted when compared to irradiated GY.

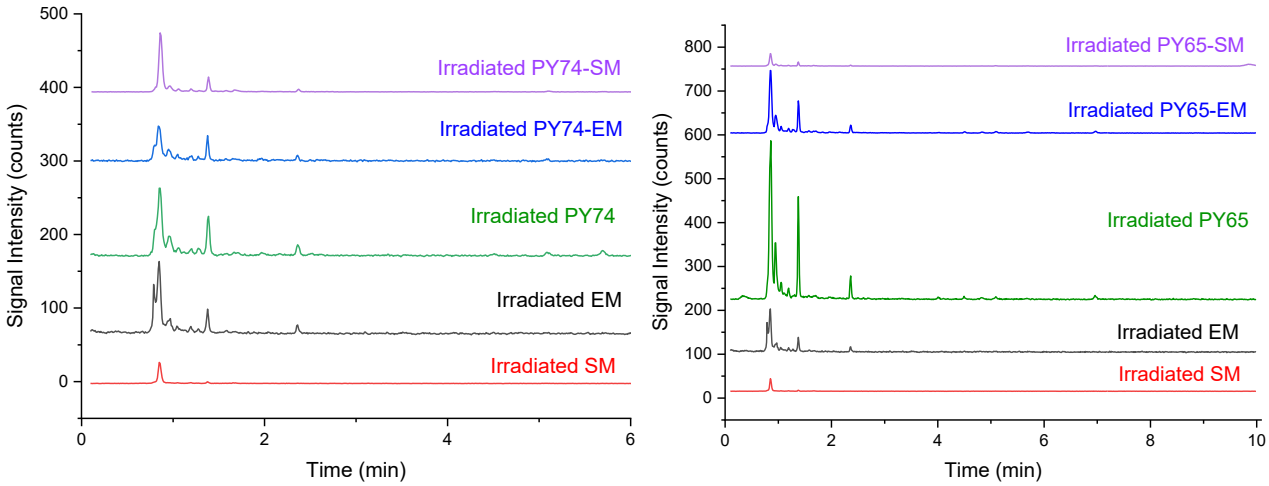



**Figure SI 4.7: GC-MS analysis of irradiated PY74, PY65, and