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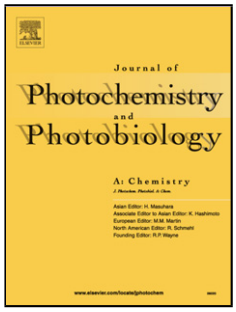

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Investigation of Benzophenoxazine Derivatives for the Detection of Latent Fingerprints on Porous Surfaces

Eman Alsolmy[a,b,*], Walid M. Abdelwahab[a,c], Vincent Martinez[a], Maged Henary[a], Gabor Patonay[a]

[a] Department of Chemistry, Georgia State University, P.O. Box 3965, Atlanta, Georgia 30302-3965, USA.

[b] Chemistry Department, Faculty of Science, University of Jeddah, P.O. Box 80327, Jeddah, 21589, Saudi Arabia.

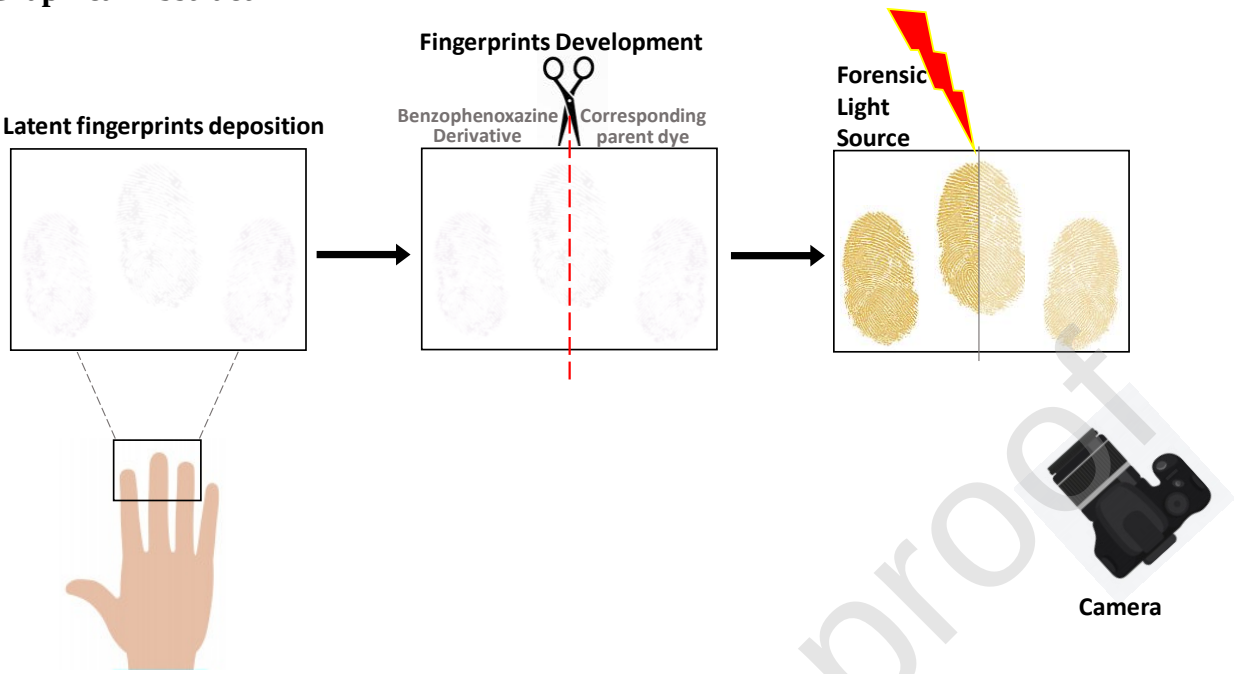
[c] Pharmaceutical Chemistry Department, Faculty of Pharmacy, Cairo University, Kasr El-Aini St., Cairo 11562, Egypt.

* Corresponding author

Tel: (+1) 404-413-5557; fax: (+1) 404-413-5505

E-mail address: ealsolmy1@student.gsu.edu

Graphical Abstract



Highlights

- The hydrophobicity of the dye plays a significant role for developing fingerprints.
- The fingerprints detection is also influenced by the chemical structure of the dye, hydrogen bonds, and interactions with solvents.
- Nile red and Nile blue detect fingerprints with excellent to acceptable definition due to the great hydrophobicity.
- Highly hydrophobic benzophenoxazine derivatives could be considered dual-fingerprint reagents because they develop luminescent and visible fingerprints.
- Less hydrophobic benzophenoxazine derivatives or hydrophobic derivatives containing interference substituents develop weak luminescent fingerprints.

Abstract

In this report, two classes of benzophenoxazine dyes, Nile red and Nile blue derivatives, were evaluated for the detection of latent fingerprints on porous surfaces. The efficiency to develop fingerprints is influenced by the physical properties of the dye molecules including hydrophobicity as characterized by distribution coefficient value (logD), and other factors such as chemical structure of the dye and hydrogen bonds. Both Nile red and the basic form of Nile blue showed excellent to acceptable ability to detect fingerprints due to their great hydrophobicity at 515 and Crime Scene Search (CSS) settings of the forensic light source. Higher hydrophobicity derivatives of Nile red and Nile blue (in the basic form) improved both quality and sensitivity of fingerprint detection in comparison with their corresponding parent dyes. They developed strong luminescent and visible fingerprints, and a better contrast was achieved between impressions and the background surface suggesting the potential use of these compounds in forensic investigation. Therefore, the hydrophobic derivatives are considered dual-fingerprint reagents because the developed prints can be seen by the naked eye and under illumination process. However, lower hydrophobicity derivatives of Nile red and Nile blue and derivatives with different substituents developed weak or non-luminescent fingerprints with poor contrast. A complete analysis of what dye properties are the most important in fingerprint detection is discussed along with dye optimization for improved performance.

Keywords: Benzophenoxazine derivatives; Nile red; Nile blue; Fingerprint detection; Hydrophobicity; Luminescent

1. Introduction

Fingerprint identification is considered one of the most crucial clues of physical evidence in forensic investigation. The common form of fingerprints found at a crime scene is latent

fingerprints, the invisible residue deposited by a person's hand when touching a surface. Due to the complexity of fingerprint residue, revealing the prints requires physical or chemical treatment to enable their detection. Several developing reagents have been used for fingerprint detection that target various components such as amino acids or lipids in fingerprint residue. Amino acid-sensitive reagents such as ninhydrin, 1,8-diazafluoren-9-one (DFO), and 1,2-indanedione are insufficient for revealing fingerprints on porous surfaces that have been wetted or found in humid conditions [1-3]. In this case, amino acids are likely to be dissolved or washed away due to their solubility in water. Therefore, lipid-sensitive reagent is the reagent of choice to detect fingerprints on wet porous substance. Physical developer (PD) is the standard technique for developing fingerprints in such applications. However, this technique has been recognized as a complex and time-consuming process due to high sensitivity to contaminations and the need for freshly prepared working solutions [4]. Many studies have been conducted on alternative lipid sensitive reagents such as dyes [5-7]. Oil Red O is a diazo dye used in histological staining for lipidic compounds and belongs to the family of Sudan dyes. It has been studied extensively for recovering fingerprints as red visible prints on wet porous surfaces due to the high affinity of the dye to lipids [8, 9]. Solvent black 3 is another diazo dye that comes from the same family of Sudan dyes. Similarly, the dye has been investigated in revealing fingerprints but to a lesser extent than Oil Red O. Solvent black 3 is more effective in developing fingerprints on greasy contaminated substances [10].

Fingerprints developed by the previously mentioned reagents are visible to the naked eye which limit their applications to light-colored surfaces. However, the use of organic dyes that fluoresce upon exposure to the light source has the advantage of revealing fingerprints with sufficient contrast on dark-colored surfaces. The fluorescent dye eosin-blue with a phase transfer catalyst was used to detect fingerprints on many surfaces [11]. Moreover, a formulation based on

Coomassie brilliant blue G-250 dye has been proposed as a developing reagent to differentiate between male and female fingerprints [12]. Furthermore, several fluorescent dyes have been reported as dusting agents for detecting fingerprints including acridine orange, acridine yellow, crystal violet, coumarin 6, and rhodamine B [13]. In addition, micro-structured fluorescent powders based on benzazole dyes have been applied for the development of latent fingerprints on many surfaces [14]. Moreover, the fluorescence properties of such compounds enhance the sensitivity of fingerprints developed with regular reagents. Rhodamine 6G, for example, was used as post-treatment of fingerprints developed with Oil Red O on dark porous surfaces [15].

