CHAPTER 10

OXIDATION MONITORING BY FLUORESCENCE SPECTROSCOPY REVEALS THE AGE OF FINGERMARKS


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ABSTRACT

No forensic method exists that can reliably estimate the age of fingermarks found at a crime scene. Information on the time passed since fingermark deposition is desired as it can be used to distinguish between crime related and unrelated fingermarks and to support or refute statements made by the fingermark donors. We introduce a non-contact method that can estimate the age of fingermarks. Fingermarks were approached as protein lipid mixtures and an age estimation model was build based on the expected protein lipid oxidation reactions. Two measures of oxidation are required from the fingermark to estimate its age: 1) the relative amount of fluorescent oxidation products 2) the rate at which these products are formed. Fluorescence spectroscopy was used to obtain these measures. We tested the method on 44 fingermarks and were able to estimate the age of 55% of the male fingermarks, up to three weeks old with an uncertainty of 1.9 days.
1. INTRODUCTION

Accurate recording and analysis of fingermarks is essential to the processing of a crime scene. Establishing the time passed since fingermark deposition can be of crucial importance. Crime scene investigators can use it to find and select relevant evidence and to discard marks that are not crime related. Also, such information can be used to assess statements from witnesses, victims and suspects [1-2]. Currently, age estimation of fingermarks is impossible. In the past, the age of fingermarks has been estimated from the quality of the mark and the ease of development with, for instance, dactyloscopic powder; but fingermarks appearing “fresh” were in fact old [3-4]. The main progress made so far is classifying fingermarks as younger, or older than 5 hours using white light imaging [5]. The obstacle that has prevented the development of an age estimation method for fingermarks is their highly variable chemical composition which affects the aging process [6]. In contrast, the age estimation of blood stains is possible based on the quantification of the oxidation products of hemoglobin [7-8]. We propose that for fingermarks oxidation processes can also be used to estimate their age.

2. THEORY

Generally, fingermarks contain proteins and lipids [6]. Tryptophan-containing proteins (Tryp) have been proposed to be the main contributor to fresh fingermarks’ autofluorescence (Figure 1-A) [9]. When exposed to air, unsaturated lipids oxidize and form reactive oxidation products (LipOx), which react with proteins to form fluorescent oxidation products (FOX) [10-12]:

\[
\text{LipOx} + \text{Tryp} \rightarrow \text{FOX} \quad \text{Eq.(1)}
\]

LipOx and FOX (Equation 1) are complex mixtures of known and unknown oxidation products; thus, we have set the stoichiometric constants to 1. As the amount of tryptophan does not always fully decay to zero over time, we split [Tryp] into two parts (brackets are used to indicate concentration):

\[
[\text{Tryp}] = [\text{Tryp}_R] + [\text{Tryp}_C]
\]

where \([\text{Tryp}_R]\), is the fraction of Tryp that will react with LipOx and \([\text{Tryp}_C]\) is the fraction that does not. As LipOx is both generated by oxidation of unsaturated lipids and consumed by oxidation of proteins, we assume \([\text{LipOx}]\) to remain constant, indicated by \([\text{LipOx}]_0\). Thus, the reaction follows pseudo first-order reaction dynamics (Equation 2):

\[
\frac{d[\text{FOX}]}{dt} = -\frac{d[\text{Tryp}]}{dt} = -\frac{d[\text{Tryp}_R]}{dt} = k'[\text{LipOx}]_0[\text{Tryp}_R]_t = k[\text{Tryp}_R]_t
\]

\text{Eq.(2)}

From Equation 2 follows that the rate constant \(k\) (day\(^{-1}\)), representing the aging rate of the fingermark, is proportional to \([\text{LipOx}]_0\). FOX is generated as described by Equation 1 with \([\text{FOX}]_0\) being the fraction of FOX already present at the time of fingermark deposition. The solution to the differential equation (Equation 2) is:
Thus:

$$f(t) = \frac{[\text{Tryp}]_0 \cdot e^{-kt} + [\text{Tryp}_c]}{[\text{FOX}]_0 \cdot (1 - e^{-kt}) + [\text{FOX}]_0}$$

