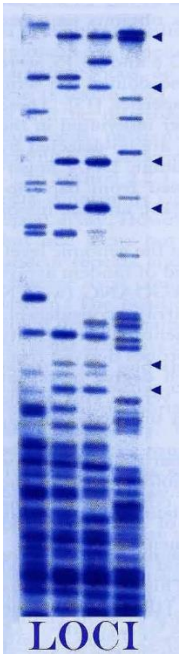

Application of the Visual Blood Enhancement Techniques Hungarian Red, Amido Black, Aqua Leuco Crystal Violet and Iron Oxide at temperatures below zero degrees Celsius

Graduation Thesis

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I Preface

I am presenting you my research thesis, entitled: Application of the Visual Blood Enhancement Techniques Hungarian Red, Amido Black, Aqua Leuco Crystal Violet and Iron Oxide at temperatures below zero degrees Celsius. This thesis is written because of my graduation research for my Bachelor study Forensic Science at the Amsterdam University of Applied Sciences, which was such a great opportunity to learn a lot more about doing research and about blood enhancement techniques.

I would like to thank some people in particular for giving me the opportunity to work on this subject. Starting with my supervisor from the Dutch Police, Jeffrey Haas, and my supervisors from Loci Forensics B.V., Martin Eversdijk and René Gelderman. I am very grateful for their efforts, their help and feedback this interesting thesis was written. But above all, thanks to everything that they have taught me about forensics on a real crime scene and in simulated circumstances. All these valuable information and experiences made my interest in forensics even bigger. In short, I had a great, fun and educational time which I will always positively look back on and will cherish forever.

I would also like to thank Robin Geitenbeek, my supervisor from the Amsterdam University of Applied Sciences, for his guidance, help and his refreshing chemistry lessons. Lastly, thanks to Myrthe Metselaars, Marlène Többen, Chinook Rijk, Lieke van Houten, Beau Leijten, Vanessa Ubeda, Kiki Verbeek, Sammyjo Hiele, Sem Hummelink, Raoul de Graaff, Ingmar de Heiden, Fetze Venema, Leon Reus, Rosan Janssen, Marije Tinhout and Thalassa Valkenburg for scoring the results.

I hope you will enjoy reading this thesis.

Manon Gout

Amsterdam/Nieuw-Vennep, June 2022

II Index

III List of abbreviations/definitions

AB – Amido Black

(A)LCV – Aqua Leuco Crystal Violet

Background – The appearance of the surface, where the shoeprint is present under the same conditions, used to visualize the print. The appearance of the background is influenced by the surface properties, like material, texture, colour, reflectivity and any present patterns.[39,40,41]

Characteristics – Unique features, caused by wearing a shoe (cracks, cuts, holes, lack of profile, dirt between the profile)[39,40,41]

Depletion series – Series of prints with a decreasing amount of substance that is printed on the surface, without moistening the object between the prints in.

Detection limit – The smallest amount of samples or lowest concentration of the component in the sample that can be quantified with a certain precision and correctness with the analysis method, in other words, the measured value of which can still be determined with a certain certainty.[42]

Development – A subset of visualisation where a process applied to the prints, resulting in becoming visible, producing a gradual change from (partially) invisible to clearly visible.[29]

Enhancement – The improvement of a partial visible blood print by the application of an additional process that either reveals additional characteristics or makes visible prints more distinguishable from the background.[40,41]

Ethanol/water solution - A solution of ethanol and demineralized water.

FO – Forensische Opsporing (English = Forensic Investigation Service)

HR – Hungarian Red

Identification – One possible end result of a comparison between a print and the corresponding reference. The result is considered an ‘identification’ when the examiner considers the level of agreement in the features occurring in the print/reference being compared, is sufficiently high that they must have originated from the same donor.[39,40,41]

Individualisation – A print that has been found can undeniably be associated with one specific and individual origin.[39,40,41]

Intraclass Correlation Coefficient – A descriptive statistic that can be used when quantitative measurements are made on samples that are organized into groups.[49,50,58]

IO – Iron oxide

Latent print – A print that has been formed on a surface as a result of contact with an object and is not visible for the human eye to conduct a visual examination. Those prints require the application of visualisation before they can be detected.[39,40,41]

Negative control – Sample that needs to give no result.[42]

NFI – Nederlands Forensisch Instituut (English = Dutch Forensic Institute)

Paired t-test – A paired samples t-test is used to compare two means of paired samples. Paired samples depend on each other.[58]

Positive control – Sample that needs to give a positive result.[42]

Power – The probability of finding a difference that actually exists (in the population).[58]

Reliability – The extent to which a measurement is independent of chance.[42]

Reproducibility – The precision obtained from performing all relevant measurements by different analysts, in different lab rooms, with different devices and batches of reagents/standards, at different times at longer intervals.[42]

Sample size – The size of the sample, which is determined by the size of the population, the desired reliability and the accuracy.[58]

Shoeprint – A reproduction of the pattern of a shoe sole obtained from a known donor under controlled conditions.[39,40,41]

Significant difference – It is unlikely that it can be explained by chance or random factors alone. In other words, there is only a very small chance of a statistically significant result occurring if there was no real effect in the study.[58]

Validation – Checking a value or method for trueness and precision to check if an analysis method is suitable for the intended application.[42]

VBET – Visual Blood Enhancement Technique

95% Confidence Interval (CI) - The range of values within which the true value in the population lies with a certain degree of probability (95%).[58]

IV Summery

Blood traces are very relevant forensically, because they can be crime related. Therefore, blood traces can help to identify the people involved and to reconstruct what happened. One method of making partial, latent blood traces visible is using Visual Blood Enhancement Techniques (VBETs), such as Amido Black (AB), Hungarian Red (HR), Aqua Leuco Crystal Violet ((A)LCV) and Iron Oxide (IO). The method of these techniques is based on enhancing the blood traces with sufficient detail and contrast with respect to the background. Therefore, they must be fixed and coloured with one of these techniques by means of a chemical reaction with components in the blood. A rinsing solution will then be applied to remove the excess dye, after which only the enhanced blood traces will remain. However, these techniques are water-based, which means that they will freeze in an environment with temperatures below zero degrees Celsius. So, when a frozen blood trace is found on a car at temperatures below zero degrees, these traces cannot be enhanced with those solutions. In addition, frozen blood traces will thaw and run out as soon as the car is confiscated to a warmer place. In order to be able to enhance these traces, the composition of the VBETs must be adjusted. For this study, an ethanol/demineralized water solution was used. Therefore, the main question is: 'To what extent are four different Visual Blood Enhancement Techniques with an ethanol/water solution applicable and yields useful and visible results with sufficient detail on prints, set with blood, at temperatures below zero degrees Celsius?' To conduct this research, the appropriate percentage of ethanol for the solution had to be determined, after which it was investigated whether the modified VBETs can enhance blood traces at all and whether they can at temperatures of minus twenty degrees Celsius. The suitable percentage of ethanol must not freeze and it needed to be as low as possible for safety reasons. To investigate whether the modified VBETs can enhance blood traces and also at minus twenty degrees Celsius, stamps of a partial shoe sole in a depletion series of four prints were placed on ceramic tiles and paper. After they were photographed, they were enhanced with the VBETs and treated with the rinsing solution and photographed again. These were compared with the untreated series by sixteen evaluators, who scored the results using a scoring system. Based on these scores, a paired t-test was used to investigate whether there is a statistically significant difference between the untreated and enhanced prints. In order to be able to make reliable statements, an Intraclass Correlation Coefficient had also been calculated to determine the extent to which the evaluators agree with each other. In addition, blood dilutions have been used to determine the detection limit of the modified VBETs.

Results showed that 40% ethanol is suitable for adjusting the techniques, after which they were adapted and the blood traces were treated with them. Visually, the processed traces turned out to be much more visible, in terms of contrast and number of characteristics, up to the greatest dilution factor. Statistically, a significant difference was shown for the four VBETs for all four print positions on the surfaces. This statement could be made, since statistics showed that the evaluators agreed with each other.