Recently, benzophenoxazine dyes such as Nile red and Nile blue have been applied in the field of fingerprint detection. Nile red is a neutral fluorescent dye used in histology for staining of some lipid compounds such as triacylglycerols, phospholipids, cholesteryl esters, and lipoproteins [16]. Nile blue is a cationic dye used in the histological staining of neutral fats, phospholipids, and fatty acids [17]. These compounds are present in fingerprint residue as a result of sebaceous gland secretions [18]. The optical properties of Nile red and Nile blue are highly influenced by the solvent polarity and dielectric constant. In non-polar media, the fluorescence spectrum of both dyes is in the visible to the near-infrared region, and high quantum yield values are recorded. In polar media, however, the fluorescence spectrum of Nile Red and Nile blue in its basic form shows a red shift with low quantum yield values due to hydrogen bonding [19-21]. The early use of Nile red in fingerprint detection was a post-treatment for cyanoacrylate fuming. It was reported that the performance of Nile red for the enhancement of cyanoacrylate-developed latent fingerprint was considerably superior to that for rhodamine 6G [22]. Similarly, Nile blue was initially used to improve the contrast of latent fingerprints developed by cyanoacrylate [23]. Both dyes have been used for the detection of latent lip prints on multicolored surfaces, and Nile red performed better

than Nile blue especially with old prints [24]. Nile red has been used effectively to develop fingerprints on different porous surfaces in a methanolic-aqueous formulation. In addition, Nile red performance was compared to that of PD with fresh and aged fingerprints [25]. On the other hand, an aqueous solution of Nile blue has been proposed as an alternative fingerprint reagent to the previous formulation of Nile red. The procedure was described as simple, less toxic, and lower cost and can be applied on different surfaces [26]. The efficiency of this solution is due to the small presence of Nile red resulting from the spontaneous hydrolysis of Nile blue [27]. The presence of the two dyes in one formulation stains both neutral lipids and acidic moieties [17]. Recently, an aqueous microemulsion of Nile red has been developed for visualizing both natural and charged fingerprints. The performance of the solution was comparable to the methanolic-aqueous formulation of Nile red and superior to the aqueous solution of Nile blue [28]. More recently, Nile red has been encapsulated into mesoporous silica nanoparticles for developing fingerprints on thermal paper without the need of organic solvents [29].

Most fluorescent dyes used in forensic detection as developing reagents are hydrophobic. The hydrophobicity of a compound is measured by the partition coefficient (logP) or the distribution coefficient (logD). Both terms describe the ratio of the concentration of a compound in organic and aqueous phases (octanol and water) at equilibrium. However, logD is more extensively used than logP to express such property because logP deals with unionized form only while logD takes into consideration both ionized and unionized forms. Moreover, logD is pH-dependent whereas logP is pH-independent [30, 31]. LogP value was first mentioned in forensic science as an indication of the great hydrophobicity of Oil Red O explaining the extensive use of the dye in histological staining [9]. The hydrophobicity characterized by logD for common developing reagents is summarized in Table 1.

Fingerprint reagents can be classified based on the appearance of fingerprints after treatment process into three types: visible-fingerprint reagent, luminescent-fingerprint reagent, and dual-fingerprint reagent. Visible-fingerprint reagents such as ninhydrin, and Oil Red O bind to fingerprint components and the resulting impression can be seen only by the naked eye. Luminescent-fingerprint reagents such as Nile red and Nile blue interact with fingerprint residue to produce fluorescent impressions which can be seen only upon exposure to a light source. Dual-fingerprint reagents produce fingerprint impressions that are both visible and luminescent. Few reagents have been known as dual-fingerprint reagents, one of which is a natural pigment called Genipin. It is extracted from gardenia fruit and produces blue color with various amino acids [32]. This reagent detects amino acids in fingerprint residue to produce visible blue impressions and red impressions under illumination [33]. Our preliminary results indicated that some benzophenoxazine derivatives synthesized by our group were dual reagents as they developed colored and luminescent fingerprints.

To the best of our knowledge, most of the fingerprint detection reports have studied the common developing reagents and compared their performance with new analogous compounds for enhanced detection [34-37]. However, very few reports have studied the relationship of the chemical structure of a reagent and its efficiency for fingerprint development. The purpose of our work was to compare the performance of two fingerprint reagents Nile red and Nile blue (in the basic form) in terms of their chemical and physical properties such as hydrophobicity. The study aimed also to evaluate several modified structures of benzophenoxazine derivatives as reagents for fingerprint detection with respect to the two parent reagents. The use of benzophenoxazine derivatives in forensic investigation has the advantage of revealing luminescent fingerprints on

porous surfaces. In addition, some derivatives of higher hydrophobicity developed visible and luminescent prints and thus are considered as dual-fingerprint reagents.

2. Materials and methods

2.1 Chemicals and reagents

Nile red (NR) and Nile blue (NB) were obtained from Sigma-Aldrich (St. Louis, MO). The synthesis of all Nile red and Nile blue derivatives except **NR1** and **NR2** will be illustrated in Martinez et al. (in preparation). The chemical synthesis of **NR1** and **NR2** is described in the following section. The chemical structures of all dyes are shown in Fig. 1. Ethanol (200 proof ethanol) was obtained from Pharmco-AAPER (Brookfield, CT). Sodium hydroxide (99%) was obtained from Fisher Scientific (Fair Lawn, NJ). Absorbance spectra were acquired using a Cary 3G UV-visible spectrophotometer (Varian Inc., Palo Alto, CA) interfaced to a PC. All measurements were performed in disposable plastic cuvette with a path length of 1.0 cm. Nanopure water was obtained using an ELGA Purelab Classic water purification system. The nuclear magnetic resonance spectra were obtained by high quality Kontes NMR tubes (Kimble Chase, Vineland, N*J*) rated to 500 MHz and were recorded on a Bruker Avance 400 MHz spectrometer interfaced to a PC using Topspin 3.1. High-resolution accurate mass spectra were obtained at the Georgia State University Mass Spectrometry Facility using a Waters Q-TOF micro (ESI-Q-TOF) mass spectrometer.

2.2. Synthesis of the dialkylated Nile red derivatives (NR1 and NR2)

All chemical reactions were maintained under a positive pressure of nitrogen unless otherwise stated. The reaction progress was monitored using silica gel 60 F254 thin layer chromatography plates (Merck EMD Millipore, Darmstadt, Germany). Open column

chromatography was utilized for the purification of all final compounds using 60−200 μm, 60A classic column silica gel (Dynamic Adsorbents, Norcross, GA).

According to Scheme 1, compounds (**2a-b**) were prepared by dissolving 3-aminophenol, compound **1**, (2.00 g) with two molar equivalents of potassium carbonate in isopropanol/water (1:1, v/v). Two molar equivalents of iodobutane were added to the mixture of **2a** and two molar equivalents of 1-bromo-3-phenylpropane were added to the mixture of **2b**, both mixtures were heated to 70°C for 3 h. The **2b** solution was evaporated under reduced pressure then extracted with ethyl acetate. For both compounds, the organic layer was separated and concentrated under reduced pressure to yield brown oil. This was purified by regular phase column chromatography using hexane /ethyl acetate (95:5, v/v). 0.6 g of Compounds (**2a-b**) were dissolved in isopropanol (2.0 mL) to facilitate its dissolution. Then 4.0 mL (32% HCl) and 8.0 g of crushed ice were added. The mixture was chilled in an ice bath and a solution of $NaNO_2$ (0.4 g in 6.0 mL water) was added dropwise over one hour. This was continuously stirred for 3 additional hours at 0º C. For compound **2a**, the liquid medium had changed color to dark orange which was then concentrated under reduced pressure. The nitroso product, compound **3a**, was dried under reduced pressure and used in the next step without further purification due its instability. For compound **2b**, a yellow-orange precipitate had formed by this time. This precipitate was filtered and dried under reduced pressure. Due to instability of nitroso compounds, the product was used in the next step without purification. Compounds (**4a-b**) were prepared by dissolving compounds (**3a-b**) and one molar equivalent of 1-hydroxynaphthalene in 15.0 mL ethanol. The mixture was heated to 70°C for 3 h to afford a highly impure dye. The solution was neutralized with ammonium hydroxide and then extracted with CH_2Cl_2. The organic layer was separated and then removed under reduced pressure. This was

purified by regular phase column chromatography using CH_2Cl_2/methanol (99:1, v/v) and (90:10, v/v) for compounds **4a** and **4b,** respectively.