Eq.(3)

The limiting behavior of this ratio is:

$$f_\infty = \lim_{t \to \infty} f(t) = \frac{[\text{Tryp}_c]}{([\text{Tryp}]_0 - [\text{Tryp}_c]) + [\text{FOX}]_0}$$

Substitution of

$$[\text{Tryp}_c] = \frac{f_\infty([\text{Tryp}]_0 + [\text{FOX}]_0)}{1 + f_\infty}$$

into $f(t)$ gives:

$$f(t) = \frac{([\text{Tryp}]_0 - f_\infty[\text{FOX}]_0) e^{-kt} + f_\infty([\text{Tryp}]_0 + [\text{FOX}]_0)}{([\text{Tryp}]_0 - f_\infty[\text{FOX}]_0)(1 - e^{-kt}) + [\text{FOX}]_0 (1 + f_\infty)}$$

With $f_0 = \frac{[\text{Tryp}]_0}{[\text{FOX}]_0}$, which represents the $[\text{Tryp}] / [\text{FOX}]$ ratio of fresh fingermarks, it follows that:

$$f(t) = \frac{[\text{Tryp}]}{[\text{FOX}]} = \frac{(f_0 - f_\infty)e^{-kt} + f_\infty(f_0 + 1)}{-(f_0 - f_\infty)e^{-kt} + f_\infty + 1}$$

(aging function) Eq.(4)

with $f_0$ being the value of $f(t)$ at $t=0$ and

$$f_\infty = \lim_{t \to \infty} f(t) = \frac{[\text{Tryp}_c]}{([\text{Tryp}]_0 - [\text{Tryp}_c]) + [\text{FOX}]_0}$$

As $[\text{Tryp}]$ and $[\text{FOX}]$ cannot be determined in fingermarks, we assumed them to be proportional to their autofluorescence intensity integrated over the wavelength range 313-550 nm (excitation at 283 nm) and 400-500 nm (excitation at 365 nm), called $\text{Tryp}_n$ and $\text{FOX}_n$ (shaded area, Figure 1-B) respectively [10-12].

Fluorescence originating from non-protein fluorophores may lead to an overestimation of the amount of Tryp. We therefore constructed a reference spectrum using six fingermarks washed with a chloroform/methanol mixture known to elute fluorescent oxidation products and other unknown fluorescent components while retaining proteins [13]. This reference spectrum was fitted to the Tryp fluorescence spectra using a weighted least-squares fitting method. The area under the curve of the fitted spectrum was $\text{Tryp}_n$ (Figure 1-C). The $\text{Tryp}_n/\text{FOX}_n$ ratio could now be calculated and plotted against time (Figure 1-D).
3. MATERIALS AND METHODS

3.1 Chemicals, instruments and software.
Chloroform, methanol and squalene were purchased from Sigma-Aldrich, USA. Thin layer chromatography (TLC) Silicagel 60 aluminum sheets were obtained from Merck, Germany. Emission and excitation spectra were measured with a LS55 Luminescence spectrometer equipped with fiber optic accessory (Perkin Elmer, USA). MATLAB® was used for data analysis.