The modification of the VBETs with this 40% ethanol therefore seems to have potential to be applied at temperatures below zero degrees Celsius. However, since no previous publications had been published about this subject, this research consisted of pilot experiments with a minimum number of variables. Further research should therefore be done on other variables, such as different materials and colours of surfaces, what influence 40% ethanol has on DNA, whether the use of human blood and the difference in blood composition between individuals influences the results and whether HR and IO can still be lifted and HR can still fluoresce. The findings that will be obtained, will help to gain insight of whether and when the VBETs with 40% ethanol can be used on crime scenes at temperatures below zero degrees Celsius.

V Introduction

Occasion

During a crime scene investigation, a partial transfer pattern, set with blood, is found on a car. This can be a fingerprint or a shoeprint. However, this investigation was conducted in February and the temperature was well below zero degrees Celsius. This means that the shoeprint, set with blood, also had a temperature of below zero degrees Celsius and is therefore frozen.[38] But, the scientific literature and the Dutch Forensic Guidelines[43,44] do not discuss what to do with such a print on a crime scene where temperatures below zero degrees Celsius apply. Nevertheless, the forensic investigators wanted to enhance the shoeprint with water-based Visual Blood Enhancement Techniques (VBETs) for the purpose of reconstruction and identification of the case. However, if you take the car to a warmer place, the shoeprint will start to defrost and possibly run out. This will contaminate and destroy the print and that is something a forensic investigator absolutely wants to avoid. Therefore, confiscation is not an option under these circumstances.

In addition, if the print was to be upgraded with the VBETs on the crime scene, it can also cause the print to defrost and run out, because its composition is warmer than the print and its surroundings. The same result will appear as when you take the car to a warmer place. This is because those techniques are water-based and are therefore only liquid above zero degrees Celsius. Besides, as soon as these VBETs are present in a freezing environment for a longer period of time, they can freeze themselves, making them impossible to apply. Therefore, a different base fluid is required for these techniques in order to be able to make them applicable to crime scenes, where the ambient temperature is below zero degrees Celsius.

Issue

The main issue in this study is that the current VBETs are not expected to be applicable at temperatures below zero degrees Celsius, because they freeze themselves or they cause the print to defreeze and run out. In order to make them applicable, it is required to replace the basis fluid in the solution. For this study, ethanol was chosen to adjust the base fluid. The accompanying main question is, which this research focuses on, to what extent are four different Visual Blood Enhancement Techniques with an ethanol/water solution applicable and yields useful and visible results with sufficient detail on prints, set with blood, at temperatures below zero degrees Celsius?

Aim

The aim of this study is to determine to what extent four different Visual Blood Enhancement Techniques with an ethanol/water solution, which is a quite safely workable base fluid, are applicable and yields useful and visible results with sufficient detail on prints, set with blood, at temperatures below zero degrees Celsius. To achieve this goal, the following empirical sub questions needs to be answered.

Empirical sub questions

Table 1: Overview of the empirical sub questions regarding the research of the Application of the Visual Blood Enhancement Techniques Hungarian Red, Amido Black, Aqua Leuco Crystal Violet and Iron Oxide at temperatures below zero degrees Celsius

Sub question 1	How do the current, water-based Visual Blood Enhancement Techniques behave under circumstances where the temperature is below zero degrees Celsius?
Sub question 2	To what extent is an ethanol/water solution suitable for adapting the base fluid of the Visual Blood Enhancement Techniques ?
Sub question 3	To what extent does an ethanol/water solution as a base fluid influence the chemical reaction between Visual Blood Enhancement Technique and the blood?
Sub question 4	What percentage of ethanol in the ethanol/water solution is most suitable to achieve the desired result (making the trace visible) with the Visual Blood Enhancement Techniques at temperatures below zero degrees Celsius?
Sub question 5	What percentage of ethanol in the ethanol/water solution is most suitable to achieve the most desired detection limit when using a dilution series?
Sub question 6	Which substrates were used for the research and what influence do these substrates have on the chemical reaction between the Visual Blood Enhancement Techniques and the blood?
Sub question 7	To what extent needs the current protocol of the application (fixation, VBET application, rinsing) of the Visual Blood Enhancement Techniques be adjusted when an ethanol/water-based technique needs to be used?

Thesis structure

The structure of this thesis is as follows. First of all, it started with a preface. After this, a list of abbreviations/definitions was described, in which some abbreviations and terms are explained, followed by a summary of the entire thesis, which can be read in itself, with the aim of giving a first insight into the research. The introduction was then written down with the occasion, the issue, the aim of the research, the main question and the sub questions.

Subsequently, the theoretical background and the research design with accompanying methodology are presented. Thereafter, the research results are discussed and a critical discussion and interpretation of those results took place. The thesis concludes with the conclusion and recommendations for possible follow-up studies in the field of the application of the Visual Blood Enhancement Techniques Hungarian Red, Amido Black, Aqua Leuco Crystal Violet and Iron Oxide at temperatures below zero degrees Celsius. All references used are also displayed in a literature list, to which the references are made, followed by the appendices.

VI Theoretical Background

Blood is of great value in forensic science, because of its possibility of individualization with DNA and as a sample itself. This can be crime-related, which can be very helpful in establishing the truth. For example, there are various blood techniques, such as Visual Blood Enhancement Techniques, which are necessary to contribute to finding out the truth of the cases.

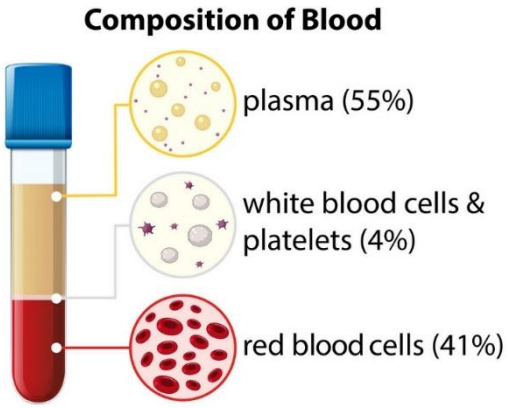


Figure 1: Composition of blood [46]

Blood plays a significant role in the human body. It is an opaque and viscous liquid that occupies about 8% of the human body weight.[16] The main functions of blood are to transport materials throughout the body and to help to maintain the body temperature. Materials transported to and from tissues and organs include oxygen, hormones, nutrients and waste products. The transport of heat by blood from warmer areas in the body to colder areas helps to maintain the body temperature (homeostasis).[6,16,40]

Blood is a complex fluid. It consists of plasma (figure 1); an aqueous component, consisting of proteins, amino acids, salts and other chemical components. One of the proteins in the blood is haemoglobin, which is stored in the red blood cells (erythrocytes). Erythrocytes are responsible for the transport of oxygen and carbon dioxide and for the red colour of blood. When the blood is a bright red colour, it is usually oxygenated. If it has a dark red colour, it is usually low in oxygen.[6,16,40] In addition, in a healthy person, there are, on average, besides the 4 to 5.8 billion erythrocytes, 4 to 10 million white blood cells (leukocytes) and 150 to 350 million platelets (thrombocytes) in every millilitre of human blood (figure 1).[16] Leukocytes are responsible for the defence against diseases and foreign materials. Platelets cause blood to clot when injuries occur. Erythrocytes and leukocytes are valuable for the microscopic examination of deposits to determine whether a blood-like stain is indeed blood. However, when the blood dries, most of the cells are destroyed and the contents are released. Furthermore, the plasma in the blood outside the body separates from the other components through clotting. At that time, plasma is called serum. Amino acids and proteins, such as haemoglobin, which were present in the plasma can then be used to stain the blood.[6,16,40]

