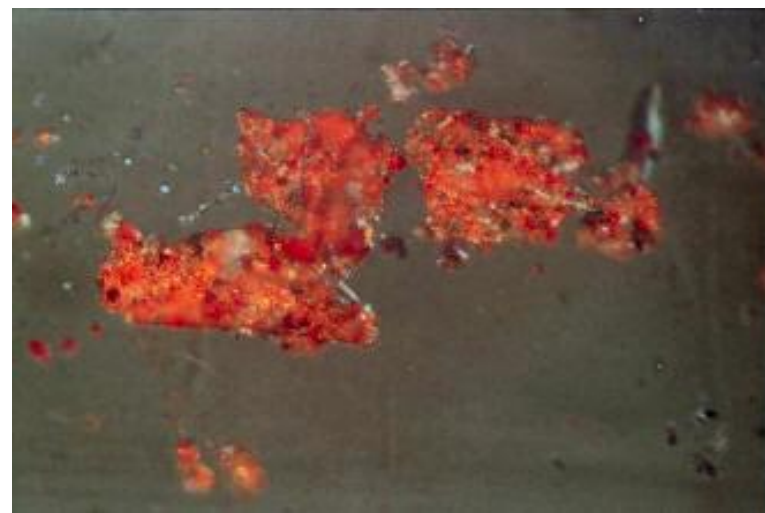
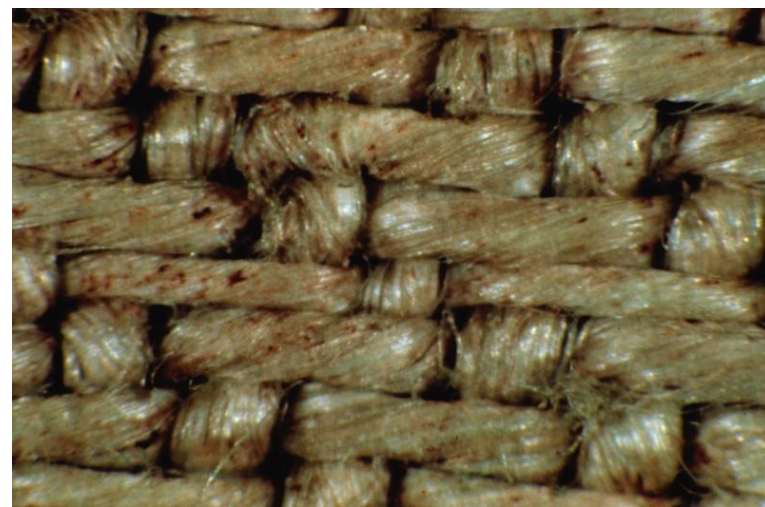


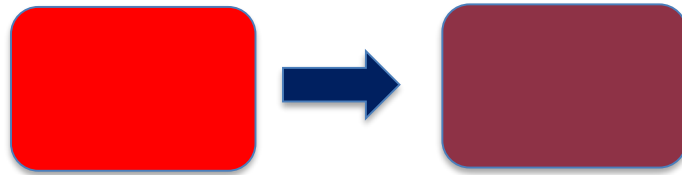
# **Hemolysis, bilirubin, and the color of the bloodstains on the Shroud of Turin**



**It has been noted by numerous investigators that the Shroud bloodstains are reddish in color**



When blood dries and ages  
it converts from red to dark brown



# Hemoglobin Conversion

Oxygenated      Deoxygenated



Methemoglobin

# Hemoglobin Conversion

Oxygenated      Deoxygenated



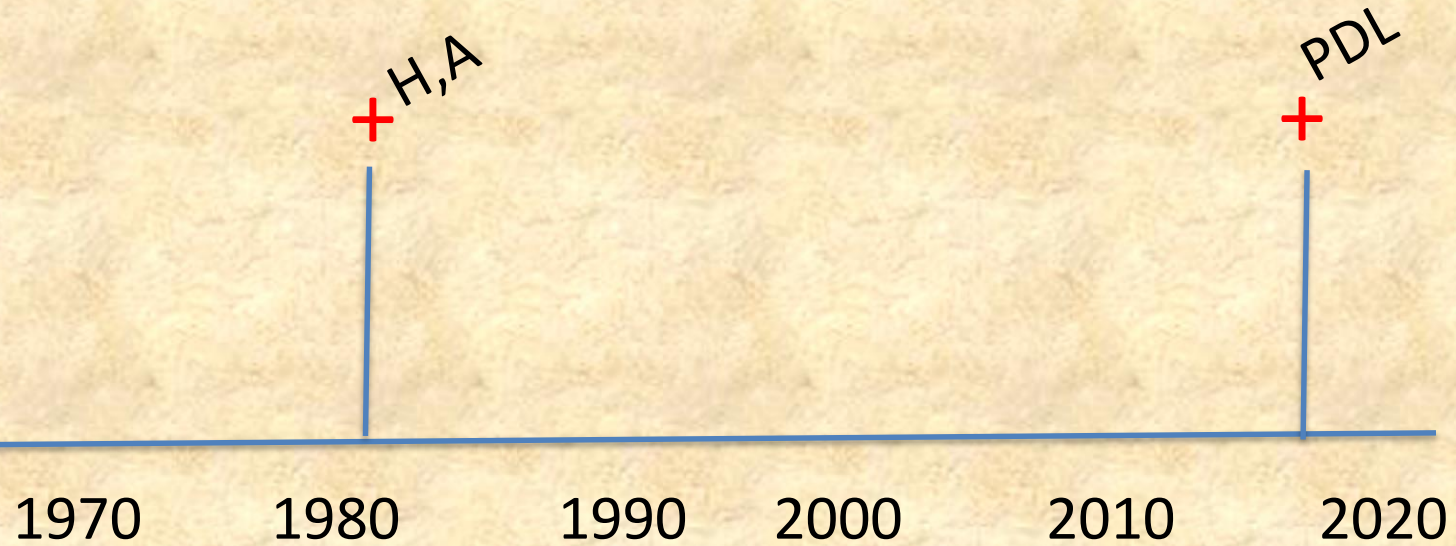
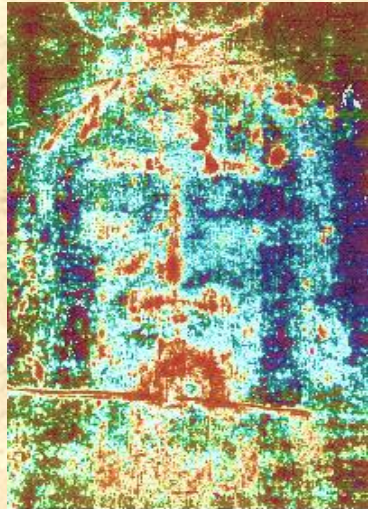
Methemoglobin

$\text{Fe}^{2+}$

$\text{Fe}^{3+}$



# Shroud blood stains exist in the Met-Hb form



Spectroscopy Methods

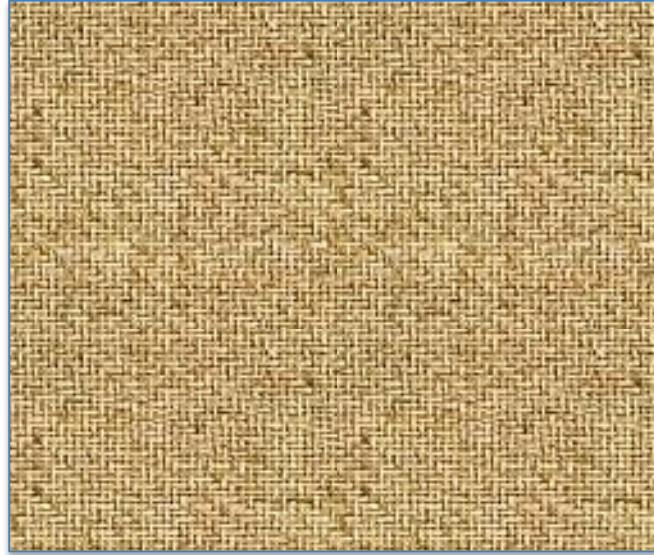
**Why are the bloodstains reddish  
in color?**