9-(dibutylamino)-5H-benzo[a]phenoxazin-5-one (**NR1**): 41% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.68 (d, 1H, *J* = 8 Hz), 8.33 (d, 1H, *J* = 8 Hz), 7.75 (t, 1H, *J* = 7.6 Hz), 7.70 (t, 1H, *J* = 7.6 Hz), 7.63 (d, 1H, *J* = 8.8 Hz), 6.65 (d, 1H, *J* = 8.8 Hz), 6.48 (s, 1H), 6.42 (s, 1H), 3.43 (t, 4H, *J* = 8 Hz), 1.69 (m, 4H, *J* = 8 Hz), 1.44 (m, 4H, *J* = 8 Hz), 1.03 (t, 6H, *J* = 8 Hz); MS (ESI): 375 (M^+); m.p. >210 °C.

9-(bis(3-phenylpropyl)amino)-5H-benzo[a]phenoxazin-5-one (**NR2**): 23% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.68 (d, 1H), 8.33 (d, 1H), 7.74 (t, 1H), 7.68 (t, 1H), 7.58 (t, 2H), 7.31-7.39 (m, 4H), 7.21-7.30 (m, 5H), 6.48 (d, 1H), 6.41 (s, 1H), 6.26 (s, 1H), 3.40 (t, 4H), 2.71 (t, 4H), 2.00 (m, 4H); MS (ESI): 499 (M^+); m.p. >220 °C.

2.3. Classification of benzophenoxazine derivatives

Nile red derivatives can be classified into three types according to the substituents attached to benzophenoxazine backbone in two positions: the 2-position and the 9-position. The first type is dialkylated derivatives containing a tertiary amine at the 9-position including **NR1** and **NR2**, the second type is 2-hydroxy-dialkylated derivatives containing a hydroxyl group at the 2-position and a tertiary amine at the 9-position including **NR3**, **NR4** and **NR5**, and the third type is 2-hydroxy-monoalkylated derivatives containing a hydroxyl group at the 2-position and a secondary amine at the 9-position including **NR6** and **NR7** as shown in Fig. 1a. Nile blue derivatives can be classified into two types according to the type of amine attached to benzophenoxazine backbone at the 9-position. The first type is dialkylated derivatives containing a tertiary amine at the 9-position including **NB1** and **NB2**, and the second type is monoalkylated derivatives containing a secondary amine at the 9-position including **NB3** and **NB4** as shown in Fig. 1b.

2.4. Preparation of working solutions

Stock solutions of Nile red and Nile blue and their derivatives (4.0×10^{-4} M) were prepared in ethanol and sonicated for 15 minutes to ensure complete dissolution of the dye. Sodium hydroxide solution (0.01 M, pH 12.0) was prepared in deionized water. The working solution was prepared by slowly adding 15.0 mL of sodium hydroxide solution to 15.0 mL of the dye with constant stirring and used immediately after preparation. Final concentration of the dye in the working solution was 2.0×10^{-4} M in ethanol: aqueous sodium hydroxide (1:1, *v/v*).

2.5. Fingerprint detection procedure

This work aims to provide a better understanding of the effect of chemical structure of the dye on fingerprint development. Therefore, one donor and one porous substrate (paper) were selected for the study. Fingerprints were collected on a white copy paper from a 26-year-old male donor and left 24 hours before treatment. Fingerprints were deposited after the donor was instructed to wipe his fingers with his nose prior to fingerprint collection. The donor was asked not to wash his hands within the previous two hours. Fingerprints were obtained using the three middle fingers holding for 8 seconds. The paper was cut in half before treatment, then the left side of the paper was developed using derivatives of Nile red or Nile blue and the right side of the paper was developed using the corresponding parent dye (Nile red or Nile blue) for comparison purposes. The sample was first immersed in deionized water for 5 minutes, then it was removed and immersed in the working solution for 30 minutes. The excess solution was removed by immersing the sample in deionized water for 5 minutes. The sample was left to dry for about one hour on a paper towel at room temperature in a dark place.

2.6. Sample visualization and evaluation

The sample was visualized by the forensic light source (FLS), Mini-CrimeScope 400, (MCS-400) (Spex Forensics, Edison, NJ) at an excitation wavelength of 515 nm for Nile red derivatives, CSS for Nile blue derivatives, and white light for all dyes. The two halves for each sample were placed side by side and photographed together using a digital camera (Nikon D3300) with a 550 nm long-pass barrier filter. University of Canberra (UC) scale [38] was used to compare the performance of the two detection methods: A and B in which A is the proposed method and B is the reference method. In this study, method A is the derivatives of Nile red or Nile blue and method B is the corresponding parent dye, Nile red or Nile blue. Samples were given scores between (-2) and (+2) depending on the improvement in detection performance including fingerprint ridge details and/or contrast between the fingerprint impressions and background substrate. UC scores: (+2) and (+1) were given when the half fingerprint developed by method A shows far greater or slightly greater improvement in detection performance, respectively, compared to the half fingerprint developed by method B; (0) was given when no significant difference between the two half fingerprints; (-1) and (-2) were given when the half fingerprint developed by method B shows slightly greater or far greater improvement in detection performance, respectively, compared to the half fingerprint developed by method A [38].

3. Results and discussion

3.1. Photophysical properties of benzophenoxazine derivatives

The absorbance spectra of Nile red and Nile blue is influenced by the surrounding environment as shown in Fig. 2. The presence of sodium hydroxide (1:1, *v/v*) in the working solution resulted in a red shift of absorbance spectrum of Nile red of about 30 nm. However, the absorbance spectrum of Nile blue displayed a blue shift of about 100 nm due to the deprotonation of the amine group at the 5-position (pK_a = 11.14) and formation the basic form of the dye. The

deprotonation process co-occurs with a color change of Nile blue solution from blue color in pure ethanol to red color in the working solution, ethanol: sodium hydroxide (1:1, *v/v*). The high pH of the working solution was selected as indicated in previous studies [9, 25] and to increase logD value of benzophenoxazine derivatives. Moreover, improvement of fingerprint detection is observed for hydrophobic dyes due to interaction with sebaceous secretions in fingerprint residue as discussed in the following sections. Given the fact that logD is pH dependent, the hydrophobicity of Nile blue alters by pH changes whereas that of Nile red does not. Therefore, Nile blue is more hydrophobic in alkaline solution at pH 12.0 (logD = 3.79) compared to neutral solution at pH 7.4 (logD = 0.60).

The chemical structure and physical properties such as hydrophobicity have an impact on spectroscopic studies of Nile red and Nile blue derivatives. The best tool to describe the hydrophobicity is the partition coefficient (logP) or the distribution coefficient (logD). LogP is defined as the ratio of concentrations of the neutral species of compound in two immiscible phases such as n-octanol and water at equilibrium. On the other hand, logD takes into account all neutral and charged species of compound [39]. Regarding the absorption spectra, a general and small blue shift was observed across Nile red derivatives (Fig. 3a) and the order of shifting was Nile red, dialkylated derivatives N**R1** and **NR2**, 2-hydroxy-dialkylated derivatives **NR3**, **NR4**, and **NR5**, and 2-hydroxy-monoalkylated derivatives **NR6** and **NR7**. Similarly, the absorption spectra of Nile blue and dialkylated derivatives **NB1** and **NB2** showed a blue shift compared to those monoalkylated derivatives **NB3** and **NB4** (Fig. 3b). A similar behavior was observed for Nile blue derivatives after the addition of sodium hydroxide to ethanol for the preparation of working solutions. There was a noticeable color change of the working solutions from blue to red color due to deprotonation of the amine group at the 5-position (pK_a = 11.14) and formation the basic form

of the dye. Examination the emission spectra (Fig. 4a) indicated that Nile red, dialkylated derivative and 2-hydroxy-dialkylated derivatives were blue-shifted with low fluorescence intensity compared to 2-hydroxy-monoalkylated derivatives. In a similar manner, the emission spectra of Nile blue and dialkylated derivatives showed a blue shift compared to monoalkylated derivatives. However, the fluorescence intensity of Nile blue derivatives were similar and emission bands were wider compared to those of Nile red derivatives. The photophysical properties such as quantum yields (Φ_f) of some derivatives of benzophenoxazine dyes are summarized in Table 2, and the complete determination of photophysical properties will be illustrated in Martinez et al. (in preparation).