However, on crime scenes, traces of blood can be found that are not coloured enough, but are (partially) latent. Examples of such blood traces are fingermarks and/or shoeprints, set in/with blood.[1,3,4] To make these blood traces (more) visible for reconstruction purposes and for identification with DNA, for instance, the Visual Blood Enhancement Techniques (VBETs) can be applied. Those VBETs are chemical methods that react with components in the blood. For example, there are visual techniques that react with the proteins in the blood and there are visual techniques that react with the haemoglobin via an oxidation reaction, because of its peroxidase-like properties, as can be seen in the reaction below.[4,13,14,19]

$$reagent + H_2O_2 \xrightarrow{haemoglobin} reagent^{ox*} \rightarrow reagent^{ox} + hv$$ [19]

But not all of the existing VBETs may be applied on crime scenes due to their base fluid.[3,5,6,15] The reason for this is because some, such as methanol, are unsafe and toxic. Only the waterbased VBETs are safe enough to use on crime scenes. The VBETs used by the Dutch Police on crime scenes are: Amido Black (waterbased), Hungarian Red (Acid Fuchsin) and (A)LCV (Aqua Leuco

Crystal Violet).[1,6,15] The first two react with proteins in the blood and (A)LCV with the haemoglobin.[13,14] The choice for which technique will be used partly depends on the material of the surface, the colour of the surface, whether further investigations are required, et cetera.[12,30]

Amido Black

Amido Black (Acid Black 1) is a chemical dye (figure 2) that colours proteins, such as those present in blood, blue-black (figure 3). However, it does not react with 'normal' eccrine and/or sweat components in fingerprints.[3,5,7,11] Amido Black is supplied on a methanol basis as well as water, water/ethanol/acetic acid and water/ethanol/acetic acid/2,5%-5-sulfosalicylic acid bases. Amido Black stains more strongly in methanol, but is also more dangerous due to its toxicity and therefore cannot be used on crime scenes. Especially for the use on a crime scene, the Amido Black on a water/ethanol/acetic acid, water or water/ethanol/acetic acid/2,5%-5-sulfosalicylic acid base is recommended.[3]

But Amido Black has the ability to provide some background coloration on porous or semi-porous surfaces, as it can also soak into the surface, which cannot be rinsed away during the rinsing step. As a result, it is mainly used on non-porous surfaces.[1,3,5,7] It is nevertheless recommended to always carry out a check on a piece of the surface (porous or non-porous), where no blood trace is expected, because of the possible background coloration.[13]

In addition, this technique is less recommended to use if (latent) blood traces on a dark surface are suspected, because it already gives a dark coloration, figure 3.[13] Also, Amido Black does not fluoresce with the help of a forensic light source, which makes it impossible to create a good contrast. Furthermore, Amido black cannot be lifted, together with the trace, with a white gelatin foil unlike Hungarian Red.[1,4,13,25] Although Amido Black has these drawbacks compared to Hungarian Red, this technique is already supplied in solutions to which ethanol has been added, which means that this technique was included in this study.

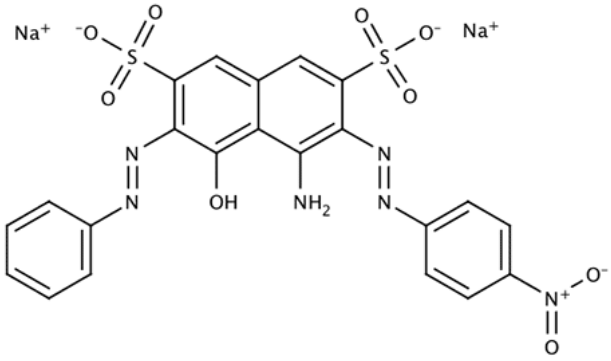


Figure 2: Structural formula of Amido Black/Acid Black 1 [7]

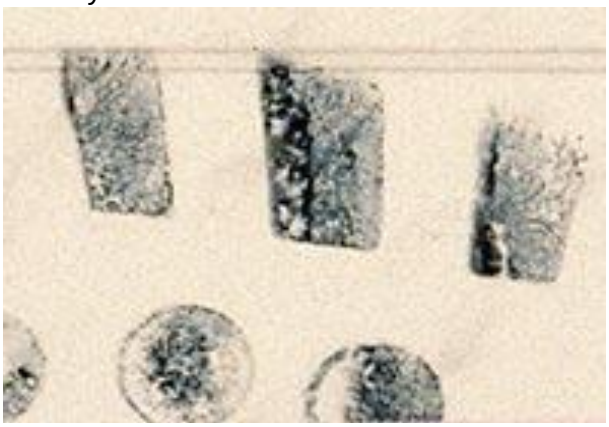


Figure 3: Image of a partial shoeprint, coloured by Amido Black [7]

Hungarian Red

Hungarian Red (Acid Fuchsin) (figure 4) is a waterbased dye solution, which is used for staining traces set with blood. These traces are then stained red and fluorescent (figure 5).[1,9,15,23,24,33] Hungarian Red reacts with the proteins in the blood, just like Amido Black, which makes it more sensitive than the VBETs that are based on the peroxidase (like (A)LCV).[4,9,13,14,35] This is because it will not only react with the protein haemoglobin, but also with the other proteins present.[33]

The advantages of this technique are[4,9,13,15,23,24,32]:

- It is safe to use because of its water base.
- It colours well.
- It can be lifted with a white gelatin foil, which makes it possible to separate background coloration from the blood trace, so that the trace can be analysed more clearly and parts of the trace on dark surfaces can be made visible.

This technique works best on non-porous, light-coloured surfaces.[32] Also, the blood must first be fixed to prevent running out and diffusion, unless it already contains a fixative.[4,9,13,15,23,24,32] (See 'Fixing traces of blood').

A characteristic of Hungarian Red is that the traces on the surfaces, which have been lifted with a white gelatin foil, can fluoresce under green light with a wavelength of 515-560 nm with a red filter of 600 nm (figure 5).[9] This allows even faint traces to become visible, even when they were present on a dark surface.[1,4,9,23,24]. However, it is still questionable and unknown whether it is possible to lift a blood trace from surfaces under temperatures below zero degrees Celsius.

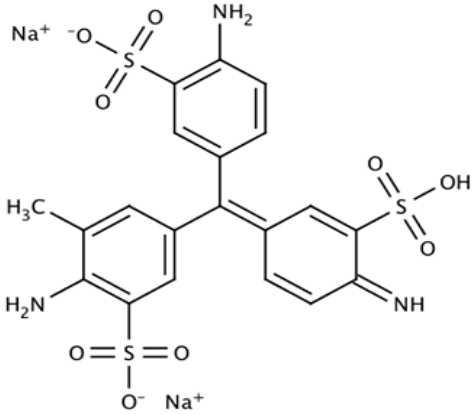


Figure 4: Structural formula of Hungarian Red [9]

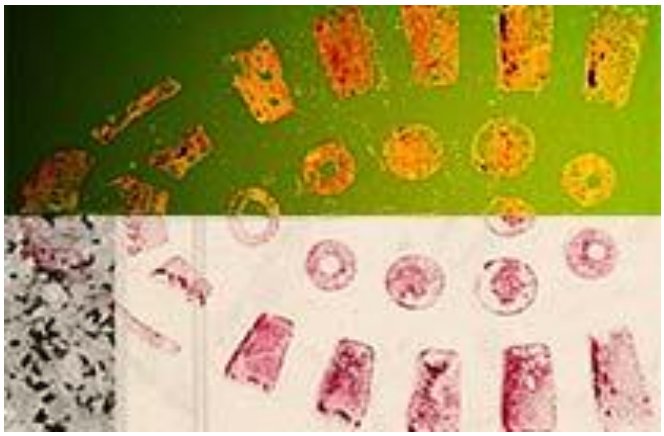


Figure 5: Image of a partial shoeprint, coloured by Hungarian Red, with a part under fluorescent light and a part under white light [9]