Figure 1. Procedure to estimate the age of a fingermark. A: Autofluorescence image of a fingermark illuminated with a UV Crime-lite® 2 (365 nm) and imaged with a Nikon D40X digital camera equipped with a Schott GG420 long pass filter. B: Tryp and FOX fluorescence emission spectra. Blue line: excitation at 283 nm, red line: excitation at 365 nm. Shaded area: integrated area for FOX determination. C: Fit of reference fluorescence emission spectrum (red line) to the measured Tryp fluorescence emission spectrum (blue line) as shown in 1B. D: Blue dots: Tryp/Fox ratio of an aging fingermark measured at consecutive time points, red line: fitted aging curve.
3.2 Fluorescence emission spectra.
Excitation of tryptophan moieties in proteins: 283 nm excitation wavelength (5 nm
bandwidth), 313-550 nm emission wavelengths (20 nm bandwidth)[10-12]. Excitation
of fluorescent oxidation products: 365 nm (5 nm bandwidth) excitation wavelength,
395-550 nm emission wavelengths (20 nm bandwidth) [10-12].

3.3 Fingermarks.
Fingermarks were collected between 9:00 and 12:00 h and stored in a dark cabinet. For
t = 0, fingermarks were measured within one to three hours after deposition. Natural
fingermarks: no special instructions were given to the donors prior to fingermark do-
nation. Stacked fingermarks: volunteers deposited five or ten fingermarks (one for each
finger) on top of each other onto a TLC plate.

3.4 Fingermark fluorescence imaging.
The fingermark in figure 1C (main text) was illuminated with a UV Crime-lite®2 (365
nm) and imaged with a Nikon D40X digital camera equipped with a Schott GG420 long
pass filter.

Data are represented as mean ± standard deviation, except for the age estimation re-
sults, which are represented as median ± inter-quartile range due to the non-normal
distribution of the results at t = 0.

3.4 Age estimation method
As [Tryp] and [FOX] cannot be determined in fingermarks, we assumed them to be pro-
portional to the autofluorescence intensity in the wavelength range 313-550 nm (excita-
tion at 283 nm) and 400-500 nm (excitation at 365 nm), called Tryp_f and FOX_f respec-
tively [10-12]. FOX_f was defined as the integration of the emission spectrum (400-500
nm) resulting from 365 nm excitation. The definition of Tryp_f is described in the next
section.

3.4.1 Reference spectrum.
A complicating factor is that fingermarks contain a wide variety of fluorescing endog-
eous and exogenous components [6, 9]. Thus, the fluorescence originating from non-pro-
tein fluorophores may lead to an overestimation of the amount of Tryp. To estimate the
extent of contamination, a reference spectrum was constructed of washed fingermarks.
Stacked (10x) natural fingermarks from 5 female (average age 28.0 ± 4.4 years) and 7
male donors (average age 37.3 ± 12.3 years) were placed onto a TLC plate, which was
developed using a chloroform/methanol (60 ml/240 ml) mobile phase [9, 13]. After
drying, fluorescence emission spectra (excitation wavelength 283 nm, emission wave-
length 313-550 nm) were measured at three randomly chosen locations of the finger-
mark residues. The fingermark residues are the washed fingermarks. The reference
spectrum was created by taking the average spectrum of all measurements.
This reference spectrum was fitted to the fingermark fluorescence spectra by a weighted least-squares algorithm. The area under the scaled reference spectrum was used as Tryp reference.

3.4.2 Intercept \( (f_0) \) estimation.
Stacked natural fingermarks from 21 male (average age 35.6 ± 13.9 years) and 17 female (average age 28.1 ± 4.1 years) donors were measured for \( f_0 \) at one randomly chosen location 1 to 3 hour after deposition. Ten samples were stacks of 5 fingermarks, 28 samples were stacks of 10 fingermarks. Twenty-three stacked fingermarks passed the inclusion criteria and were used for determination of the intercept, \( f_0 \) of the aging function, which is the ratio of [Tryp] to [FOX] content, estimated as described in the previous section (Figure 2). The mean ratio, 3.9 ± 3.5, was used for \( f_0 \).