3.2. Comparison between Nile red and Nile blue for fingerprint detection

To investigate the detection efficiency of the two benzophenoxazine dyes, the working solution of Nile red and Nile blue (basic form) was used for developing fingerprints on a porous surface (paper) as shown in Fig. 5. The sample treated by Nile red produced fingerprints more luminescent than those with Nile blue at two settings of FLS, 515 nm and CSS, both viewed with 550 nm long-pass barrier filter. Although both dyes have similar hydrophobicity at pH 12.0 (logD for Nile red and Nile blue is 3.83 and 3.79, respectively), the efficiency of fingerprint detection is attributed to the difference of chemical structure of the dyes. Both molecules have similar benzophenoxazine backbone except the attached groups at the 5-position, a carbonyl group and an amine group for Nile red and Nile blue, respectively. The amine group is a hydrogen bond donor forming hydrogen bonds with hydroxide ions present in the working solution. The hydrogen bonds weaken the hydrophobic interaction of Nile blue with sebaceous portion of fingerprint residue resulting in weak development.

FLS setting is an important factor to develop fingerprints with clear ridge details and good contrast, especially for comparison purposes. Nile red developed luminescent fingerprints and clear ridges in both settings of FLS as shown in Fig. 5 (left half). However, at CSS which corresponds to 455 nm the background appeared more bright providing poor contrast. Moreover, the maximum wavelength of the working solution of Nile red and Nile blue were 576 and 525 nm, respectively (Fig. 2). Consequently, excitation wavelength at 515 nm is considered the optimum condition of fingerprint visualization for performance comparison of Nile red and its derivatives. On the other hand, weak fingerprint detection and poor contrast were observed for Nile blue in both settings of FLS as shown in Fig. 5 (right half). Moreover, the sample treated by Nile blue at 515 nm seemed more pale due to lack of contrast between fingerprints and the substrate. Therefore, CSS setting of FLS could be the best for fingerprints visualization for performance comparison of Nile blue and its derivatives.

3.3. Application of Nile red derivatives for fingerprint devolvement

Fingerprints developed by the dialkylated derivatives **NR1** and **NR2** were compared to those developed by Nile red as shown in Fig. 6. Both dyes as well as Nile red showed excellent ability to detect fingerprint in which luminescent impressions and detailed ridges were observed at excitation wavelength of 515 nm of the FLS (Fig. 6a), and the results of Nile red are consistent with work of Braasch et al. [25]. Moreover, the underlying substrate of the sample treated by **NR1** was darker compared to Nile red providing more contrast between the prints and background and thus improvement in fingerprint detection. When using white light of FLS without long-pass filter, fingerprints appeared as pink impressions with complete ridge details for **NR1** and **NR2**, whereas the prints were not visible for Nile red (Fig. 6b). However, solid particles were precipitated on the background of the sample treated by **NR2** which could be due to the aqueous nature of the working

solution and the great hydrophobicity of the dye. Nonetheless, the precipitation process did not prevent fingerprint development. The improvement in fingerprint detection is attributed to the fact that both compounds are more hydrophobic than Nile red as indicated by logD values in Table 3. It has been hypothesized that Nile red tended to interact with lipids secreted by sebaceous glands and not with epidermal lipids [25, 28]. Therefore, the hydrophobic interaction of **NR1** and **NR2** with sebaceous secretions of fingerprint residue is stronger relative to Nile red making the two derivatives dual-fingerprint reagents developing visible prints seen by the naked eye (pink impressions) and luminescent prints (yellow-orange impressions) when using FLS at excitation wavelength of 515 nm.

The use of the 2-hydroxy dialkylated derivatives of Nile red resulted in fingerprints that produced various degree of luminescence according to their chemical and physical properties (Fig. 7). Blurred and non-luminescent fingerprints were obtained by the less hydrophobic derivative, **NR3** due to the weak interaction with fingerprint components. On the other hand, fingerprints developed by **NR4** and **NR5** exhibited comparable results in which fingerprints were luminescent with clear ridge details. However, fingerprints developed by Nile red displayed a higher degree of luminescence, ridge details, and clarity. Despite the fact that **NR4** and **NR5** are hydrophobic and the latter dye is more hydrophobic than Nile red (Table 3), there was no improvement in the developing of fingerprints. This could be due to the unwanted interaction of the dye molecules with the working solution. At pH 12.0, the hydroxyl group at the 2-position is deprotonated (pK_a = 5.37) and the negatively charged hydroxide ion can form a bond with positively charged sodium ions present in the working solution. Therefore, the hydrophobic interaction of both dyes with fingerprint secretions is weakened by interference effect. The ability of the dye to develop fingerprints is mainly influenced by its hydrophobicity and other factors such as type of

substituents attached to benzophenoxazine backbone as indicated in the results of dialkylated derivatives and 2-hydroxy dialkylated derivatives. The chemical structures of Nile red, **NR1** and **NR2** are analogous to those for **NR3**, **NR4** and **NR5**, respectively with the exception that the latter derivatives contain a hydroxyl group at the 2-position. The presence of hydroxyl group decreases the hydrophobicity of dyes and has adverse impact on fingerprint development.

Fingerprints developed by the 2-hydroxy monoalkylated derivatives were faint and appeared as smudged prints compared to those developed by Nile red (Fig. 8). The inefficiency of **NR6** and **NR7** is referred to the low hydrophobicity as characterized by logD resulting in weak interaction with fingerprint residue and poor detection.

It was observed that the fluorescence properties of the final product (fingerprint impressions) resulting from the interaction between the dye and fingerprint residue were not influenced by photophysical properties of the dye. For instance, the performance of dialkylated derivatives **NR1** and **NR2** were comparable in which fluorescent fingerprints with good contrast were obtained (Fig. 6) even though the fluorescence intensity of **NR2** was higher than **NR1** as shown in Fig. 4a. Moreover, samples treated by 2-hydroxy monoalkylated derivatives **NR6** and **NR7** resulted in non-fluorescent fingerprints as shown in Fig. 8 regardless their high fluorescence intensity compared to other derivatives (Fig. 4a).

3.4. Application of Nile blue derivatives for fingerprint devolvement

Samples treated by the dialkylated derivatives were compared to those treated by Nile blue (all in the basic form of the dyes) as shown in Fig. 9. The fingerprints developed by **NB1** and **NB2** appeared more luminescent with clear ridge pattern than those developed by Nile blue at CSS setting of the FLS. Furthermore, visible fingerprints were obtained as dark blue impressions with clear definition for dialkylated derivatives samples with the use of white light of FLS. However,

no fingerprints were visible for Nile blue sample at the same setting of FLS. Thus, the two compounds are considered dual-fingerprint reagents due to their ability to reveal fingerprints that are visible to the naked eye (dark blue impressions) and luminescent when using FLS (yellow-orange impressions). The enhancement of fingerprint performance for **NB1** and **NB2** is due to the great hydrophobicity as characterized by logD (Table 3) resulting in stronger interaction with lipid components of fingerprint residue.

On the other hand, non-luminescent fingerprints were obtained for the monoalkylated derivatives samples relative to those for Nile blue sample (all in the basic form of the dyes) as shown in Fig. 10. The inferior performance of **NB3** and **NB4** is attributed to the low hydrophobicity and type of groups attached to benzophenoxazine backbone as previously mentioned. **NB3** is less hydrophobic than Nile blue and thus unsuccessful fingerprint detection was obtained due to the weak interaction with fingerprint reside. Despite the similarity in hydrophobic properties of **NB4** and Nile blue, no development in fingerprint detection was observed. The chemical structure of **NB3** and **NB4** is analogous to Nile blue and **NB1** respectively with the exception that an extra alkyl group is attached to the amine at the 9-position in the latter dyes. **NB3** and **NB4** contain secondary amine at the 9-position which is considered a hydrogen donor that has the potential to form hydrogen bonds with hydroxide ions present in the working solution. The hydrogen bonds suppress the interaction of the dyes with fingerprint residue. Therefore, the use of these dyes to detect fingerprints is not suitable in forensic detection due to the hydrogen bonds and/or low hydrophobicity.

It was shown that the photophysical properties of Nile blue derivatives did not have impact on the intensity of the developed fingerprints. For example, **NB2** with the lowest fluorescence intensity (Fig. 4b) was considered a dual reagent due to its ability to develop fluorescent and visible

fingerprints (Fig. 9). On the other hand, **NB4** had the highest intensity as indicated in Fig. 4b despite its poor detection (Fig. 10). Furthermore, the fingerprint performance of **NB**, **NB1**, and **NB3** was different regardless their similarity in fluorescence intensity.