The word acid in Acid Fuchsin does not refer to the acidity of the dye, but to its ability to colour.[14] Acidic dyes possess coloured anions. The SO3-groups of the acidic dye help the reaction, because of their negative charge (anion).[24] Under acidic conditions, the negative portion of the protein can react with the acid to form a neutral portion, which creates a net positive charge through the NH3+. This makes it a cation, with which the negative, coloured anion can react. In addition, hydrogen bonds and other physical forces, such as Van der Waals forces, play a role in the affinity of acid dyes with protein molecules.[21,22,34]

To chemically show how people can perceive the red colour of Hungarian Red, there must be looked at the π-π*-transitions. Hungarian Red is an aromatic cyclic conjugated system, which can be explained as a system of atoms in an organic compound with single and double bonds, which alternate with each other. The electrons within those conjugated double bonds are delocalized. This means that they no longer belong to one bond, but they can be distributed among all neighbouring bonds in the conjugated system. This is because a suitable orbital overlap can arise between the p-orbitals.[41,55,56] A property of conjugated systems is that they often interact with electromagnetic radiation. As a result, many conjugated compounds have a specific colour. Looking at Hungarian Red, it has a red colour, as it already indicates. The reason is that Hungarian

Red is a large molecule with three aromatic rings and seven functional groups, among other characteristic groups (figure 4). These all play a role in how much energy is needed to transfer an electron from a π-state to a π*-state (π-π*-transition). The rule that applies is that the larger the molecule, the shorter the distance the electrons have to travel to go from π (ground state) to π* (excited state). When that distance is smaller, less energy is needed to make that transition take place and the wavelength number is larger.[41,55,56] As mentioned, Hungarian Red is a large molecule, which means that less energy is needed for the π-π*-transition and the wavelength number is larger. This corresponds to the colour red, because red lies between 650 and 780 nanometres.[9]

(A)LCV

(A)LCV ((Aqua) Leuco Crystal Violet) is a blood staining dye, which is the fully reduced form of crystal violet, which is denoted by the word Leuco. The reaction is based on the catalytic reaction of blood (haemoglobin) with hydrogen peroxide, turning an uncoloured substance (LCV) into a dye (the blue-purple crystal violet) (figure 7).[2,10,15,19,20] When the reagent comes into contact with blood, the hydrogen peroxide will be broken down by the haemoglobin.[14] The now oxidized haemoglobin can in turn oxidize the colourless Leuco Crystal Violet to the purple dye Crystal Violet (figure 6). The haemoglobin is then back in its original form and can be oxidized again by hydrogen peroxide. So, this is a catalytic reaction with the haemoglobin as the catalyst.[21,22,25,31]

Figure 6: Image with partial shoeprint, set with blood, that is coloured with (A)LCV [10]

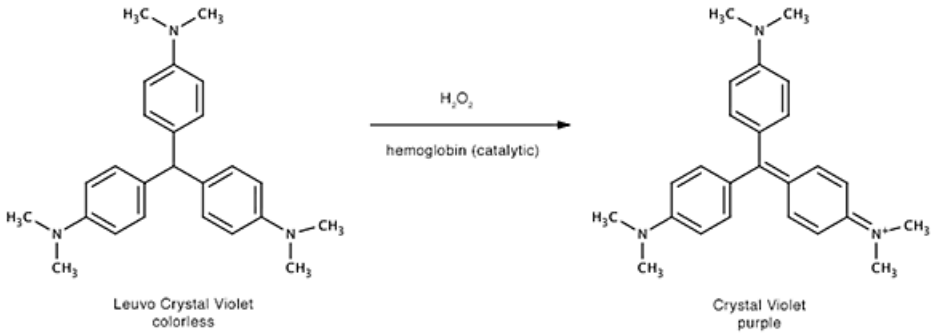


Figure 7: Reaction between (A)LCV and haemoglobin, where LCV changes from a colourless substance to a violet-coloured substance [2,10,21,31]

Because this colouring takes place slowly under the influence of light and oxygen from the atmosphere, the contrast of the visible traces is not permanent and the environment will also turn purple over time. As a result, the processed blood traces must be quickly photographed for further use.[2,4,20,39,40] The traces can also be post-treated with Amido Black.[10,31]

(A)LCV already contains a fixative of 2-2,5% of 5-sulfosalicylic acid in water, which ensures that the trace is fixed and therefore does not run.[2,10] This is advantageous, because no additional pre-fixation step is required. In addition, (A)LCV is very suitable for both porous and non-porous surfaces, unlike the other VBETs and usually it does not need to be rinsed, which is much easier

to work with. Also, (A)LCV does not contain volatile solvents and it is a safe technique in terms of its chemical composition.[2,10,14,20,31] All these advantages make (A)LCV the most widely used Visual Blood Enhancement Technique on crime scenes.[1,15,39,40] But when investigators are dealing with an excessive amount of blood, such as large blooddrops, or with a high percentage of hydrogen peroxide, there is a chance that the blood will bubble up completely after the (A)LCV has been applied, resulting in a destroyed blood trace.

Iron Oxide

Iron Oxide (Fe_2O_3) is a powder suspension method, where Iron Oxide is a solid, which is generally mixed with a detergent and water. In the case of VBETs, Iron Oxide is dissolved in the fixation fluid: 2-2,5% of 5-sulfosalicyclic acid in water, where the Iron Oxide particles remain in the fixative and therefore do not react with a component in the blood, but only attach to the fats and proteins in it. This makes it possible to lift the trace with a white gelatin foil and/or treat it with another VBET, such as Hungarian Red.[24,26,44,51,52]

Figure 8: Example of Iron Oxide in a solid state [51]

The colour that Iron Oxide has, and thus colours the blood trace, is rust coloured (figure 8), which gives a contrast with light coloured surfaces. Since only light-coloured surfaces are used in this study, it is very advantageous to use Iron Oxide as the suspension method, instead of a Titanium Oxide solution, which colours into white. Furthermore, Iron Oxide is mainly applicable on non-porous surfaces.[24,26,44,51,52]

Fixing traces of blood

Blood and traces of blood are soluble in water. Before coloring can be started, the traces of blood must therefore be fixed, which means that the traces are well adhered to the substrate on which it is located. This is to prevent the trace from running out (dissolving/spreading the blood), because it can cause loss of details and loss of the original shape/appearance of the trace, as a result of which it is contaminated and/or can no longer be used for follow-up investigations. It is therefore crucial that the trace is fixed in order to keep the quality as good as possible.[1,12,15,17] Fixation takes place by precipitating the proteins in the blood, preventing diffusion or running out. However, usually this concept takes place with liquid blood (precipitation reaction), but for this study one is dealing with solid, frozen blood. What effect this has on fixation should be investigated.

In general, before using a VBET, the trace is always fixed, with the exception of (A)LCV, which already contains a fixative.[2,9,10,20] Fixation can be done with a 2-2,5% solution of 5-sulfosalicylic acid in water.[1,12,15] Fixation with 2-2,5%-5-sulfosalicylic acid is based on the formation of insoluble salts/complexes and on the disruption of the protein structure.[17] However, it will not be possible to use this liquid as a loose fixation step at temperatures below zero degrees Celsius, as it will freeze immediately. This is because only 2-2,5% of the acid is present in the water-based liquid, which means that it is not sufficient to further lower the freezing point of water (zero degrees Celsius). That is why VBETs were used, to which the acid has already been added and so no separate fixation step is required.