# **Hemolysis Theory**

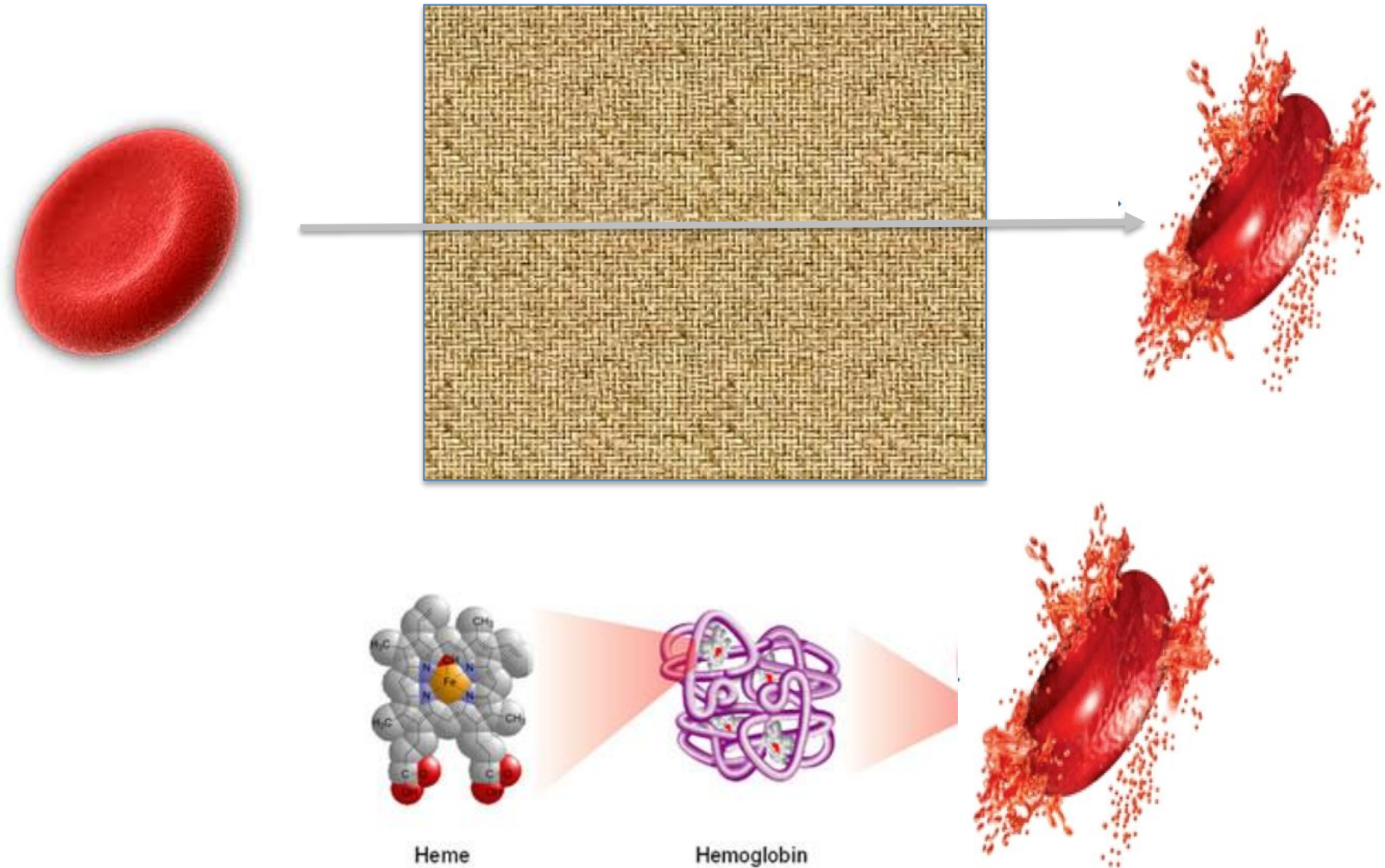
**Lysis: Break apart**



# Saponaria (Soapwort) Detergent (proposed by Rogers)

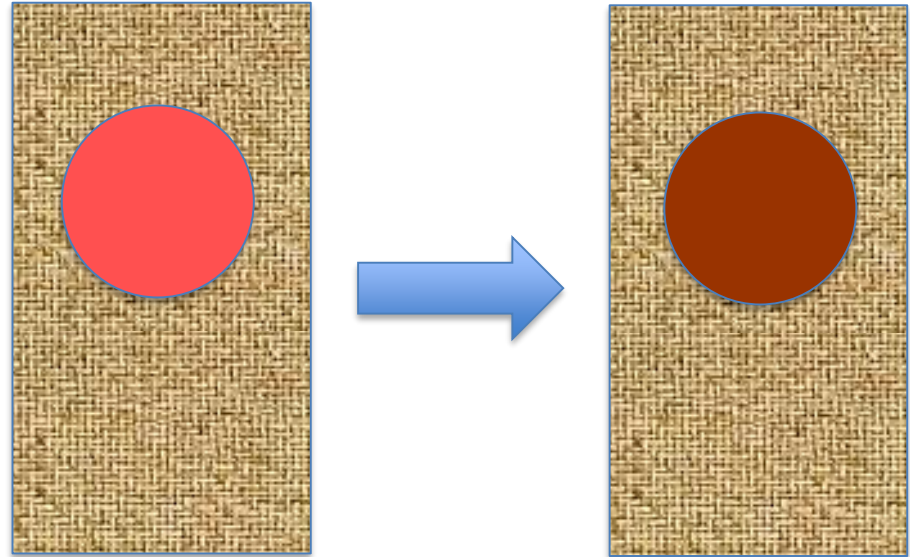


# Saponaria (Soapwort) Detergent (proposed by Rogers)



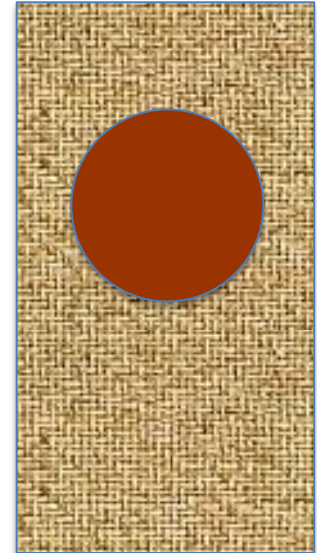
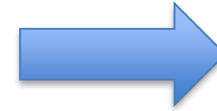
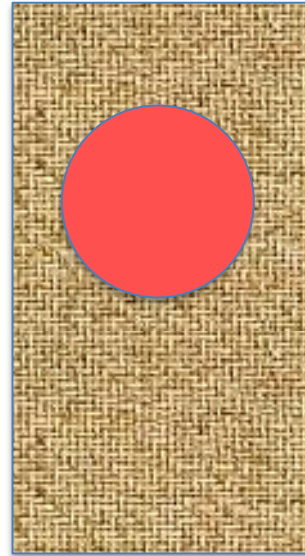
# Saponaria (Soapweed) Detergent (Rogers and Arnoldi)

**Control**

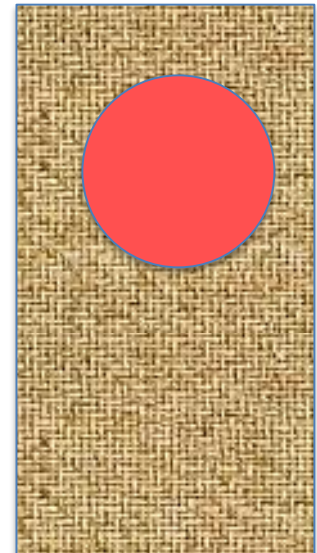
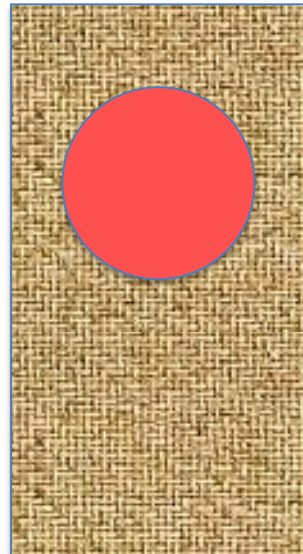