3.5. Performance evaluation of benzophenoxazine derivatives

To assess the performance of benzophenoxazine dyes as potential fingerprint reagents, UC scale [38] was applied for comparison between the two methods A and B, in which method A is derivatives of Nile red or Nile blue and method B is corresponding parent dye, Nile red or Nile blue as shown in Table 4. For Nile red derivatives performance, dialkylated derivatives **NR1** and **NR2** showed improvement in fingerprint detection relative to Nile red in which they developed visible and luminescent prints with clear ridge patterns, and **NR1** displayed better background contrast. Consequently, the two dyes can be considered as dual-fingerprint reagents due to their great hydrophobicity compared to that of Nile red. However, no improvement in fingerprint detection was observed for the 2-hydroxy dialkylated derivatives and 2-hydroxy monoalkylated derivatives due to the interference effect and/or the low hydrophobicity. Faint or non-luminescent fingerprints and poor contrast were obtained by both types of Nile red derivatives.

For Nile blue derivatives detection performance, dialkylated derivatives **NB1** and **NB2** exhibited enhancement in developing fingerprints relative to Nile blue. Both dyes are considered dual-fingerprint reagents due to their ability to reveal visible and more luminescent prints with clear ridge details. However, weak fingerprints were developed by monoalkylated derivatives **NB3** and **NB4**. Therefore, these dyes are not useful reagents for fingerprint detection due to the low hydrophobicity and/or hydrogen bonds.

Besides the improved results of dialkylated derivatives of Nile red and Nile blue, these compounds exhibit high sensitivity to produce visible prints at dye concentration of 0.2 mM. This

concentration is 5-fold less than the concentration needed for the dual-fingerprint reagent genipin, to develop visible prints at concentration of 10 mM [33]. In addition, the combination of color and luminescent properties of the developed prints by a single reagent offer visualization in the field as well as in the laboratory. Moreover, the fluorescent prints are more practical as they can be recovered from dark-colored surfaces. Other than immersing the sample in water before and after the treatment, no pretreatment or post-treatment is needed to produce luminescent prints.

4. Conclusions

The use of two types of benzophenoxazine dyes as developing reagents to reveal latent fingerprints on porous surfaces was investigated. It was found the most significant property of the dye to develop fingerprints is hydrophobicity as indicated by logD value. In this work, hydrophobic dialkylated derivatives of Nile red and Nile blue (in the basic form) showed enhancement in developing fingerprints. **NR1**, **NR2**, **NB1**, and **NB2** detected fingerprints more effectively than their corresponding parent dyes producing more luminescent and visible prints. Therefore, these dyes are considered as dual-fingerprint reagents. Furthermore, the application of the reagents is simple, sensitive, and effective suggesting their potential use in forensic detection. Besides the significant role of hydrophobicity for fingerprint development, other parameters such as the functional groups attached to the benzophenoxazine backbone and their ability to form hydrogen bonds or interact with solvent should be taken into consideration. Therefore, the use of less hydrophobic derivatives such as **NR3**, **NR6**, **NR7**, and **NB3** or hydrophobic derivatives containing interference substituents such as **NR4**, **NR5**, and **NB4** as fingerprint reagents was not sufficient for detection purposes. Further studies are needed to evaluate the implementation of the modified reagents to develop latent fingerprints on different substrates for a large number of donors and to reveal aged fingerprints.

Author Contribution:

E.A. designed this work and prepared the manuscript. W.A. synthesized and characterized NR1 and NR2. All authors reviewed and edited the manuscript.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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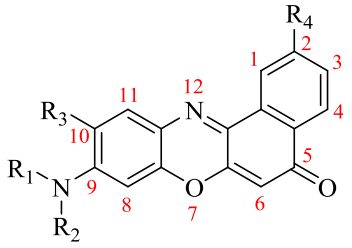
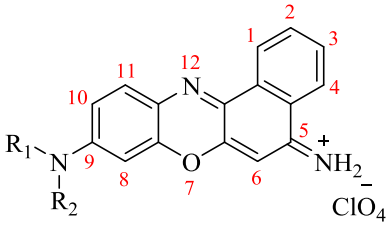
(a)



(b)



NR: $R_1 = R_2 = C_2H_5$, $R_3 = R_4 = H$
NR1: $R_1 = R_2 = C_4H_9$, $R_3 = R_4 = H$
NR2: $R_1 = R_2 = (C_6H_5)C_3H_6$, $R_3 = R_4 = H$
NR3: $R_1 = R_2 = C_2H_5$, $R_3 = H$, $R_4 = OH$
NR4: $R_1 = R_2 = C_4H_9$, $R_3 = H$, $R_4 = OH$
NR5: $R_1 = R_2 = (C_6H_5)C_3H_6$, $R_3 = H$, $R_4 = OH$
NR6: $R_1 = C_4H_9$, $R_2 = H$, $R_3 = CH_3$, $R_4 = OH$
NR7: $R_1 = (C_6H_5)C_3H_6$, $R_3 = H$, $R_3 = CH_3$, $R_4 = OH$

NB: $R_1 = R_2 = C_2H_5$
NB1: $R_1 = R_2 = C_4H_9$
NB2: $R_1 = R_2 = (C_6H_5)C_3H_6$
NB3: $R_1 = C_2H_5$, $R_2 = H$
NB4: $R_1 = C_4H_9$, $R_2 = H$

Figure 1: Chemical structures of (a) Nile red derivatives and (b) Nile blue derivatives

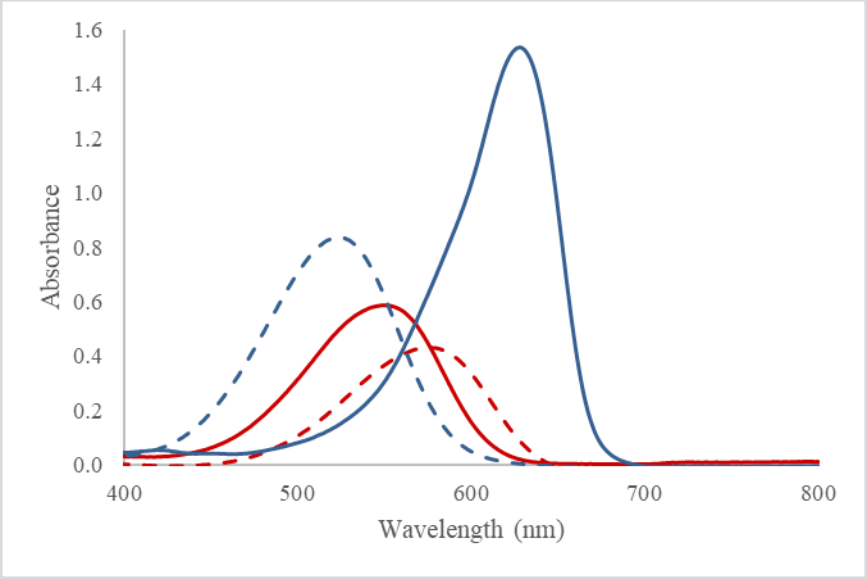


Figure 2: Absorbance spectra of 2.0×10^{-5} M Nile red (red line) and Nile blue (blue line) in ethanol (solid line) and in the working solution [ethanol: sodium hydroxide (1:1, *v/v*)] (dashed line)