Procedure

Scientific literature has shown[10,15,30] that the procedure for applying VBETs means that the traces of blood must always be fixed in advance to prevent the traces from spreading or even being washed away, provided that the technique itself contains no fixative (see 'Fixing traces of blood') and then dried.[10,12,13,14,15,30]

Then the fixed trace is treated with the chosen blood enhancement technique. The VBET is selected on the basis of the nature of the surface (porous or non-porous), the color of the surface and the nature of the trace itself. The choice therefore depends on the expected final result; providing the highest contrast between the surface and the trace of blood to be treated. Optionally, a second VBET can be applied after application of the selected substance, but this depends on the initial selected technique and the factors as mentioned above.[1,10,13,14,15,30] After that, the post-treatment takes place, which means that the excess of the Visual Blood Enhancement Technique will be rinsed away by treating the trace with a post-treatment solution suitable for that particular VBET. This is often the same solvent without the added active substances. It is important that each step is photographed (possibly with a forensic light source for possible fluorescence (Hungarian Red)).[4,6,9,13,30,47] Photographing should be performed as soon as possible after the end of the treatment, because the blood trace can continue to develop together with the VBET via the chemical reaction, resulting in less contrast.[7,8,9,10,12]

What could be beneficial for some VBETs, such as with Hungarian Red and Iron oxide, is to lift the trace onto a white gelatin foil and capture it photographically. In this way, the distinction between the background and the trace can be preserved.[1,4,9,13,47]

In addition, in the Netherlands there is a, so called, FO guideline (Forensic Investigation Guideline) for the Police, which describes the use of Visual Blood Enhancement Techniques. This FO guideline is FO guideline 02.70: “Treatment methods for traces placed with blood”[43,44], which shows how to proceed step by step in the treatment of the blood traces and the accompanying explanations are provided. For example, the preparation steps are described, the fixation step, the treatment with the chosen technique, the post-treatment step and removing the trace with the gelatin foil (optional). It is also expressly stated that every action must be recorded photographically.[43,44,47]

Those accompanying explanations clearly explain what should be paid attention to, what should be done and what the options are. Thus, this FO guideline corresponds to what is broadly described in the scientific literature[1,6,12,13,14,15,30] about the method and the advantages and disadvantages of these techniques for traces, which are placed with blood.[43]

What is not in the FO guideline?

The FO guideline 02.70[43,44] is actually only applicable for traces of blood found under non-deviating environmental conditions, such as dry weather conditions with temperatures above zero degrees Celsius and on dried traces that have been set with blood. However, it does not discuss what to do on a crime scene where deviating environmental conditions apply, such as temperatures below zero degrees Celsius. Nothing is described about temperatures in general. It is therefore also unknown whether the temperature influences the applicability and effectiveness of the VBETs, with the result that the desired outcome cannot be achieved. The question is therefore whether the current waterbased VBETs can be used as a technique for research? There are also no publications on this topic in the scientific literature, which suggests that no research has been done.[1,6,12,13,14,15,30]

The prevailing expectation is that waterbased VBETs, such as Amido Black, Hungarian Red and (A)LCV, cannot be used at temperatures below zero degrees Celsius. The freezing point of water is in fact zero degrees Celsius[45] under normal conditions, which means that the solution will freeze and thus become solid. This temperature of zero degrees Celsius can deviate slightly when the investigator is dealing with, for example, polluted water and atmospheric pressure. When the VBET is frozen, it can no longer be applied to a blood trace. On the other hand, if one wants to keep the waterbased VBETs liquid, it needs to be kept at a temperature above zero degrees Celsius at all times.[45] But in that case, when the technique is then applied to the trace, there is a chance that the trace will thaw and spread, causing trace loss.

In order to make the VBETs applicable at temperatures below zero degrees Celsius, a different solvent was used, which changes the composition of the current VBETs. The substance properties, such as the freezing point, must then be considered. For this research, an ethanol/water solution was used, since ethanol is already present in some Amido Black solutions.

Ethanol

Ethanol is a chemical and the most common primary alcohol, because it is found in alcoholic beverages and solvents. As can be seen from the structural formula (figure 9), the molecular formula of ethanol is C2H5OH.[53,54] Ethanol is a colorless and slightly flammable liquid with a characteristic odor and must therefore be handled with care. For example, ethanol should not be used in hot environments, such as sparks, open flames or other ignition sources.[45,48] The freezing point of ≥96% ethanol is minus one hundred and fourteen degrees Celsius, which is very low.[45,53] Furthermore, the physical properties of ethanol largely originate in the hydroxyl group, because this allows ethanol to participate in hydrogen bonds, making ethanol less volatile than other organic compounds of similar molar mass.[53,54]

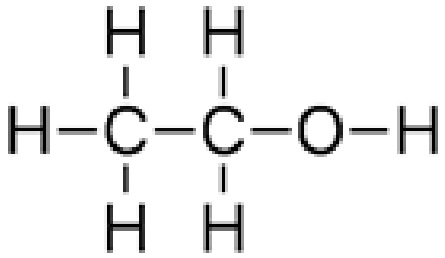


Figure 9: Structural formula of ethanol [53]

In this research, ethanol was chosen to adapt the VBETs, because it is a safe, workable substance, in contrast to, for example, methanol, which is contained in anti-freeze solutions. This safety is very important to make the VBETs applicable on a crime scene and not just in controlled environments, such as an accredited laboratory.[3,39,40,43,44] In addition, ethanol is already used on crime scenes as, for example, a disinfectant and it does not affect the DNA in the leukocytes in blood. Besides, ethanol is used during the isolation process of DNA, because DNA dissolves less well in it and thus precipitates, after which it can be removed.[57]

As mentioned, ethanol is a slightly flammable liquid. As a result, it must be investigated which percentage of ethanol in the ethanol/water solution is suitable enough for a desired result, that corresponds to the results of the current VBETs under non-deviating conditions, to keep it as safe as possible.[45,53,54]

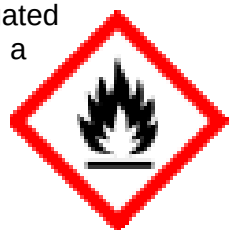


Figure 10: Sign slightly flammable [53]

VII Research Design/Methodology

Goal

The aim of this study is to determine whether an ethanol/water solution in the Visual Blood Enhancement Techniques are applicable at temperatures below zero degrees Celsius. It was achieved by placing bloody shoeprints on surfaces, followed by enhancing it with the four modified VBETs. Which percentage ethanol in the solution is both suitable and safe to use on the crime scene, is determined in advance. All the results are captured with a camera (Sony) and white light sources (studio lights), (Appendix 1 and 2). The grading of the photos with the shoeprints is performed by objective evaluators. The photos that have to be graded are of two stages; the bloody shoeprints prior to enhancing and the shoeprints post enhancement. In this way, a conclusion can be drawn about to what extent the modified VBETs are applicable and yields useful and visible results with sufficient detail at temperatures below zero degrees Celsius.

Strategy

To determine whether the VBETs with an ethanol/water solution can be used at temperatures below zero degrees Celsius, the percentage of ethanol has been determined and evaluated by several tests, prior to the preliminary experiments and the main research, where bloody depletion series have been made with a partial shoe on surfaces. These series have been enhanced with the VBETs with an ethanol/water solution. A conclusion about the useful and visible results with sufficient details of the enhanced shoeprint, is drawn based on both the contrast between the print and the surface, as the number of characteristics in the prints, compared to the same prints prior to enhancing.[29] This conclusion could be drawn, because all results are analysed and a selection of shoeprint series is graded by a group of sixteen evaluators. The grading was based on a grading system (Centre of Applied Science and Technology – CAST) and focuses on the surface area of developed characteristics relative to a full shoeprint. Originally, this grading system is based on fingermarks, but with some modifications, it has now been used for partial shoeprints. The grading is based on scores varying between 0 (No development) and 4 (Full development).[29] Every rater graded all the selected shoeprint series for consistency. Therefore, two stages were included for grading: the original shoeprint series and the enhanced shoeprint series. This allows for a comparison to be made between the original prints and the effect of enhancing with the modified VBETs. Based on the data gained on the grading, it is determined whether a significant difference between the two stages is present.