# **Saponaria (Soapweed) Detergent (proposed by Rogers and Arnoldi)**

**Control**



**Saponaria  
Treated**



# Hemolysis Theory:

**No data or pictures presented**





# **Hemolysis Theory**

**Saponin**

**Other Detergents**

**Other Lysis Methods**

**Filter Paper, Linen**



**Soak in Saponin, other  
[Rinse]**



**Add blood**

**Blood**



**Treat with Saponin, other**



**Add to filter paper, linen**

# Saponin Treated Linen

$t_0$



24hrs



# Hemolysis treatment 1st

$t_0$

5 days

Control



Saponin



Freeze-  
thaw





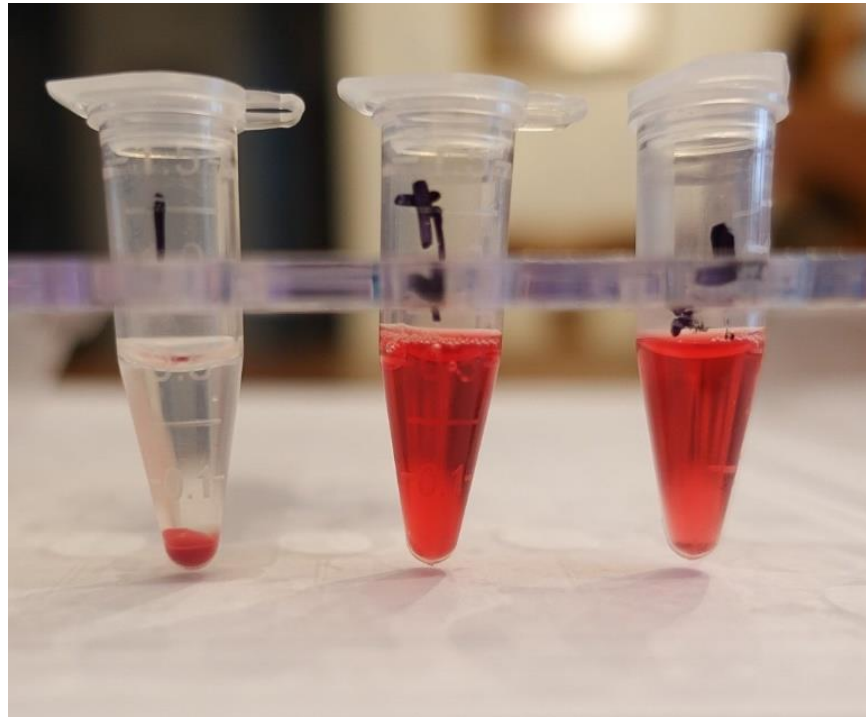
**Treat cells**



PBS

**Spin down, remove Supernatant (Sn)**

**Sn**

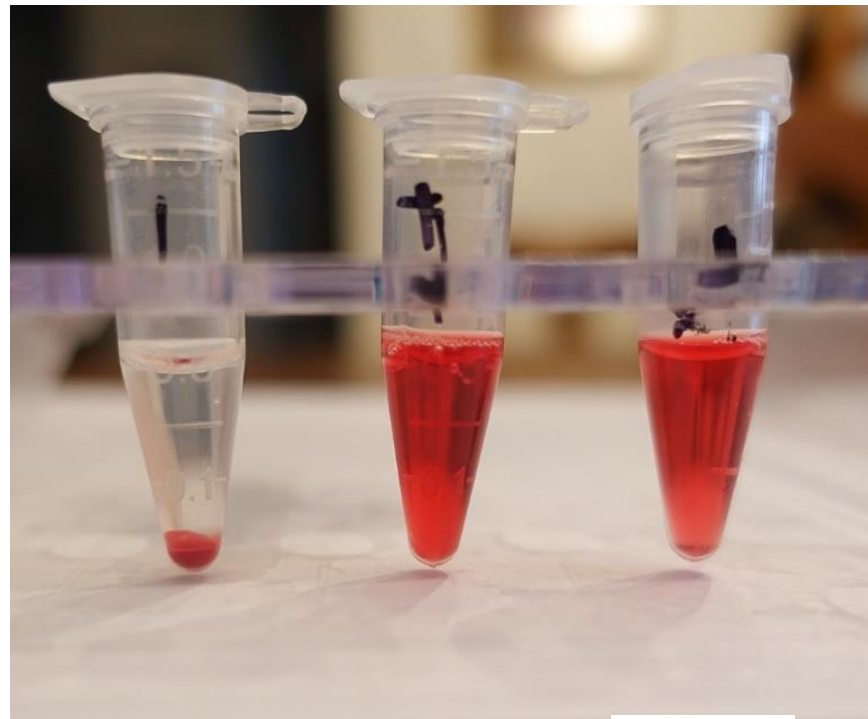