mixture of these pigments with melanin.**

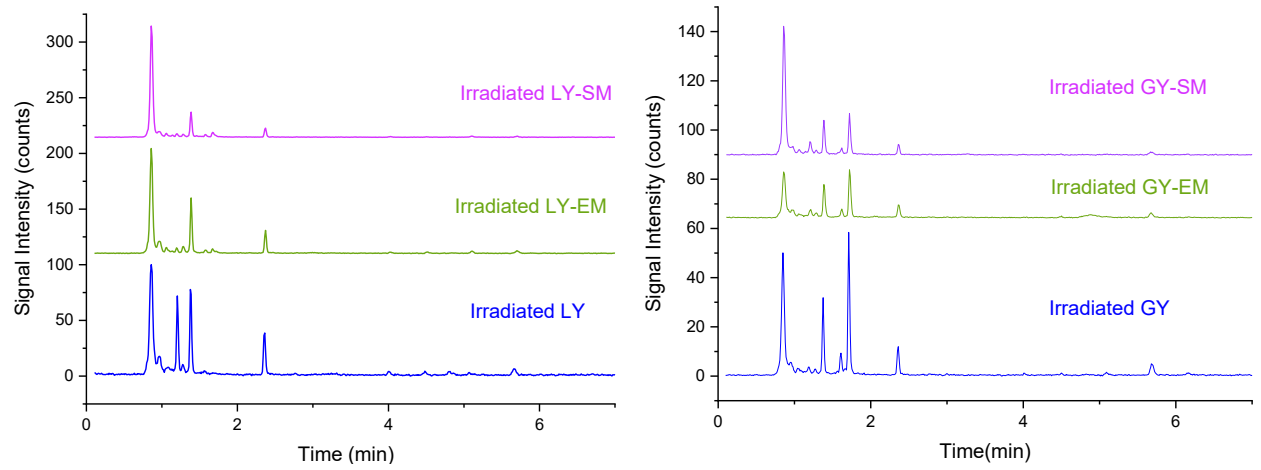



**Figure SI 4.8: GC-MS analysis of irradiated