Before starting the study, various tests and experiments were conducted to gain insight in the problem and in the functionality of the strategy that was determined beforehand. For example, it was evaluated whether the determined substance in the solution, ethanol, is able to fix frozen traces of blood in the first place. It appeared that it worked, so it was decided to work with ethanol in the further experiments.

Prior to the main experiment, three preliminary studies were performed. The results of these experiments helped to provide a narrowed method for the main study. The preliminary studies were focused on gaining insight in the problem and on determining the optimal conditions of ethanol, for example. The preliminary studies were conducted on the same surfaces as the main research was carried out to make sure that that is a constant factor. For other constant factors that were taken into consideration to lower the variability and maximize the reproducibility, see Appendix 5. The results of these preliminary studies were used to provide a specific, optimal method for the main research.

Throughout the main study, the shoeprints, set with undiluted blood, were placed as depletion series on the tiles and paper by the same person to make sure that that is a constant factor as well. On the other hand, the variation that can exist between different persons is not included in the result, which means that deviant results cannot be distinguished. The depletion series with the partial shoe were placed on the surfaces in a series of four in the same order every time to imitate practice. The first print had the most blood and it reduces exponentially with every successive stamp. More than four were not possible on the tiles and when the blood volume is too low, blood enhancement will not occur, which means that the blood stays latent. It is important to say that it is not possible to dose exact amount of blood on the shoe sole every time, so the depletion series do contain small variations of blood in volume. This also applies to the amount of pressure that was used to set the prints, but it was tried to keep it as constant as possible.

To investigate the detection limit[1], a preliminary study was performed with four dilutions. In practice, shoeprints placed with diluted blood can be found. They can either be visible (low dilution factor) or invisible (high dilution factor). When a print is invisible to the human eye, it will not be recognized and therefore not enhanced, hence why dilutions factors, higher than 1/500, were not considered for this study. The dilution factors that were included are 1/50, 1/100, 1/250 and 1/500.

Blood

The blood that was used in the experiments, is approved and certified blood from one cow, as it is much safer to use than human blood. Because the blood of one cow was used, minor differences by medicine or food in the blood between individuals, that could potentially affect the results, was not investigated, since it was not relevant for the variables that were studied. In addition, it contains the same components that the VBETs react with as the components in human blood, because humans and cows are both mammals. A 3% anticoagulant (EDTA) had been added to the blood, so that it clots less quickly and is therefore easier to work with. Furthermore, the blood is stored in the freezer, but the cellular components and the morphology of the blood cells are well preserved. Prior to using the blood, it was thawed and then respectively put into a blender and sieved to create a homogeneous substance of blood cells and serum and remove possible clots formed by defrosting. To keep it homogeneous, a stirrer was used. Afterwards, the blood is stored in the freezer again, when it is not used in the experiments. During the experiments, the blood was brought to room temperature (± twenty degrees Celsius) and used immediately after.

Chemicals

During the research, the following chemicals were used: Amido Black[7], (A)LCV[9], Hungarian Red[10] and Iron Oxide[52]. These are all supplied and made by Loci Forensics B.V., according to the recipes in Appendix 3. This contributes to the validation of this study, because existing VBETs were used for this study. These have already been validated and are being applied in practice. Only the solvent had to be adapted to these techniques. These four techniques were chosen, because Amido Black and Hungarian Red react with proteins, (A)LCV reacts with the haemoglobin and the Iron oxide attaches to the fats and proteins in the blood but does not react.[1,2,3,15] In this way, all blood components are investigated, with which the techniques react/adhere. This allows better insight to be obtained. These VBETs were made with an ethanol/water solution, because of the freezing temperature of ethanol of minus one hundred and fourteen degrees Celsius.[45]

All of these VBETs were produced such that they already had an internal fixative of 2,5%-5-sulfosalicylic acid, eliminating the need for the extra step of fixation of the prints, as the FO guideline indicates.[43,44] This is more efficient, because now the fixative does not also need to be adjusted with an ethanol/water solution to make it freeze-resistant. In addition, the prints were treated with one step less, which is more beneficial for the prints and which reduces the chance

of thawing, spreading and/or contamination. At the moment, no VBETs with internal fixer are used on crime scenes, except for (A)LCV, and a fixation step must therefore be performed.[43,44] This could possibly be investigated in a follow-up study.

All VBETs were applied to the blood traces with a handpump micro sprayer until the traces were completely covered by the dye. The use of a micro sprayer was chosen, because there was a relatively large amount of VBET needed to enhance the traces and it is not possible to achieve that with an airbrush. Also, on crime scenes, such sprayers are more readily available or are already being used, unlike an airbrush. The reaction was proceeded for four minutes until it was stained to the colour associated with the technique used with sufficient detail.[43,44] After this, the traces were post-treated with the rinsing solution, which was also modified with the ethanol/water solution to make it freeze-resistant. An acetic acid/demineralised water solution of 0.2% as a post-treatment reagent does not work, because the percentage of acetic acid in the solution is insufficient to bring the freezing temperature of water down to such an extent. This will still cause it to freeze around zero degrees Celsius.[21]

For (A)LCV it is important that the solution was activated with 3% hydrogen peroxide before it could be applied to the trace. This was not supplied already mixed, as this will not benefit the effect and result of the reprocessed traces.[2,10,15,31]

All liquids were not stored under freezing conditions before being applied to the surfaces, because in practice there is no possibility for this either. The buses of the Forensic Department are not equipped with a freezer compartment, where these VBETs can be stored.

Surfaces

As indicated, this study is a pilot study. The main focus was therefore on whether it is possible and how the VBETs can be adapted to make them applicable on crime scenes, where temperatures below zero degrees Celsius apply. The research was therefore not conducted on many different surfaces. The surface used for the VBETs, is white ceramic tile as it is non-porous and does not react with the blood and chemicals in the VBETs. These tiles have been pre-degreased and cleaned with ethanol as a disinfectant to ensure that any grease and grime could not affect the reactions between the blood and the VBETs. Only (A)LCV was not used on the ceramic tiles, because prior tests showed that (A)LCV wipes away the traces of blood and stains very much. That is why it has been decided to use (A)LCV only on white paper of 250 g/m^2, since (A)LCV is well applicable for porous substrates. It was decided to use 250 g/m^2 paper, because it is much thicker than normal A4-paper, which greatly prevents the paper from waving after it had been wet. Besides, this technique was used to check whether the adaptation to an ethanol/water solution of the (A)LCV is suitable for porous substrates.

The colour white of the surfaces was chosen, because this provides the most contrast between the surfaces and the enhanced traces. After they were cleaned, these surfaces were first placed in the freezer for 24 hours before the blood and the VBETs were applied.

Temperature

To examine the VBETs under conditions below zero degrees Celsius, a freezer was used in which the substrates (tiles and paper) were placed for about 24 hours before the blood was placed on it. Placing the stamp impressions with blood took therefore place in the freezer. This is done in order to imitate the case example from the 'Introduction'. Namely, a car was parked outside, at a temperature below zero degrees Celsius, on which a trace, set with blood, had been applied to. The car had already assumed the temperature of the environment. This is imitated by first placing

the surfaces in the freezer. The freezer was set to minus twenty degrees Celsius, so it covers all temperatures between zero and minus twenty degrees. But it is important to mention that the freezer fluctuates a bit in temperature. Besides, it was necessary to open and close the doors a lot during conducting the experiments, so it was not possible to maintain constantly the temperature on minus twenty degrees Celsius.

This temperature does not seem realistic for the winter conditions in the Netherlands, but these are realistic temperatures for Scandinavian countries and Canada. However, a personal conversation with a Norwegian forensic investigator showed that they also did not know how to deal with this problem in Norway, which makes it still relevant to investigate. In addition, it is better to use a somewhat more extreme temperature, which covers all realistic temperatures in the Netherlands, than a slightly warmer temperature, which is on the edge of realistic temperatures, including the perceived temperatures.