**Treat cells**



**PBS**

**Spin down, remove Supernatant (Sn)**



**Sn**

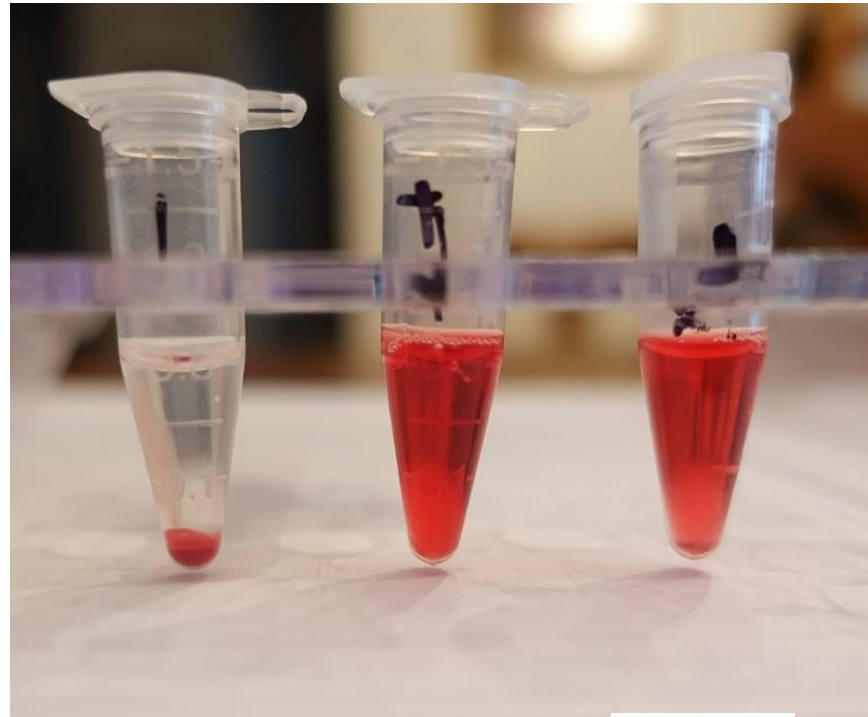


**Treat cells**



**PBS**

**Spin down, remove Supernatant (Sn)**



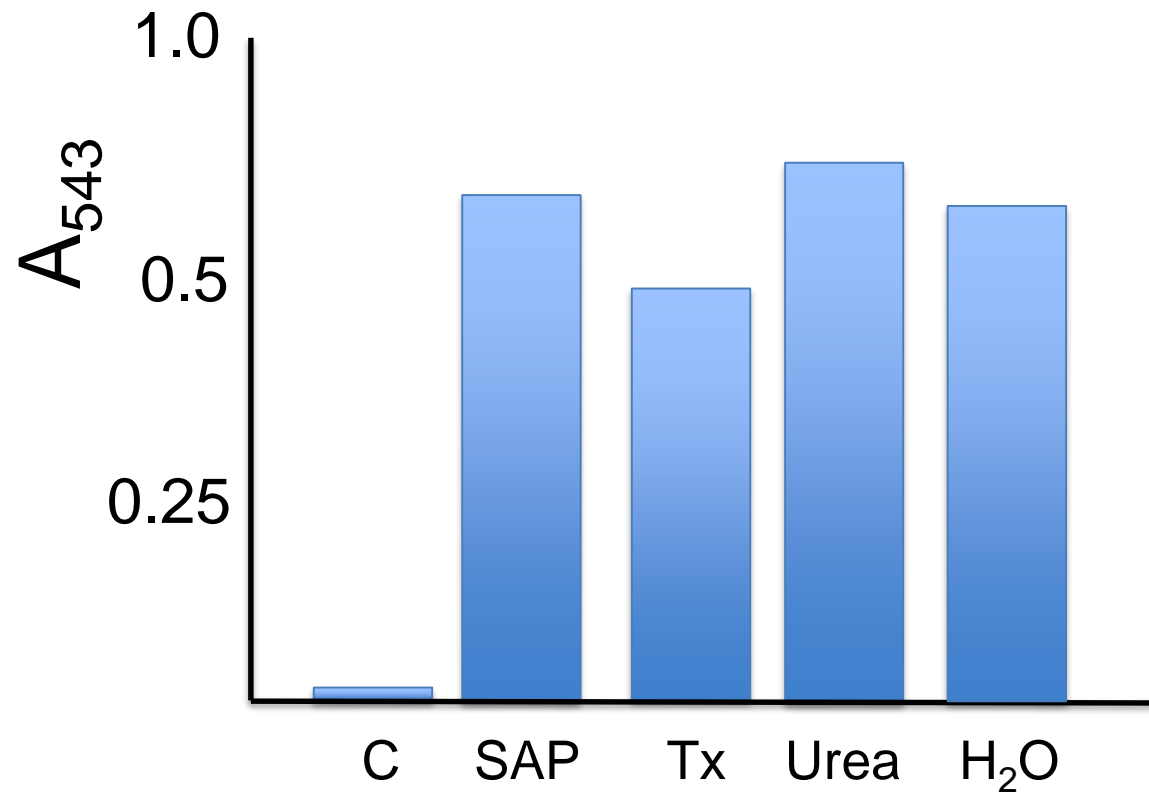
**Sn** {

**Read**

**$A_{543}$**

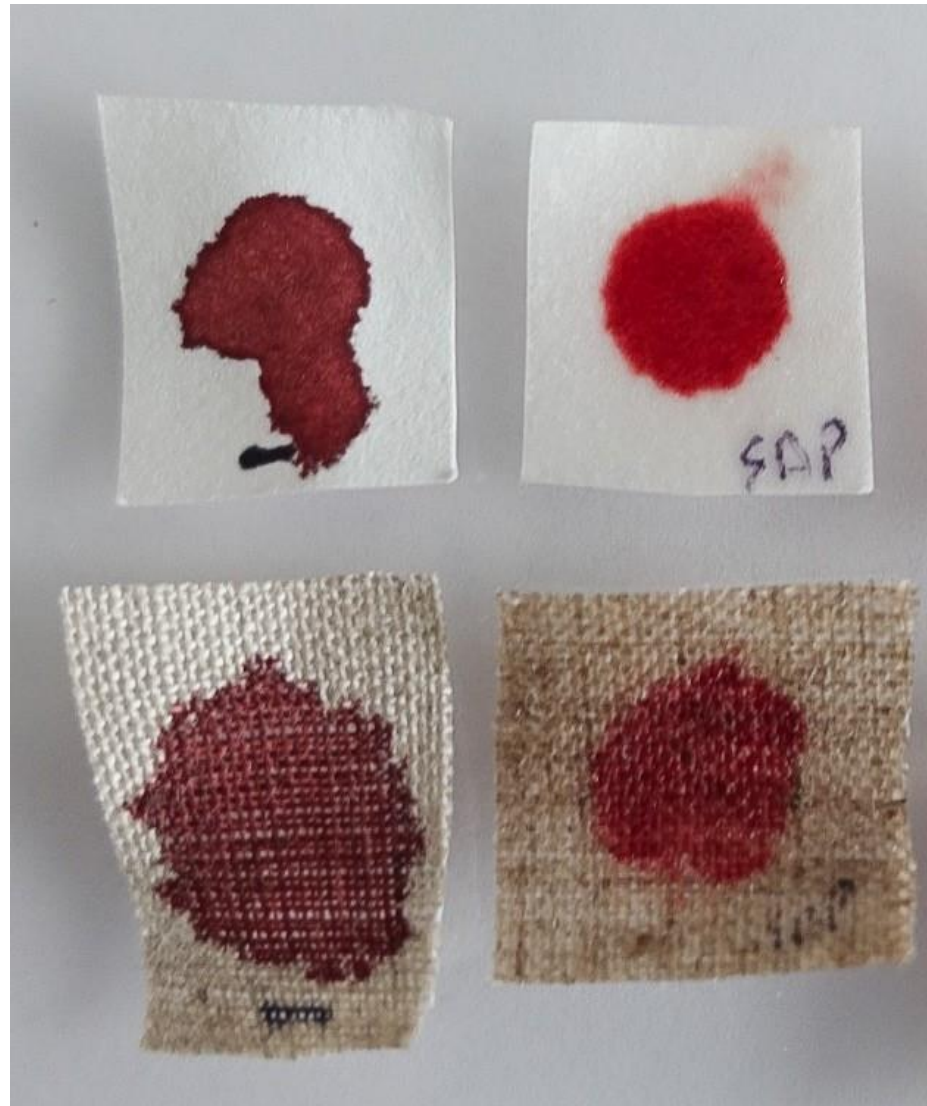


# Hemolysis



No trt

Saponin

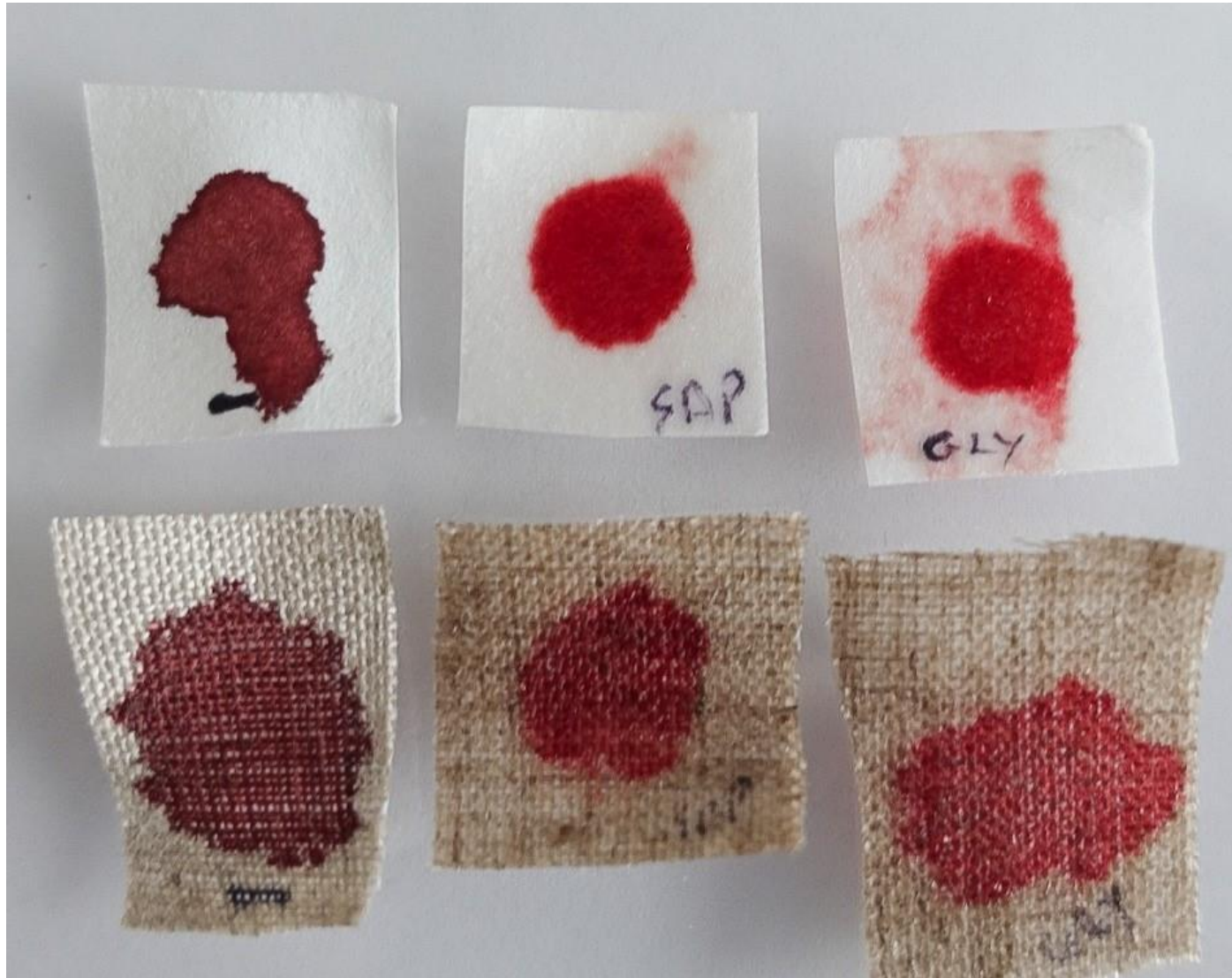




No trt

Saponin

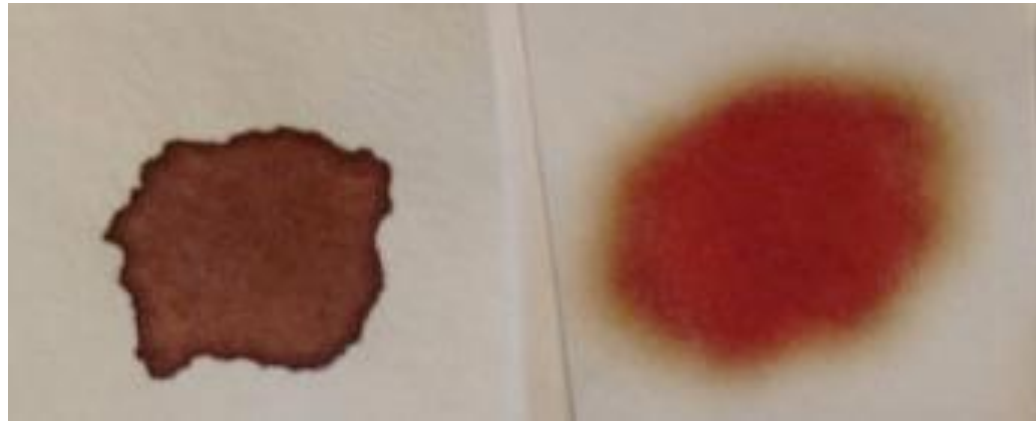
Glycerol



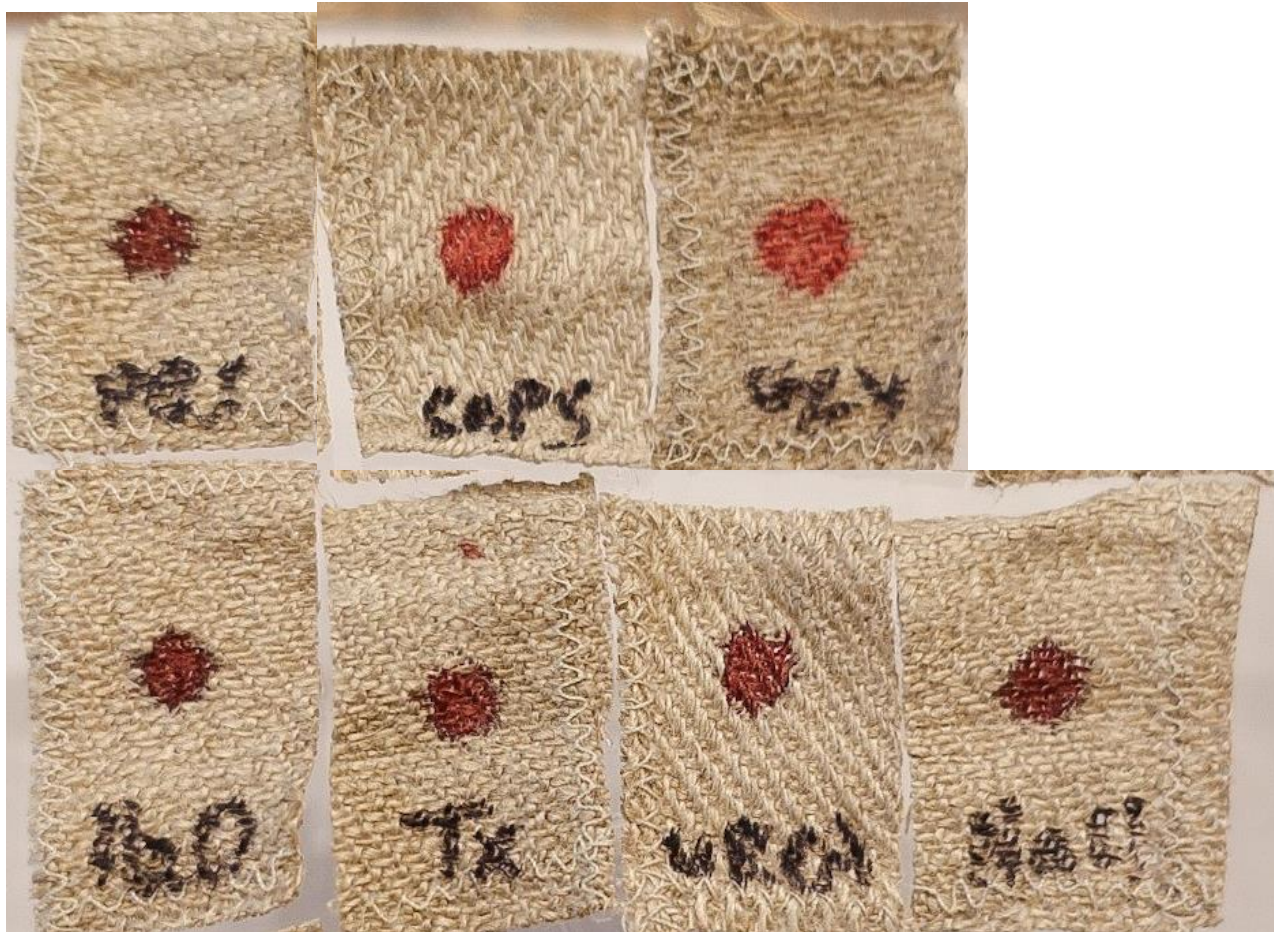
5 days

No trt

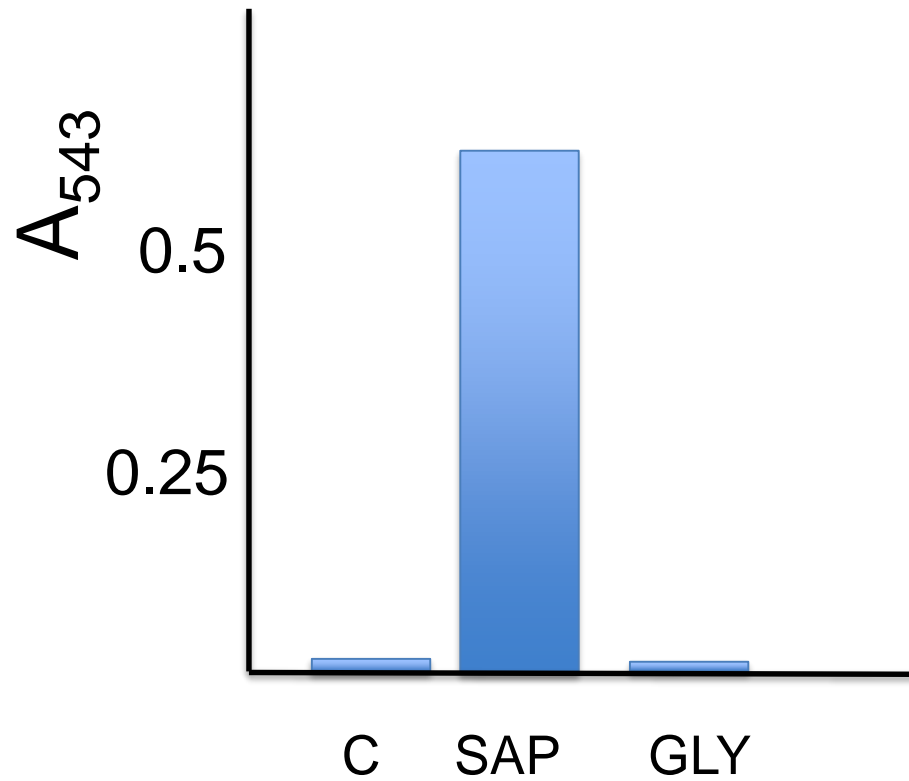
Glycerol



12 hrs



# Hemolysis







# Hemolysis Summary

Reddish color of bloodstains was not maintained following exposure to numerous hemolytic agents

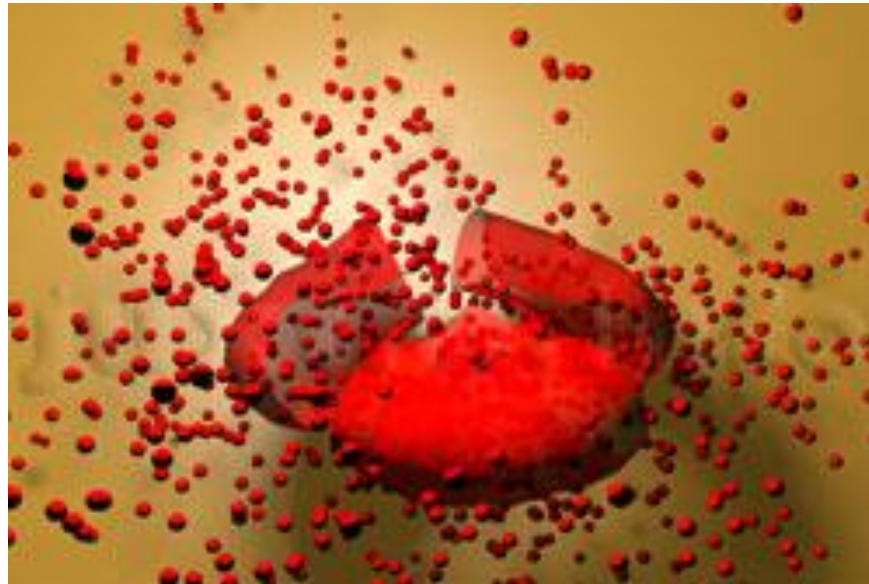