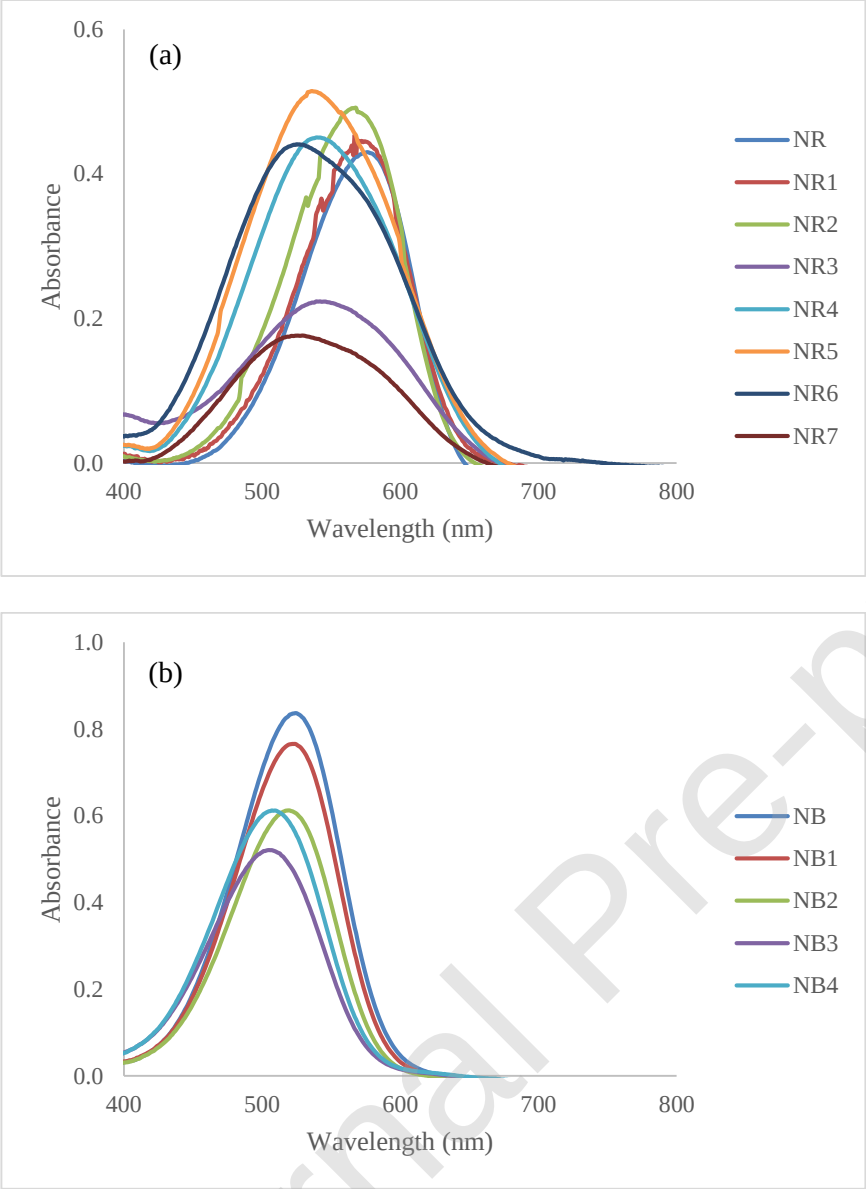


Figure 3: Absorbance spectra of 2.0×10^{-5} M of (a) Nile red derivatives and (b) Nile blue derivatives in the working solution [ethanol: sodium hydroxide (1:1, *v/v*)]

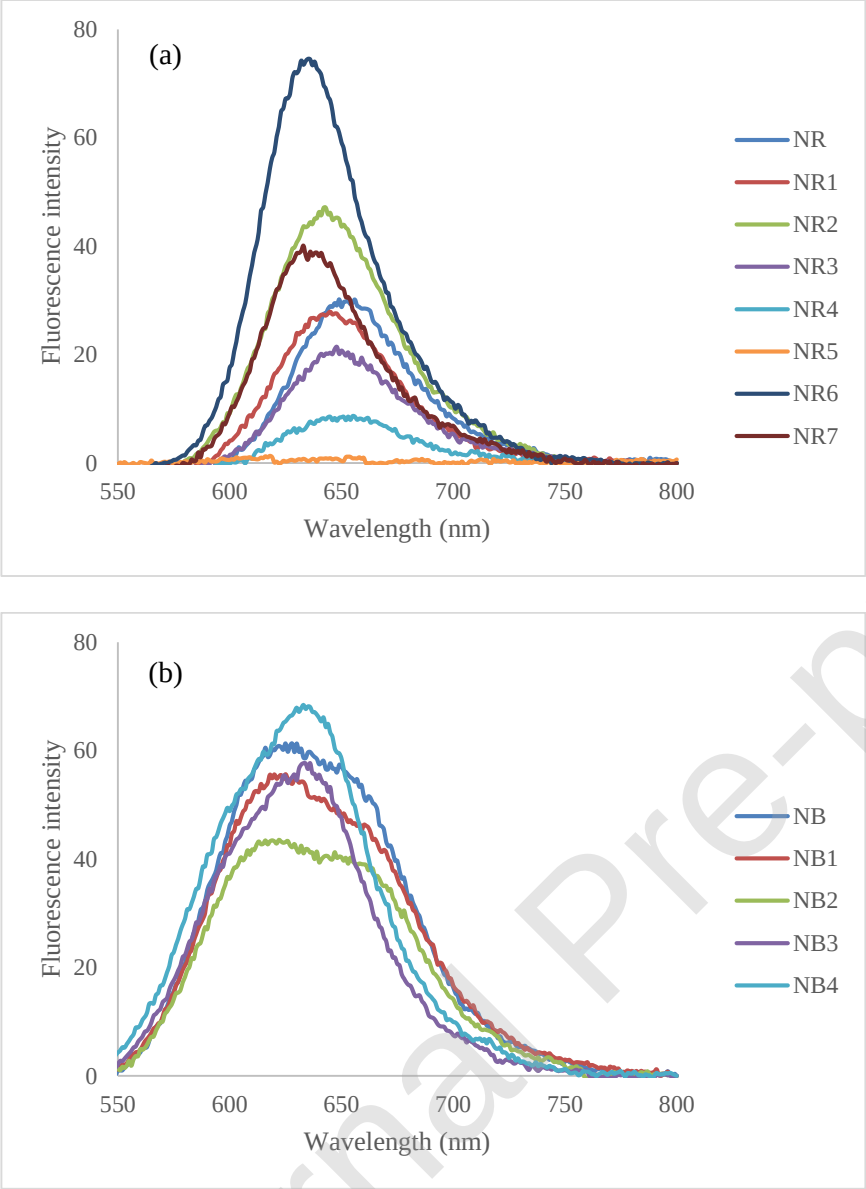


Figure 4: Emission spectra of 2.0×10^{-6} M of (a) Nile red derivatives and (b) Nile blue derivatives in the working solution [ethanol: sodium hydroxide (1:1, *v/v*)]

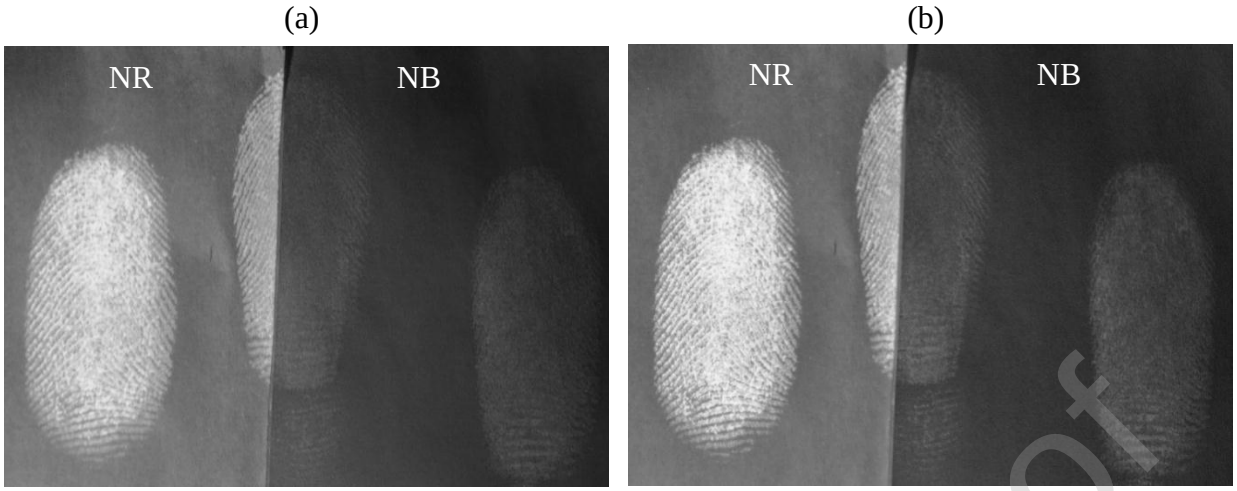


Figure 5: Comparison of fingerprints developed by NR (left half) and NB (right half) at different FLS settings: (a) 515 nm excitation wavelength and (b) at CSS, both viewed with 550 nm long-pass barrier filter