Stamping

The blood was applied using a stamp from a partial shoe sole, figure 11. The stamps were made from a sole of a new shoe, which means that there were no characteristics in it. So, some damage was manually made with a scalpel in the soles, which acts as characteristic features. These were necessary when the results of the modified VBETs of the main research were assessed.[1] Such characteristic features are important evidence for the identification and individualization of the source of the trace, which in this case is a particular shoe.

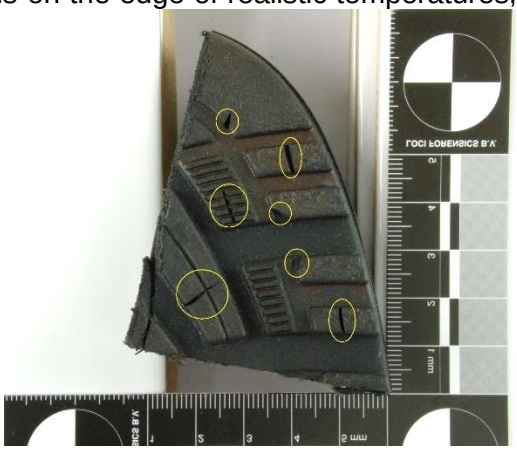


Figure 11: The partial shoe sole used for with manually applied characteristics

Photography

Throughout all the experiments, results were captured with a Sony DSC-RX10M3 digital camera in a fixed setup; fixed distance between surface and camera and fixed position of studio lamps and fixed camera settings (aperture, ISO and shutter speed) to prevent obtaining deviant results (Appendix 2, figure 26). The lens that was used was a macro lens with an 8.8-220 mm RX in RAW+JPEG format. This was done to capture optimal detail.

Also, additional light sources were used to maintain a constant lighting in all photographs. Two studio lamps (FalconEyes, 6200 Kelvin, white light) shone on the surfaces from the left and right side. A Lumatec (Superlite 400) was used to create a green light of approximately 515-560 nm, since that is the wavelength that Hungarian Red fluorescence. The room itself was free of daylight.

Methodology

As mentioned, prior to the main research, several tests for defining the optimal conditions and three preliminary experiments were performed to gain more insight in the problem and to set up the further experiments in this study, see table 2. There is no specific order for the pilot experiments, because they were performed at the same time. But they had to be done before the main research could be started.

Table 2: Study design [42]

Pilot experiments	Waterbased VBETs	Main Research	Series
	Determination ethanol		Scoring
	Conditions blood		Statistices
	Conditions rinsing solution		ICC-test
	Comparison below and above zero degrees		
	Dilutions		

Preliminary experiment: Waterbased Visual Blood Enhancement Techniques

Since this research is a basic study, it was first examined how the existing waterbased VBETs (Amido Black[7], (A)LCV[9], Hungarian Red[10] and Iron Oxide[52]) with an internal fixation of 2,5%-5-sulfosalicylic acid at temperatures below zero degrees Celsius react, (see Appendix 5 for how the current VBETs were made). First, 25 milliliters of the current VBETs were poured in a 50 milliliters centrifuge tube and placed in the freezer at minus twenty degrees Celsius horizontally to investigate whether the solutions actually freeze. In this way, some insight could be obtained and it could be tested whether the expectations are actually true. In other words, whether the current VBETs indeed have the potential to freeze. The centrifuge tubes of all the VBETs were photographed individually. Also, these results were compared to the modified VBETs, after the most suitable and safest percentage ethanol for in the solution was determined.

Preliminary experiment: Determination percentage ethanol

To investigate what percentage of ethanol in the ethanol/demineralised water solution is suitable as a solvent, some important criteria needed to be considered. Those important criteria were that the modified VBETs provide as much as possible the same desired result as the existing waterbased VBETs. Which means that they made the traces, set with blood, just as visible, and that they are safe enough to use on a crime scene. The latter is mentioned because ethanol is a flammable liquid, so it must be handled with care.[45,48]

In order to use the safest possible percentage of ethanol, the following percentages of ethanol were chosen: 20%, 30%, 40% and 50% ethanol. These percentages were initially chosen because it was suspected that these percentages ethanol would not freeze. This is based on the fact that alcoholic drinks with such ethanol percentages do not freeze either. Just like the preliminary experiment with the current VBETs, 25 milliliters of the four ethanol/water solutions were poured in the centrifuge tube and placed horizontally in the freezer at minus twenty degrees Celsius to investigate whether the solutions freeze (Appendix 5). Again, all results were photographed.

When the optimal percentage ethanol was determined, the active VBET substances and the 2,5%-5-sulfosalicylic acid were added (Appendix 3+5), to investigate if the active substances and the fixative had an influence on the solution at temperatures below zero degrees Celsius. If it did not influence the possibility of freezing, then this solution was used for testing the optimal conditions. Again, 25 milliliters of the modified VBETs were poured in a 50 milliliters centrifuge tube and placed in the freezer at minus twenty degrees Celsius to investigate whether the solutions actually freeze. Also, these results were photographed individually and in combination with the waterbased VBETs.

Optimal conditions

Optimal conditions of blood

First, the optimal conditions of blood were determined. This includes the freezing time and the ability of defrosting and running out with and without a fixation with 2,5%-5-sulfosalicylic acid. These experiments were performed on the white ceramic tiles to avoid a variable change.

The blood was prepared, as previously mentioned, and was placed with a partial shoe sole on a tile at room temperature in a depletion series of four prints. Consequently, the tile was placed in the freezer and after five minutes, it was checked whether it was already frozen. If it was not frozen yet, it was left five more minutes in the freezer. In this way, it was determined what the maximum freezing time is, so that it could be considered during the other experiments in this study. However, if there was a slight deviation from that time, it did not matter much, because the traces were still

frozen, so the drying and clotting process had been shut down. Furthermore, the Forensic Department is normally not on site in a few minutes after the blood was applied.

To investigate whether the blood indeed defrosts and runs out, an experiment was set up. The tile with the shoeprints with blood, from the previous test, was taken out the freezer and was placed diagonally on a table to see if it ran out. This tile was not treated with a 2,5%-5-sulfosalicylic acid in an ethanol/water solution.

This experiment was repeated, but now it was treated with a 2,5%-5-sulfosalicylic acid in an 40% ethanol/water solution, before it was taken out of the freezer. In this way, it could be determined whether the fixative with the determined ethanol percentage in the solution had the ability to fix the shoeprints, set with blood. The untreated and treated tiles with the fixative were photographed next to each other.

Optimal conditions of the rinsing solution

Since the current rinsing solution exists of only a 0,2% acetic acid solution in water, this solution will freeze at temperatures below zero degrees Celsius, because the amount of acetic acid in the solution is not enough to reduce the freezing temperature of water extremely. That is the reason this solution was also modified with the same percentage ethanol as was determined, to investigate if it was possible to rinse away only the excess dyes and to leave the prints on the tiles. How the rinsing solution was made, can be seen in Appendix 3.

To investigate this possibility, tiles with a series of fingerprints of the right thumb, set with blood, was left to freeze in the freezer for the determined freezing time of blood. Then, the prints were treated with Hungarian Red on an ethanol/water bases. After four minutes[43], the prints were treated with the rinsing solution (0,2% acetic acid in an ethanol/water solution) to see if it worked. The results were photographed.

Optimal conditions of applying blood

The blood was mainly applied by stamping with a partial shoe sole to create shoe prints. In order to determine how to apply the prints, a test was set up. It had been investigated if it was better to apply the blood by making a rolling motion to simulate a step while walking or by placing the entire surface of the shoe sole perpendicular on the tiles. The manner with the most characteristics visible was chosen to carry out the main research.