Reddish color of bloodstains was maintained with Saponin solution but was found to be an artifact

Glycerol treatment maintained reddish color of bloodstains, Hb was in the deoxygenated (Met-Hb) form



# **Bilirubin Theory (proposed by Adler)**

**Bilirubin is produced when RBCs  
are broken down**



**Heme -> -> Bilirubin**

**Adler could reproduce  
red color by adding  
bilirubin to blood in vitro**

**No data available/presented**

# **Other studies:**

**Goldoni 2008**

**In vitro, bilirubin 2-5x levels  
uv treatment**

**N. Svensson 2010**

**Patients with 10x bilirubin levels**

**P.D. Lazarro 2018**

**Patients with 10x bilirubin levels  
uv treatment**

## **Other studies:**

Anti-coagulant was present

Bilirubin in range of 2-10x  
above normal

Mixture of various forms of bilirubin  
was present

# Adler's Hypothesis

Hemolysis

Methemoglobin

Bilirubin

Red color



# Adler's Hypothesis

Hemolysis



Methemoglobin

Bilirubin

Red color

Hemoglobin (oxygenated)

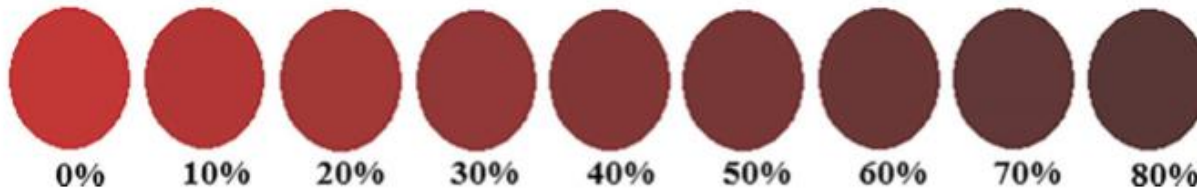
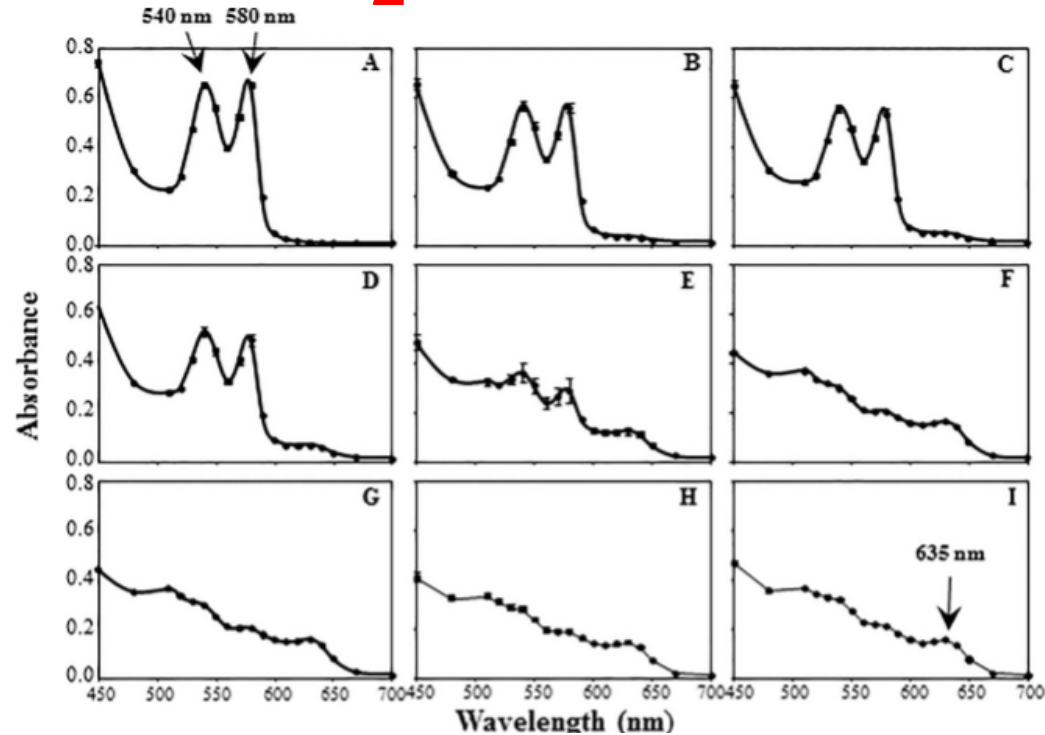


Methemoglobin (deoxygenated)

Naturally (with time)

Chemically ( $\text{NaNO}_2$  treatment)

# NaNO<sub>2</sub> Treatment



**Fig 4. Percent methemoglobin color chart.** Predicted RCV generated from the starling, duck, and vole's standard curve equations were averaged and used to generate custom color cards in MS Paint. Blue and green color values were estimated to be 40, which was based on the average blue and green color values in these samples.