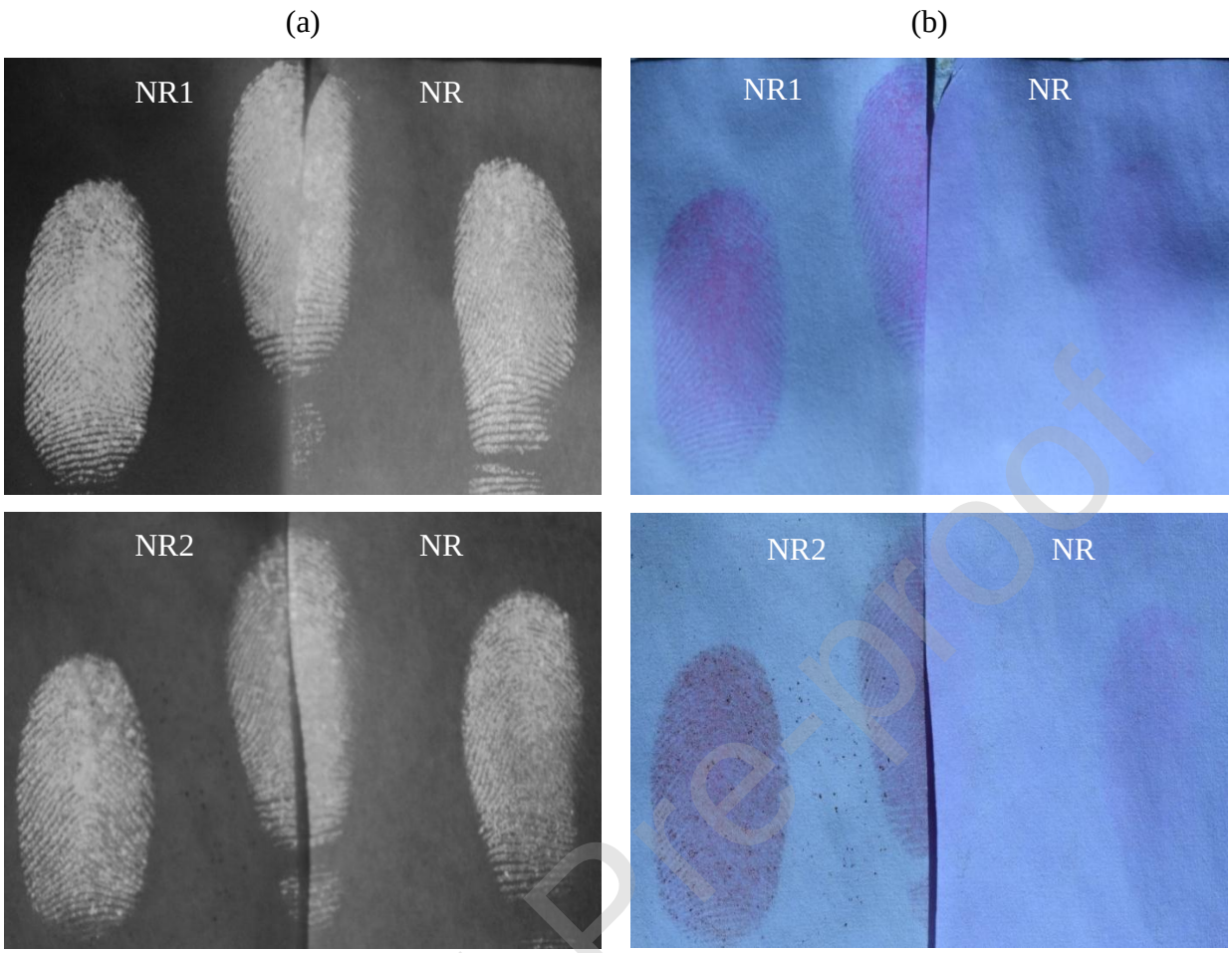


Figure 6: Comparison of fingerprints developed by dialkylated derivatives of Nile red (left half) and NR (right half): (a) at 515 nm excitation wavelength of FLS and viewed with 550 nm long-pass barrier filter and (b) under white light

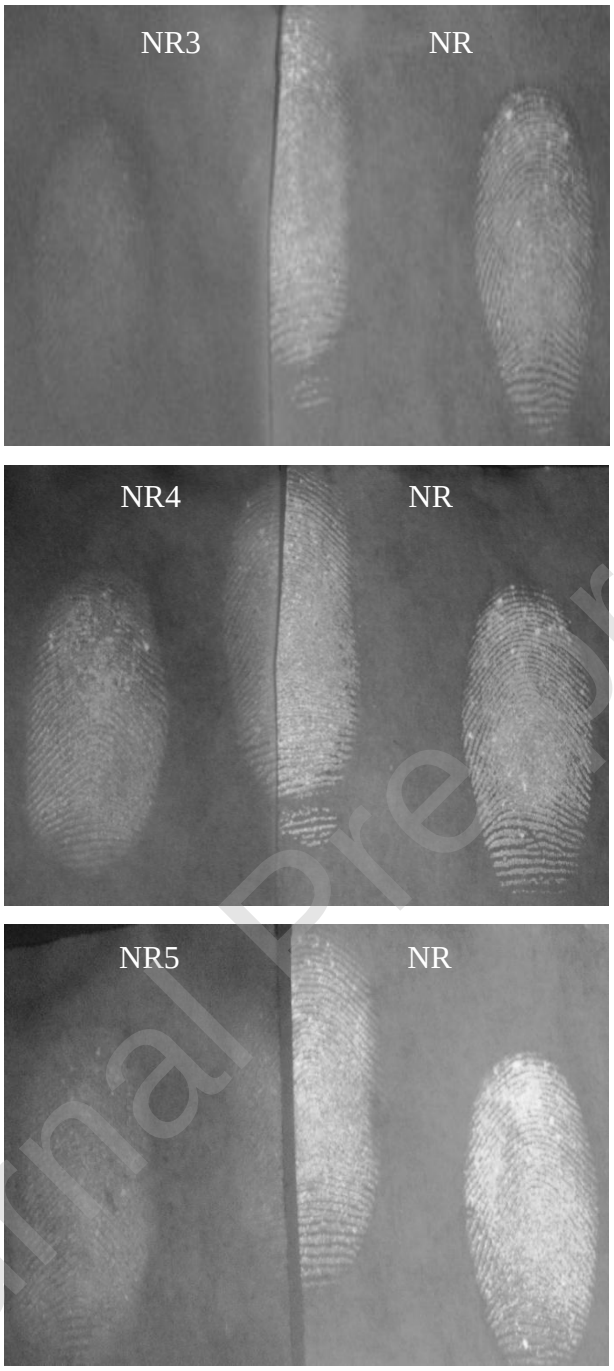


Figure 7: Comparison of fingerprints developed by 2-hydroxy-dialkylated derivatives of Nile red (left half) and NR (right half) at 515 nm excitation wavelength of FLS and viewed with 550 nm long-pass barrier filter

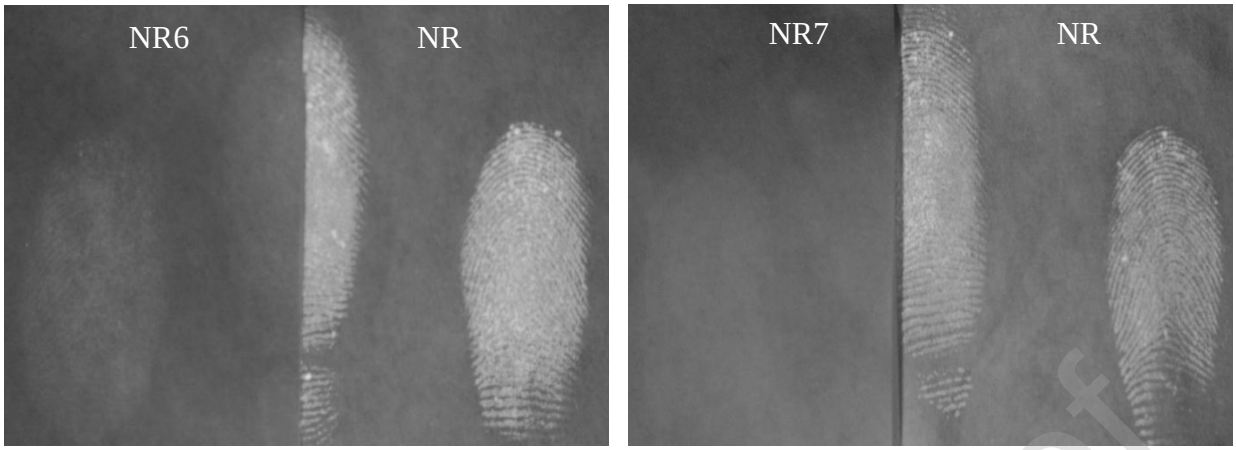


Figure 8: Comparison of fingerprints developed by 2-hydroxy-monoalkylated derivatives of Nile red (left half) and NR (right half) at 515 nm excitation wavelength of FLS and viewed with 550 nm long-pass barrier filter