The uncertainty in the time estimate, \( \Delta t \), caused by an uncertainty in the \( f_0 \)-value, \( \Delta f_0 \), follows from

\[
\Delta t = \frac{\Delta f_0}{(df_0 / dt)_{f_0}}
\]

where

\[
\frac{df}{dt}(t = 0) = -\frac{k(f_0 - f_\infty)(1 + f_0)}{f_\infty + 1}
\]

Thus,

\[
\Delta t = -\Delta f_0 \cdot \frac{(f_\infty + 1)}{k(f_0 - f_\infty)(1 + f_0)} \quad \text{Eq.(5)}
\]

Applying Equation 5 to the intercept data, a median error of 5.0 days is to be expected.

3.4.3 Inclusion criteria.
Not all fingermarks studied allowed age estimation and the following inclusion criteria were used for the first measurement \( (t_0) \) of a time series:

a. **Minimal value for Tryp**
   Fingermarks that yielded scaling factors smaller than 0.2 were excluded.

b. **Minimal value for FOX**
   The emission was measured with half nanometer steps, resulting in 200 data points for each fluorescence emission spectrum. A minimal amount of signal, defined as 50 positive values of which at least 10 consecutive, was required for data inclusion.

c. **R² of fitted reference spectrum to the Tryp fluorescence spectra had to be larger than 0.**

In order to estimate the age of a fingermark with our method, time series were generated, and the aging function was fitted by non-linear least squares to the measurements.
Important for an accurate age estimation are the start values for the fitting parameters, \( f_\infty \) and the rate constant \( k \). These were estimated as follows: the average of the last 3 data points was used for \( f_\infty \). The rate constant \( k \) was approximated from a linear fit of the first 5 data points, utilizing the first-order Taylor series of the aging function about the time of the first measurement \( t_0 \). In order to detect the global minimum, local solutions were sought for multiple start time points (every number of days in the interval of 0 days to 10 years). The fitted aging curve was only deemed reliable for age estimation when:

\[ a. \quad R^2 > 0.85. \]
\[ b. \quad Six \ or \ more \ non-constant \ data \ points \ were \ available. \]
\[ c. \quad The \ ratio \ of \ the \ first \ measurement \ (t = t_0) \ had \ to \ be \ at \ least \ 0.2. \]
\[ d. \quad Signal-to-noise \ ratio, \ defined \ as \ amount \ of \ decay \ of \ the \ aging \ curve \ relative \ to \ the \ root \ mean \ square \ error \ of \ the \ fit, \ was \ larger \ than \ 3.92. \]

### 3.4.4 Fingermarks for age estimation.

Each donor deposited stacked natural fingermarks (one mark per finger of one hand). Nineteen donors were male, 19 female. The average age of the male donors was 38.2 ± 13.0 years and of the female donors 29.4 ± 3.1 years. Each set of stacked fingermarks was measured at one location of the fingermark for various time points up to 154 days. These fingermarks together with the six natural fingermarks described in the next section were used for age estimation.

### 3.5 Squalene spiked versus natural fingermarks

Six donors provided stacked natural fingermarks (one mark per finger of one hand). Three male donors (29, 31 and 36 years old) and three female donors (27, 31 and 59 years old). Each donor deposited two sets of stacked fingermarks: natural and squalene-spiked fingermarks. Drops of squalene were applied onto a paper towel. Volunteers dabbed their index finger onto the towel and rubbed the other four fingers over the squalene-dabbed fingertip. Next, they created a stacked fingermark by sequentially placing all five fingertips onto the same spot on a TLC plate. Each set of stacked fingermarks was measured at a randomly chosen location of the fingermark for various time points up to 176 days.