LY, GY, and mixture of these pigments with melanin.**

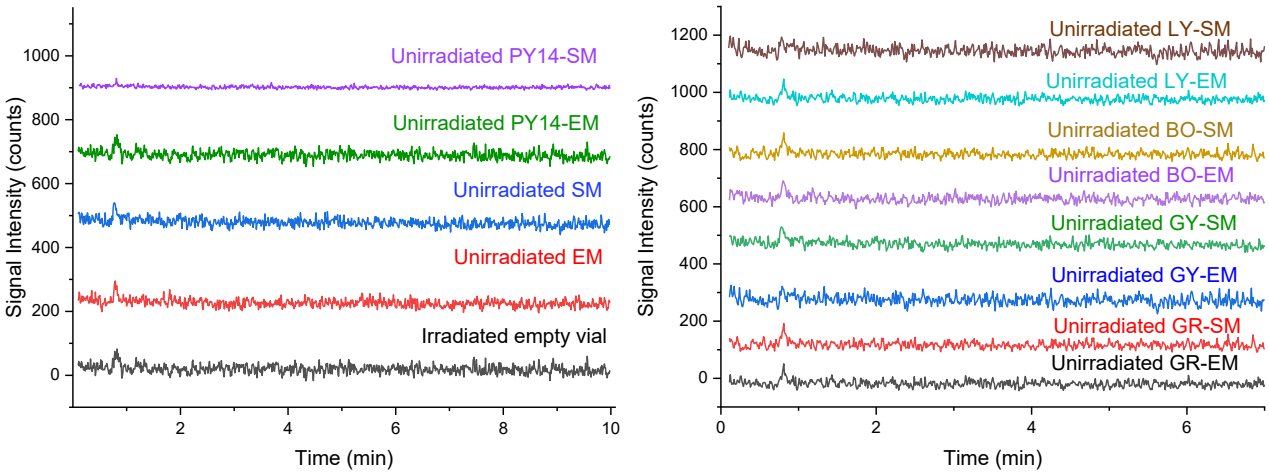
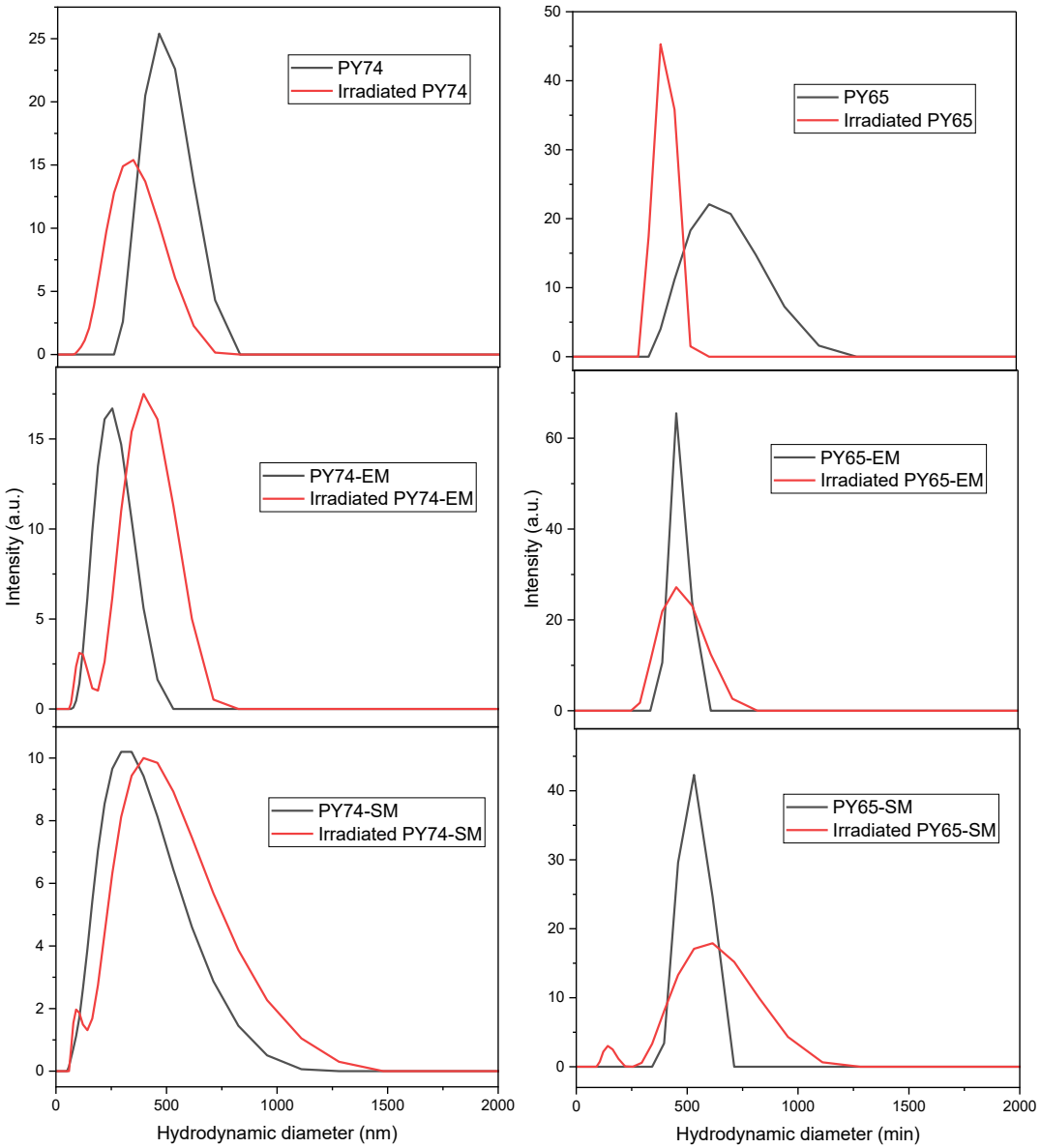