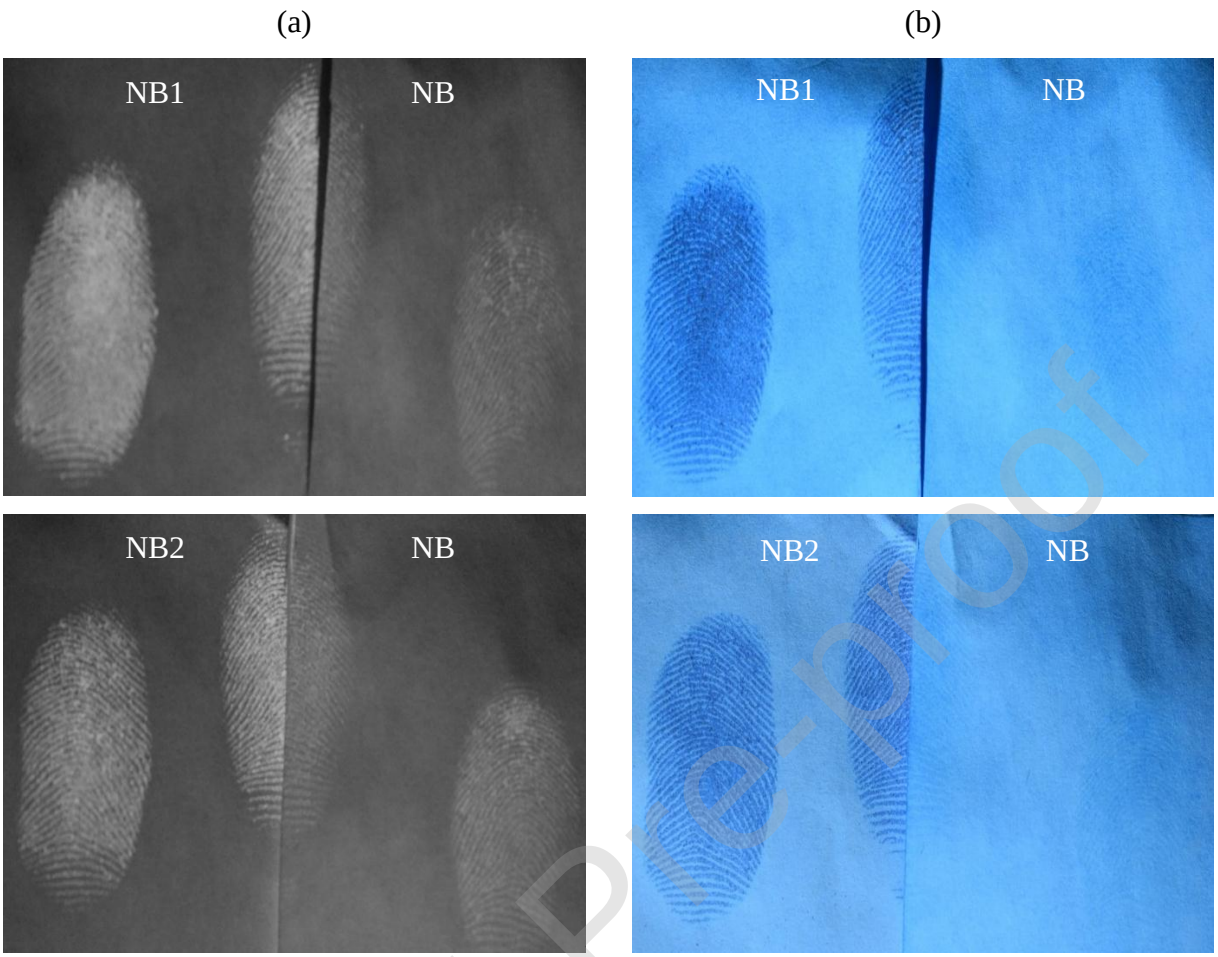


Figure 9: Comparison of fingerprints developed by dialkylated derivatives of Nile blue (left half) and NB (right half): (a) at CSS of FLS and viewed with 550 nm long-pass barrier filter and (b) under white light

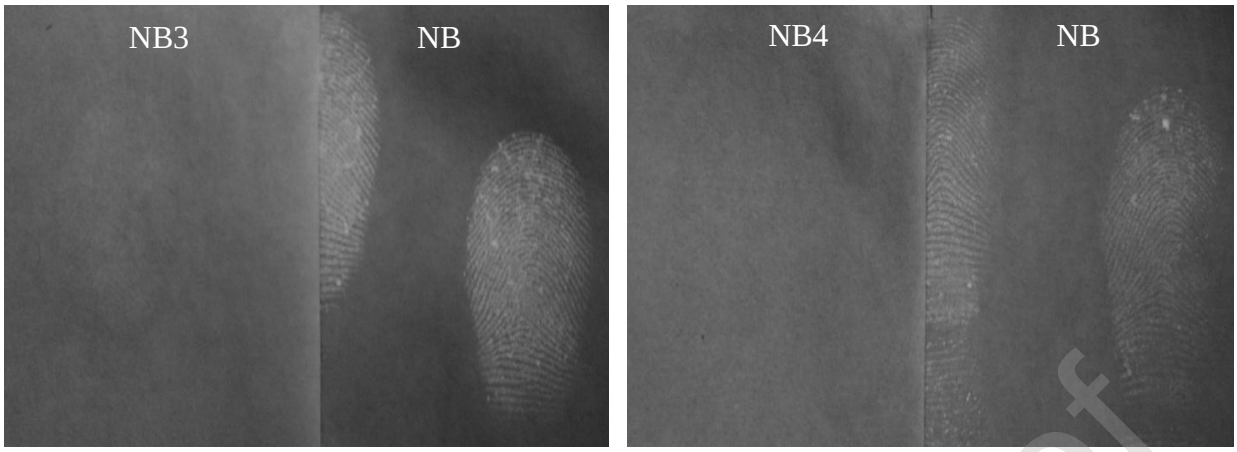
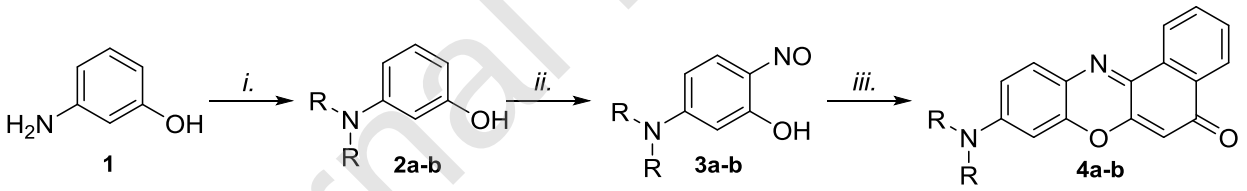


Figure 10: Comparison of fingerprints developed by monoalkylated derivatives of Nile blue (left half) and NB (right half) at CSS of FLS and viewed with 550 nm long-pass barrier filter



i. R-Br,K_2CO_3, H_2O:isopropanol, (1:1, v/v), 3 h
ii. $NaNO_2$, EtOH or isopropanol, HCl, 0º C, 3 h
iii. 1-hydroxynaphthalene, EtOH, HCl

a (**NR1**): R = C_4H_9
b (**NR2**): R = $(C_6H_5)C_3H_5$

Scheme 1: Synthetic route for the preparation of the Nile red derivatives, NR1 and NR2

Table 1: LogD values of some dyes at pH 7.4

Dye	LogD[a]
Oil Red O	9.52
Solvent black 3	8.13
Eosin-blue	6.08
Coomassie brilliant blue	5.86
Acidine orange	2.93
Acidine yellow	1.85
Crystal violet	1.39
Coumarin 6	4.79
Rhodamine B	2.34
Rhodamine 6G	1.45
Nile red	3.83
Nile blue	0.60

[a] Calculated by ChemAxon

Table 2: Quantum yield values of benzophenoxazine dyes in DMSO

Dye	Φ_f
NR3	0.43[a]
NR4	0.45[a]
NR5	0.43[a]
NR6	0.44[a]
NR7	0.45[a]
NB1	0.24[b]
NB2	0.29[b]
NB3	0.70[b]
NB4	0.65[b]

Standards used for quantum yield determination: [a] rhodamine 6G = 0.95 in EtOH and [b] Nile Blue = 0.27 in MeOH

Table 3: LogD values of benzophenoxazine dyes at pH 12

Dye	logD[a]
NR	3.83
NR1	5.77
NR2	8.04
NR3	1.37
NR4	3.31
NR5	5.57
NR6	1.86
NR7	2.99
NB	3.79
NB1	5.72
NB2	7.99
NB3	2.80
NB4	3.76

[a] Calculated by ChemAxon

Table 4: UC score of benzophenoxazine derivatives; (**NR1**-**NR7**) relative to Nile red and (**NB1**-**NB4**) relative to Nile blue

Dye	score
NR1	+1
NR2	+1
NR3	-2
NR4	-1
NR5	-1
NR6	-2
NR7	-2
NB1	+2
NB2	+2
NB3	-2
NB4	-2