### 3.6 Effect of temperature on the aging of a fingermark

Four donors provided stacked natural fingermarks (one mark per finger of both hands), two male donors (32 and 44 years old) and two female donors (25 and 34 years old). The fingermarks were stored in a dark stove set at 37°C. Each stacked fingermark was measured at a randomly chosen location of the fingermark for various time points up to 12 days. The first five days, two measurements per day were performed, after that one per day. The \( \text{TrypH}_\| / \text{FOXH}_\| \) ratio was determined for each data point and age estimation was performed.
4. RESULTS

For age estimation, the Tryp$_n$/FOX$_n$ ratio was measured for the first time at (an unknown) $t_0$ days after fingermark deposition and subsequently for several days, thereby generating multiple values for $f(t_0 + t_{\text{measured}})$, where $t_{\text{measured}}$ is the time of subsequent measurements. The parameter $f_0$, the Tryp$_n$/FOX$_n$ ratio of fresh fingermarks, was established from 23 fresh fingermarks (3.85 ± 3.48), as shown in figure 2.

By fitting the aging function [Eq. (4)] to the data points using a nonlinear least-squares method, $t_0$, $k$ and $f_\infty$ could be estimated. The first measurement of an unknown fingermark ($t=t_0$) was used to assess the suitability of that fingermark for age estimation. We included the three criteria described in the method section ‘2.4.3 Inclusion criteria: (a, b) Tryp$_n$ and FOX$_n$ had to exceed a predefined minimum; (c) fitting the reference spectrum to the measured Tryp fluorescence spectrum had to yield a positive $R^2$ value. The Tryp inclusion criteria (a, c) were incorporated to ensure dominant contribution of protein fluorescence over background fluorescence. They were only applied to the first measurement at $t=t_0$ as Tryp is expected to disappear and FOX to appear in subsequent measurements. Aging curves were included for age estimation when (a) $R^2 \geq 0.85$, (b) a minimum of 6 non-constant data points were present and (c) the ratio of the first measurement ($t=t_0$) exceeded 0.2. A signal-to-noise limit was set at 3.92 (Method section ‘2.4.3 Inclusion criteria’).

Figure 2. Intercept data. Tryp$_n$/FOX$_n$ ratio of 23 fingermarks from different donors at $t = 1\text{-}3$ hours.

Age estimation of fingermarks with our method requires a time series of Tryp$_n$/FOX$_n$ at consecutive time points ($t_{\text{measured}}$) and fitting these data to the aging function [Eq. (4)]. A set of 22 male and 22 female fingermarks was used to test our method. First, the complete time series (ranging from 65 to 176 days in length) of each fingermark was used for fitting. Then, the first time point was excluded from the time series and the aging function was fitted again and so forth. In this way, the data from each fingermark were reused to simulate increasing ages for $t_0$, varying from 1 hour to 5 months. Twelve
fingermarks allowed age estimation, and more than twelve data points were generated based on the simulation of increased ages (Figure 3).

All fingermarks suitable for age estimation were derived from male donors due to the low fluorescence signal retrieved from female fingermarks: 77% of the fresh female fingermarks did not display sufficient Tryp fluorescence as opposed to 27% of the male fingermarks. Overall, age assessments were possible up to three weeks after deposition with a median uncertainty of 1.9 days. To demonstrate that the aging rate depends on the unsaturated lipid content, six volunteers deposited both natural and squalene-spike fingermarks (fingertips rubbed with squalene prior to deposition). Squalene is a highly unsaturated lipid present in fingermarks known to readily oxidize [14-16]. Fingermarks typically contain cholesterol and unsaturated fatty acids such as oleic acid which are also expected to oxidize and thus to contribute to the aging process [14-16]. Squalene clearly accelerated the fingermark aging, which is shown in figure 4.

Preliminary experiments on the effect of temperature on the aging rate of fingermarks, demonstrated that although the aging rate is increased at higher temperature, our method can still date fingermarks, as shown in figure 5 and table 1. While the 37°C fingermarks of both female donors did not allow age estimation, those from the male donors were suitable for estimation (Table 1). The heating clearly accelerated the aging process as the rate constant of datable fresh fingermarks was much higher when stored at 37°C than at room temperature: rate constants for the two datable 37°C fingermarks were 0.44 and 0.67 day\(^{-1}\) while average rate constant for room temperature fingermarks was 0.04 ± 0.01 day\(^{-1}\).