doi:10.1371/journal.pone.0167942.g004

Patton, et al., "Detecting Methemoglobinemia in Animals with a Drop of Blood PLoS one 2016; 11 (12) e0167942

# Fresh RBCs + NaNO<sub>2</sub>

Control



NaNO<sub>2</sub>



# Fresh RBCs + NaNO<sub>2</sub>

Control      NaNO<sub>2</sub>



# Fresh RBCs + NaNO<sub>2</sub>

mM NaNO<sub>2</sub>

0

3.12

6.25

12.5



mM NaNO<sub>2</sub>

25

50

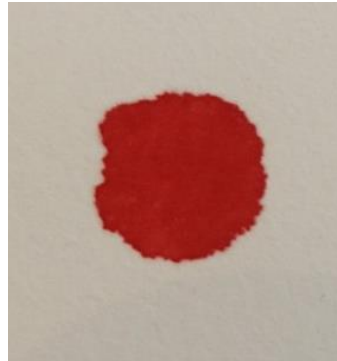
100





# RBC lysates

Control



NaNO<sub>2</sub>



# Adler's Hypothesis

Hemolysis



Methemoglobin



Bilirubin

Red color

# Heme Catabolism



Hemoglobin



Biliverdin



Bilirubin



# Bilirubin

Bilirubin  
(Unconjugated)

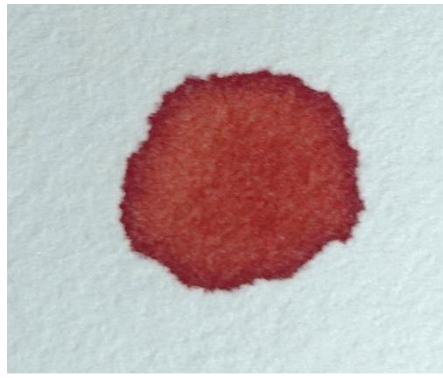


Bilirubin  
(Conjugated)

# NaNO<sub>2</sub> Treatment (lysates)

Solvent Control      Unconjugated Bilirubin 100x

No trt



NaNO<sub>2</sub>



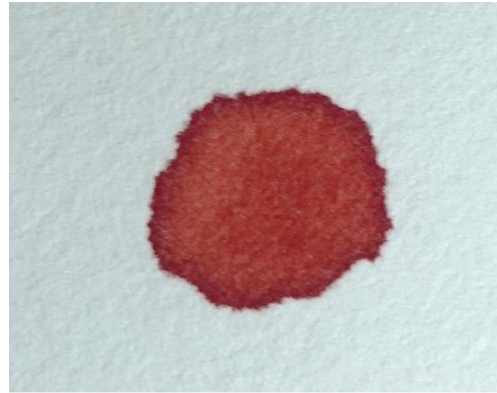


# Unconjugated Bilirubin

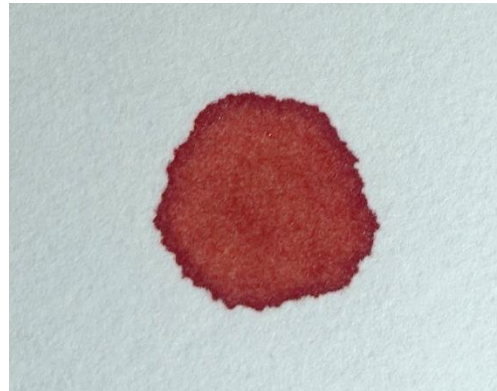
t0

1 month

Solvent  
Control



Bilirubin  
100x

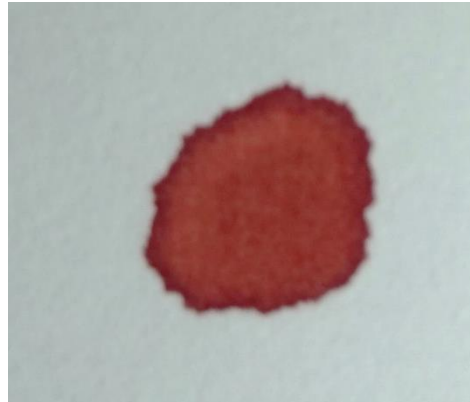


# Conjugated Bilirubin

t0

1 month

Solvent  
Control



Bilirubin  
100x



# Mix Bilirubin

t0

1 month

Solvent  
Control



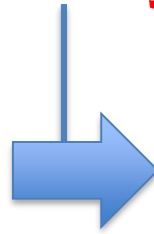
Bilirubin  
100x



# Bilirubin

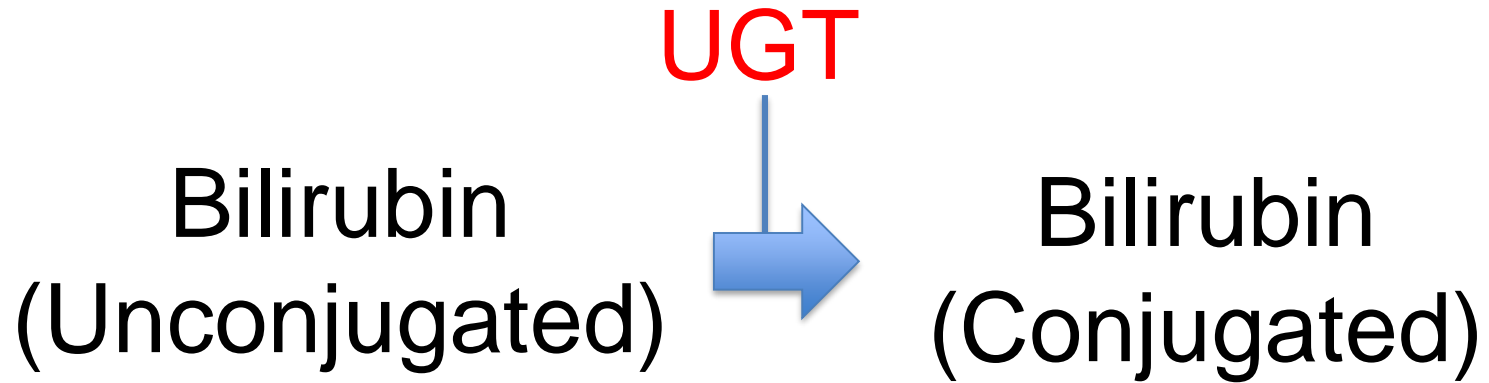
UDP-Glucuronyltransferase

Bilirubin  
(Unconjugated)



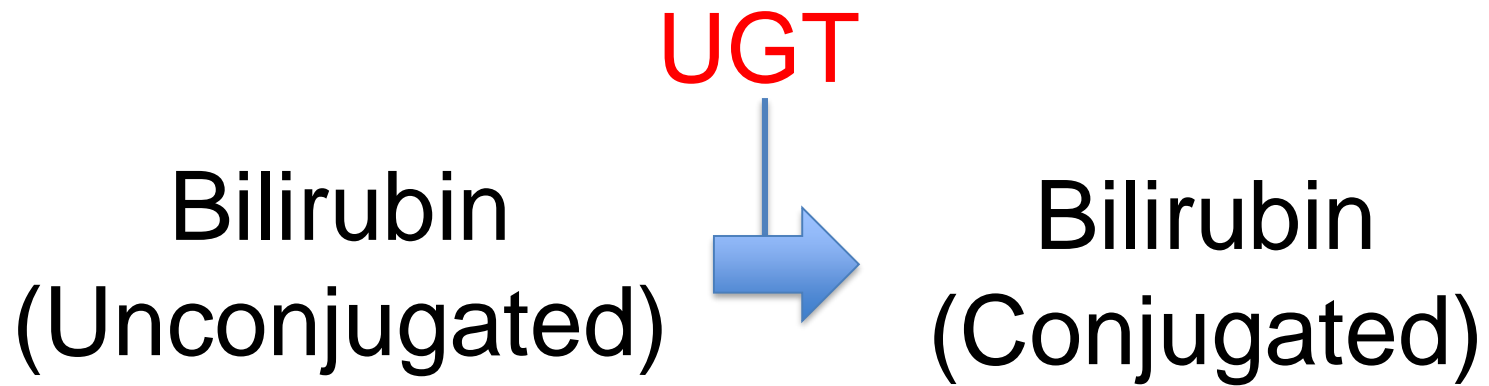
Bilirubin  
(Conjugated)