**Figure SI 4.9: GC-MS spectra of unirradiated and irradiated empty vial, melanin, pigments, and inks.**




**Figurer SI 4.10: DLS analysis of unirradiated and irradiated PY74 and PY65 – melanin mixtures .**

## 7.4 Appendix 4: (Supplementary information- Chapter 5)

### Chemical and Cytotoxic Profiling of Tattoo Ink Degradation Products Generated by Laser Irradiation

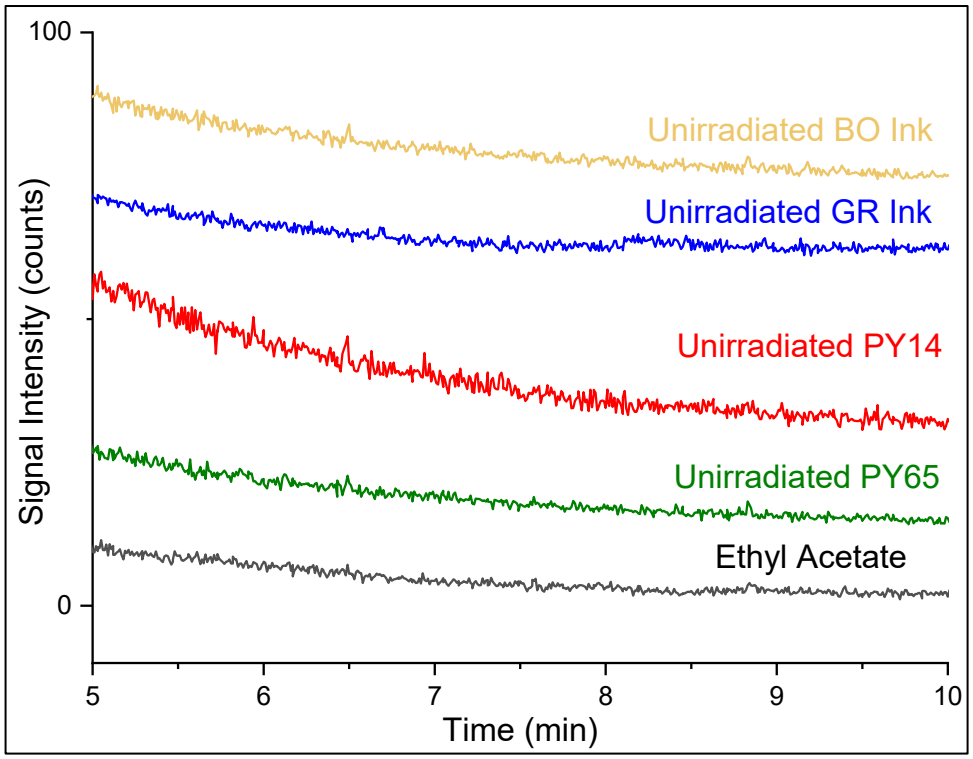



**Figure SI 5.1: GC-MS taken before laser irradiation of PY14, PY65, GR and BO ink.**

**Py-GC-MS**

Pyrolysis-GCMS (Py-GCMS) was performed using a Py-3030S single-shot pyrolizer unit (Frontier Laboratories Ltd., Japan), a 7890B GC system (Agilent Technologies Inc., USA), and a 5977B series single-quadrupole MSD system (Agilent Technologies Inc., USA). Helium was used as the carrier gas, and an electron impact ion source operating at 70 eV was used to produce the ionisation. A HP-5MS capillary column (29.4 m length, 250 µm internal diameter, 0.25 µm film) consisting of 5% diphenyl/95% dimethylpolysiloxane fused silica (Agilent Technologies Inc., USA) was equipped with the GC system. The Sartorius microbalance was used to properly weigh out pigment samples (5.0–10 µg) into 50 µL stainless steel ECO cups (Frontier Laboratories Inc., Japan) before they were analysed. The pyrolysis interface temperature was adjusted at 400 °C, and samples were pyrolyzed for 5 minutes at 650 °C. Using splitless mode and an injection temperature of 320 °C, the sample was added to the GC column. The GC column temperature was programmed as follows: an initial temperature of 40 °C maintained for 5 minutes, followed by a ramp to 280 °C at a rate of 6 °C/min, also held for 5 minutes, resulting in a total analysis duration of 50 minutes. The helium gas flow rate was 21 mL/min, whereas the column flow rate was established at 1 mL/min. Blanks were conducted

both before and after the examination of all samples, and meticulous precautions were implemented to prevent any potential contamination from items in the surrounding laboratory environment. In the mass range of 45 to 450 m/z, mass spectra were obtained at 230 °C employing electron impact as the ionisation source. Peaks associated with identified substances in the spectral database were found and compared using NIST libraries of mass spectra.