![Figure 3. Age estimation results of twelve fingermarks. By reusing the data from a single fingermark, the method was tested for a wide range of ages. Closed circles: median average of at least four data points, open circles: single results. Error bars: inter-quartile range. Line of unity was added as a visual guide.](image)
Figure 4. Effect of squalene on fingermark aging rate. Typical example of aging natural (closed symbols and blue line) and squalene spiked (open symbols and red line) fingermarks. Solid lines represent fitted aging curve.

Figure 5. Effect of temperature (37°C) on fingermark aging rate.
5. DISCUSSION

Fifty-five percent of the fingermarks from male donors were suitable for age estimation versus none from female donors. We attribute this to the lower excretion of skin components by women [17]. This does not imply inappropriateness of our ageing model, but urges for techniques of efficient deposit collection. Age estimation with our method requires sufficient fluorescence signal. Many fingermarks included in our study generated little to none fluorescence, probably as a result of an insufficient amount of deposited material. Given the gender-bias in crime statistics leaning towards males, the apparent inability to date female fingermarks does not compromise the applicability of our method [18]. Additionally, we expect fingermarks that displayed little to none fluorescence in our study to be non-detectible in forensic practice as well. Traces such as semen and vaginal fluid can also be approached as protein-lipid emulsions. Preliminary experiments with semen yielded similar aging curves as described in this work.

The use of fluorescence to establish the time passed since deposition has first been explored over thirty years ago when a “red shift” (which befits the formation of FOX) towards longer fluorescing wavelengths was observed for aging fingermarks [19-20]. The inter- and intra-person variability was too large for quantitative age inference. We tackled the variability issue by measuring the fingermark aging rate. Corrections for environmental conditions such as temperature and light exposure are needed to complete our age estimation model.

Preliminary experiments demonstrated that although the aging rate is increased at higher temperature, our method can still date fingermarks. The deposition substrate also affects the aging rate: for example a faster degradation of squalene and cholesterol in fingermarks on glass compared to those deposited on microfilter paper has been reported [16]. Our method includes the aging rate in the age estimation and thus accounts for substrate effects. Strong substrate fluorescence and the requirement to monitor the aging process over time are limitations to our method. Translation of the age estimation model to the crime scene can follow two routes. 1) Optimization of the experimental set-up to minimize noise will reduce the required monitoring time and enable onsite

Table 1. Age estimation of fingermarks stored at 37 ° C

<table>
<thead>
<tr>
<th>Real age (days)</th>
<th>Estimated age (days)</th>
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<tbody>
<tr>
<td>Donor 3, male</td>
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<tr>
<td>0.04</td>
<td>0.33</td>
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<td>0.23</td>
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<tr>
<td>Donor 4, male</td>
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<tr>
<td>0.04</td>
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measurements. The fingermark thus remains undisturbed and still available for other tests such as DNA profiling. 2) The requirement of monitoring the oxidation process over time can be abandoned if FOX components generated at different rates are identified. Establishing ratios of these components relative to un-oxidized proteins will enable age estimation in one single measurement. Relative quantification can be established either with mass spectrometry imaging or by specific labeling techniques [21-24]. These techniques do interfere with the integrity of the fingermark but also produce a high quality image of the mark which can be used for identification.

6. CONCLUSION
In conclusion, we have successfully introduced a fingermark age estimation method. Fluorescence spectroscopy was used to measure the degradation and generation rate of proteins and oxidation products, respectively, in aging fingermarks. We were able to estimate the age of 55% of the fingermarks from male donors up to three weeks old with an uncertainty of 1.9 days. The method presented is not limited to fingermarks but serves as a template for the age estimation of all protein and lipid containing traces such as semen, vaginal fluid or even tears.

REFERENCES
13. Fletcher B, Dillard C, Tappel A: Measurement of fluorescent lipid peroxidation products in biologi-