# Bilirubin





# Bilirubin



Gunn rat

# Gunn rat model

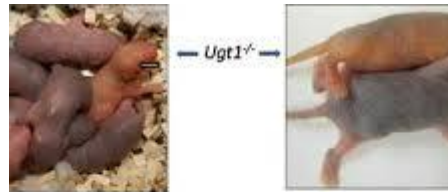
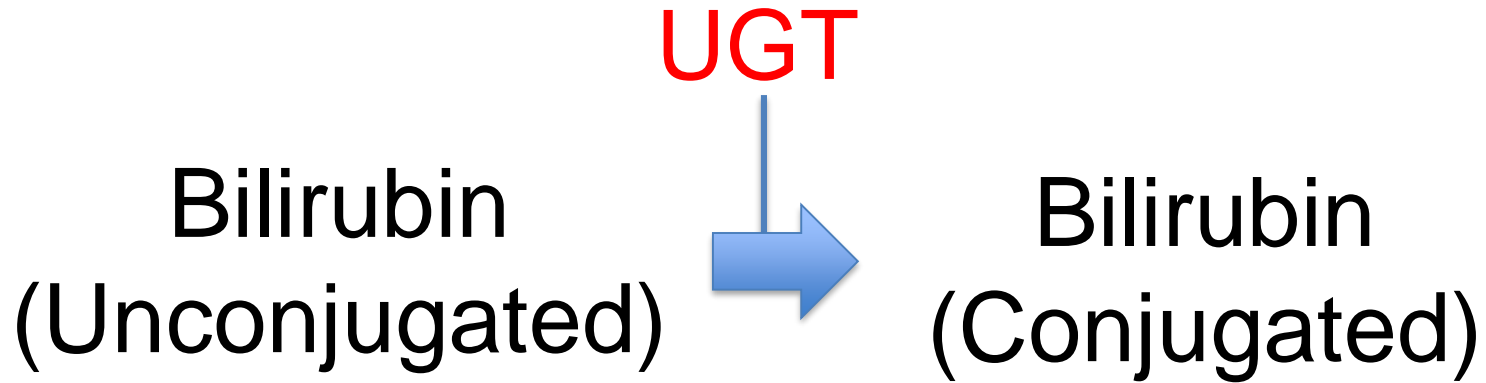
$t_0$



8 wk

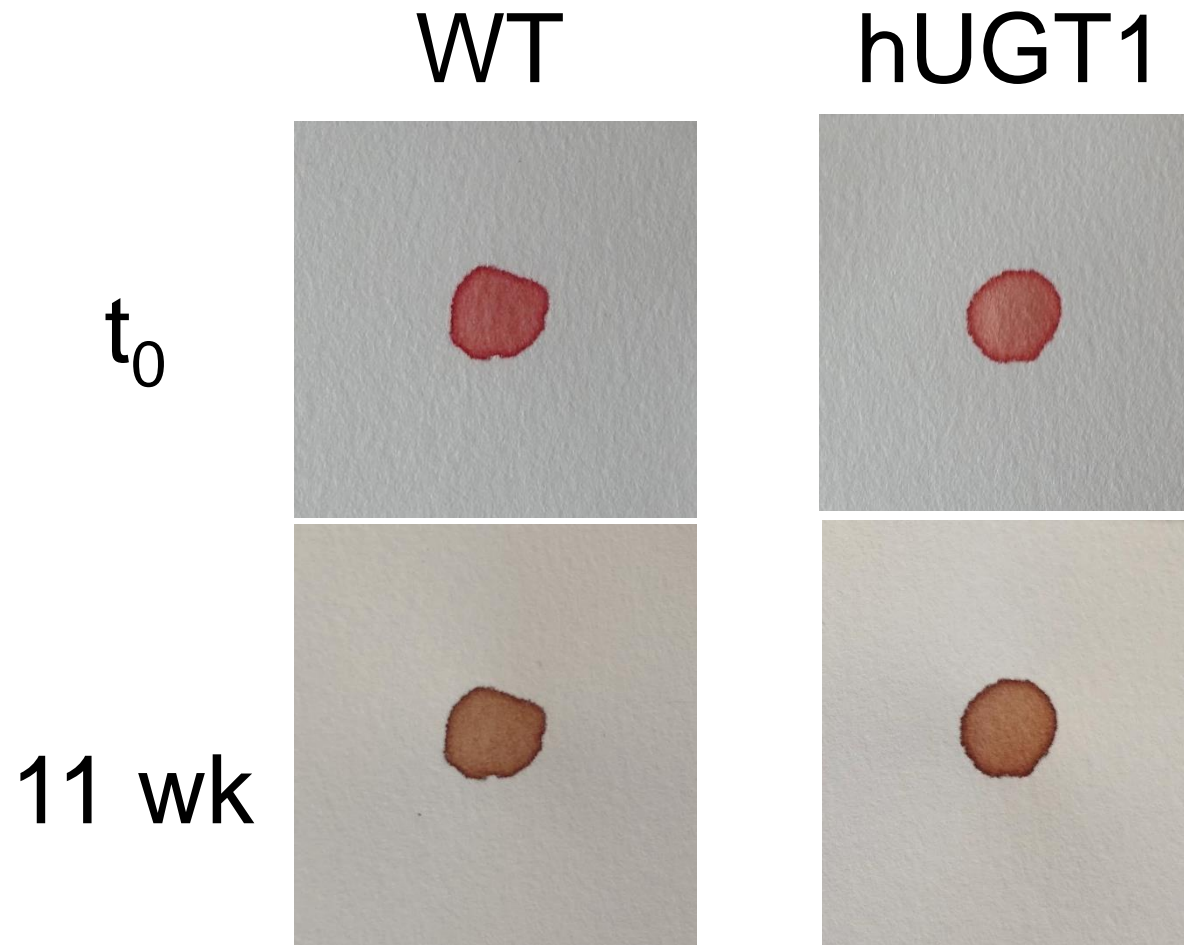


# Bilirubin



ugt -/- mice

# WT and hUGT1 mice

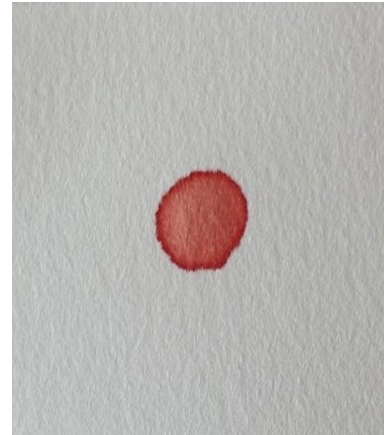
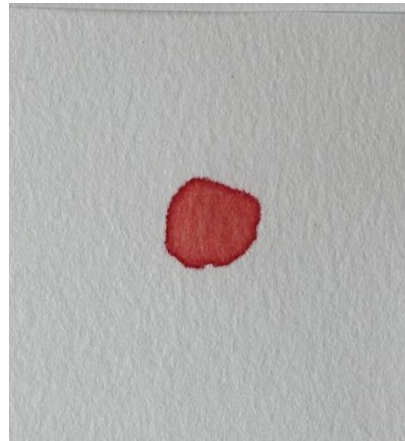


# WT and hUGT1 mice

WT

hUGT1

-



NaNO<sub>2</sub>





## **Bilirubin Summary**

Reddish color of bloodstains was not maintained in samples containing high amounts of bilirubin

This was observed using *in vitro* simulacrum of human blood containing high amounts of bilirubin

This was also observed in various genetic models (rats, mice) expressing high amounts of bilirubin





## **Bilirubin Summary cont.**

These results were performed in the absence of anti-coagulant and utilized both unconjugated and conjugated forms of bilirubin in a variety of concentrations