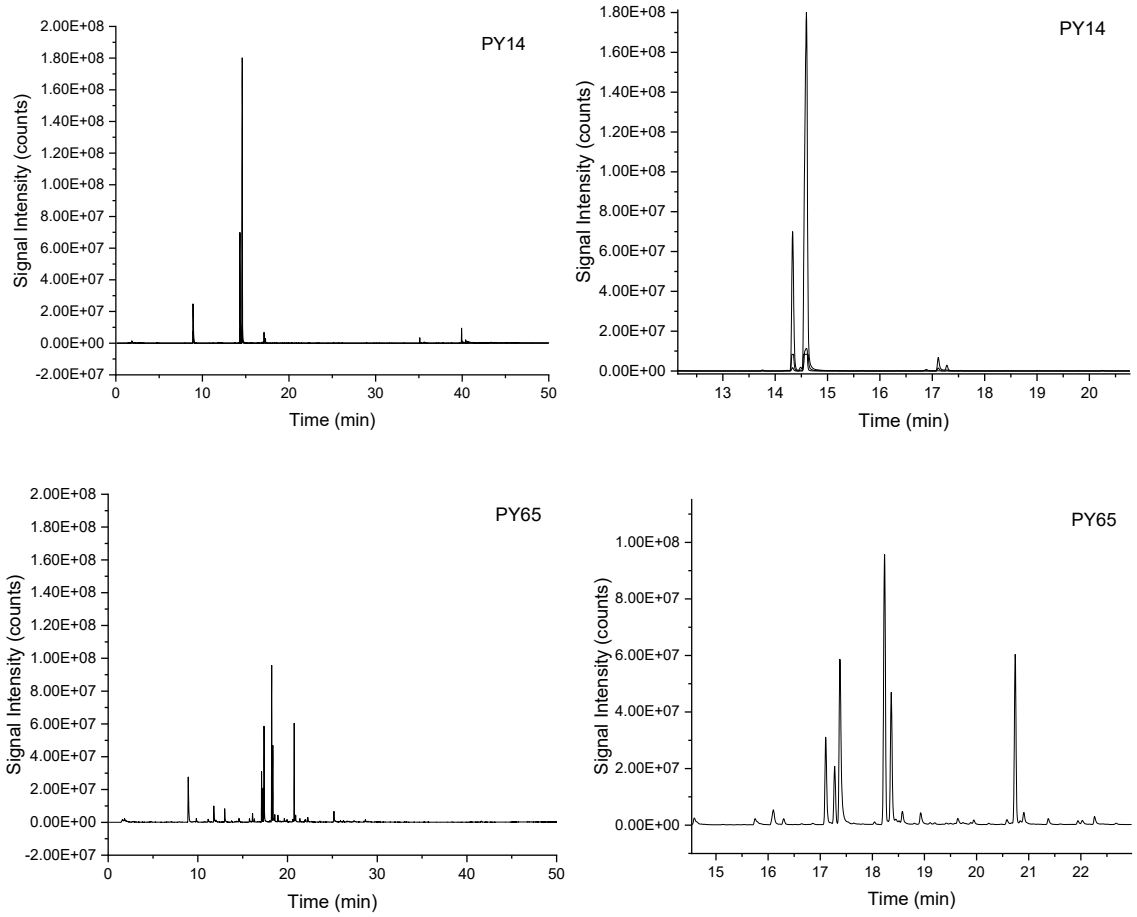



**Figure SI 5.2: Py-GC-MS data of PY14 and PY65.**

Pyrolysis of PY14 produced styrene, o-toluene isocyanate, *o*-toluidine, and m-xylidine. In contrast, PY65 fragmented into styrene, aniline, benzene isocyanate, 2-propynylbenzene, *p*-toluidine, *o*-anisidine, 2-methoxyphenyl isocyanate, 2-aminobenzaldehyde (2-formylaniline), 5-methoxybenzofurazan, and 2-benzoxazolinone.

**Table SI 5.1:Py-GC-MS data of PY14 and PY65.**

| Pigments | Time (min) | Compound | m/z |
|---|---|---|---|
| **PY14** | 8.87 | styrene | 104, 78, 51 |
| | 14.32 | o-toluene isocyanate | 133, 104, 91, 78, 51 |
| | 14.65 | o-toluidine | 106, 77 |
| | 17.10 | m-xylidine | 121, 106, 91, 77 |
| **PY65** | 8.87 | styrene | 104, 78, 51 |
| | 11.74 | aniline | 93, 66 |
| | 12.9 | benzene isocyanate | 119, 91, 63 |
| | 13.02 | 2-propynylbenzene | 116, 115, 89, 63 |
| | 14.60 | p-toluidine | 107,106 |
| | 17.1 | m-xylidine | 121,106 |
| | 17.2 | 2,6-dimethylphenyl isocyanate | 147, 132, 118, 91 |
| | 17.37 | 2-aminoanisole | 123, 108, 80, 53 |
| | 18.25 | methoxyphenyl isocyanate | 149, 134, 120, 106, 78, 51 |
| | 18.39 | 2-aminobenzaldehyde (2-Formylaniline) | 121, 93, 66 |
| | 20.76 | 5-methoxybenzofurazan | 150, 120, 105, 92, 80, 77, 65, 52 |
| | 25.18 | 2-benzoxazolinone | 135, 91, 79, 52 |


**Figure SI 5.3:Unirradited and Irradiated BO tattoo ink.**

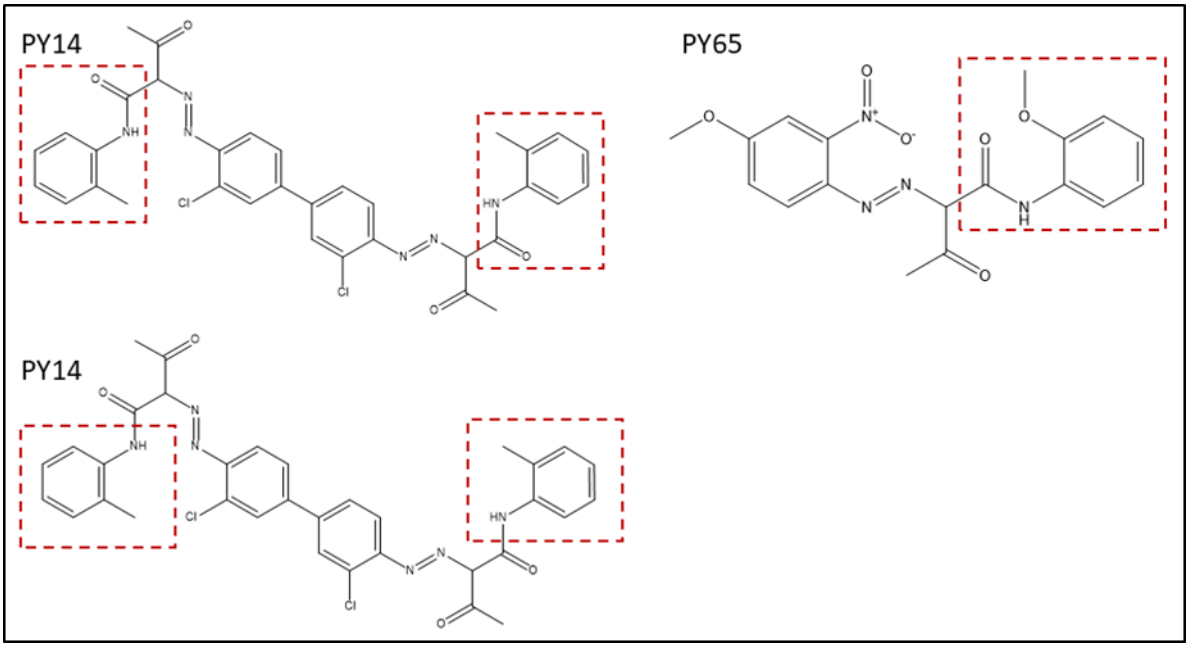



**Figure SI 5.4: The chemical structure of PY14 and PY65 along with the main laser fragments detected in the GC-MS analysis.**

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## Tattoo inks in general usage contain nanoparticles

**Author:** Høgsberg, T.; Loeschner, K.
**Publication:** British Journal of Dermatology
**Publisher:** Oxford University Press
**Date:** 2011-12-01

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**Author:**
Yanyan Cui,Mona I. Churchwell,Letha H. Couch,Daniel R. Doerge,Paul C. Howard

**Publication:** Drug Metabolism and Disposition

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**Date:** October 2005

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**Author:** Michelle D. Miranda
**Publication:** WIRES Forensic Science
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**Publication:** American Journal of Clinical Dermatology
**Publisher:** Springer Nature
**Date:** Oct 8, 2016

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**Author:** David M. L. Morgan
**Publication:** Springer eBook
**Publisher:** Springer Nature
**Date:** Jan 1, 1998

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