Besides, since the occasion of this study is a print, set with blood, on a car, it can also be the case that the print was set on the glass under a layer of ice. To cope with this issue, it was investigated if the modified VBETs still work. To be able to investigate this, a tile had been put in the freezer for 24 hours. After this, a series of fingerprints, set with blood, was placed on the frozen tile and was left to freeze. Then, a layer of tap water was sprayed on the tile with a micro sprayer and left to freeze too. Consequently, Hungarian Red was applied on the tile. Only Hungarian Red was used to maintain a constant staining throughout every preparatory test. Hereafter, the rinsing solution was applied. The results were photographed.

Comparison modified VBETs below and above zero degrees Celsius

In order to be able to compare the influence of the VBETs with the determined ethanol/water solution compared to the waterbased VBETs on the effectiveness and applicability, they were also investigated at temperatures above zero degrees Celsius. That comparison cannot take place below zero degrees Celsius, because waterbased VBETs do not work, since they will freeze. If this is not investigated, it cannot be determined whether a possible result was caused by the addition of ethanol or by freezing. So, it was investigated by applying series of fingerprints with

blood on tiles at room temperature and was left to dry. Then, the prints were enhanced with Hungarian Red. After four minutes[43], the prints were rinsed with the modified rinsing solution. Now, some statements could be made about if the modification with ethanol is working. The results were photographed.

Preliminary experiment: Dilutions

Optimal conditions of surfaces

Initially, to investigate the dilution to which the modified VBETs with ethanol react (detection limit), three-well microslides would be used, allowing a triplicate study to be performed on each microslide for the purpose of the reliability, reproducibility and detection limit to the effectiveness of the modified VBETs. However, the surface showed an even distribution of the blood dilutions was not possible in the wells and due to the transparency, no good contrast could be observed. So, the surface was changed to the white tiles and paper. The advantages are that, firstly, those surfaces are the same used in the main study (constant factor). Secondly, the surfaces can be divided into areas, so that the different dilutions could be applied to them and the results could be seen at a glance. (figure 12) The disadvantage is that it was not possible to do it in triplicate. In addition, it was noticed that the areas marked on the tiles for the division with a permanent marker were wiped away by the ethanol in the modified VBETs and rinsing solution. To prevent this, some adhesive transparent tape was used over the markings.

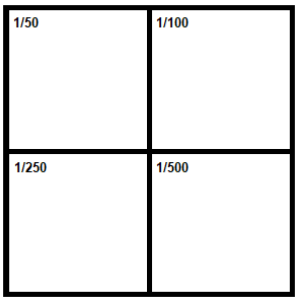


Figure 12: Division of areas for dilutions

Dilutions

As mentioned earlier, this experiment was set up to investigate the influence of diluted blood on the modified VBETs with the determined ethanol/water solution including 2,5%-5-sulfosalicylic acid and what its detection limit is. The dilutions that were used, are: 1/50, 1/100, 1/250, 1/500 times diluted. The blood was diluted with demineralised water.[1] These dilutions were applied on the frozen marked tiles and paper with a swab to create a zigzag form. It is important to mention that the distribution of the blood is also uneven, just like the droplets on the microslides. But it is more spread, so more even. The diluted blood was then given time to freeze. Subsequently, the surfaces were treated with the modified VBETs and allowed to react for four minutes.[43] Then the rinsing solution was applied and left to dry. All results were photographed. For each VBET, at least ten surfaces (tile or paper) were used. Hungarian Red, Amido Black and Iron oxide were used on the tiles and (A)LCV was used on paper.

The reason this experiment does not fall under the main research, but is considered supplementary to the depletion series, is because caution should be taken when interpreting the dilution series. The physical behaviour of diluted blood with demineralised water differs from pure blood. In addition, the stamp series better reflect practice.[1]

Since a swab with diluted blood was used, these results did not need to be evaluated according to criteria based on the characteristic features (Table 4). The results were judged by answering the question; to what dilution is a discolouration of the trace visible to the VBET-colours?

Main research: Depletion series treated with modified VBETs

After the preparatory test and preliminary experiments had been carried out, the main research took place. Here, the clean frozen ceramic tiles and the frozen paper were used for the sample series. A total of 50 sample series were carried out per VBET with four stamps per series (table 3), but the sample series were carried out one by one, so not at the same time. These 50 series,

which is a relatively large number of samples, allowed a good picture to be obtained of the effectiveness of the modified VBETs. This contributes to being able to make reliable statements about the results. As mentioned, initially, the tiles and paper were placed in the freezer for 24 hours at minus twenty degrees Celsius to reach the same temperature as the freezer. The stamps with undiluted blood were then placed on the surfaces in the freezer in the way that emerged from the preliminary experiment. The application of the blood to the surfaces was accomplished by placing a decreasing series of prints with a partial shoe sole, wetted with blood. Wetting the sole was done by moistening it once per series. Because it was applied to frozen surfaces, the blood did not dry but rather froze. (See Appendix 6 for an example). After application, the blood was left to freeze with the doors closed, after which the substrates were quickly taken out of the freezer to take a before picture and were put back in the freezer.

Table 3: Number of samples per VBET

	Ceramic tiles	Paper
(A)LCV	x	50 sample series
Hungarian Red	50 sample series	x
Iron oxide	50 sample series	x
Amido Black	50 sample series	x

Subsequently, the surfaces were treated with the VBETs (including 2,5%-5-sulfosalicylic acid) with the determined percentage of ethanol in the solution, using micro sprayers until the prints were completely covered with the dye, which was then allowed to react with the blood for four minutes.[43] The four VBETs were not applied on the same surface, but one tile/piece of paper applies for one type of VBET. Also, the drip container and the freezer were cleaned between the application of the different VBETs to prevent contamination of another technique. Then, the post-treatment step took place with the adapted rinsing solution. This step was only necessary for Amido Black, Hungarian Red and Iron Oxide, because (A)LCV does not need rinsing.[2,10,11,14] Afterwards, the surfaces were taken out the freezer and were left to dry prior to photographing. Since the reactions were rapid and the results were not permanent, they had to be captured quickly and, in a frame-filling manner, by means of photography in a fixed setup.[47] (Appendix 2) In this way, it was possible to compare the prints before and after enhancement. For the protocol, see Appendix 5.

Main research: Scoring the results

A selection of results from the main research were graded and identified. This selection regards ten sample series per VBET (so 40 in total) with the before treatment and after treatment picture. All sample series were assessed on the basis of the photographs by sixteen independent evaluators, consisting of forensic investigators from five units, forensic bachelor students and an NFI-expert. A grading form was put together (Appendix 6). The table is based on the grading system used for determining the quality of ridge detail for developed marks by Bandey and Gibson[29]. This table was developed and reported in The Center of Applied Science and Technology (CAST)[29]. It is up to the evaluators to score the series with a 0-4. Based on previously conducted research[21,22], the aim was to ask ten evaluators, but more people appeared to be willing to perform the assessment on the results. This contributes to reliability, because more evaluators provide more data, which allows reliable and reproducible statements to be made about the results, although the ratings are based on human judgement, which is subjective. But that is what the statistical paired t-tests were for. Since shoeprints were used and not fingerprints with associated papillary lines, on which the table was originally based, the criteria had been adjusted (Table 4).[21,22]

Table 4: Grading system for determining the quality of ridge detail for developed marks [29]. Table with which the scores can be given to the sample series of the shoe sole stamps by the evaluators.

Grade	Details (maladjusted)	Details (adjusted)
0	No development	No development
1	Signs of contact but <1/3 of mark with continuous ridges	Signs of contact but <1/3 of mark with characteristics
2	1/3-2/3 of mark with continuous ridges	1/3-2/3 of mark with characteristics
3	>2/3 of mark with continuous ridges, but not quite a perfect mark	>2/3 of mark with characteristics, but not quite a perfect mark
4	Full development – whole mark clear with continuous ridges	Full development – whole mark clear with characteristics

Main research: Validation using a statistical test

The data was processed and analysed in Excel. The frequency of the granted scores per print of all evaluators were put together in tables and stacked bar graphs. (Appendix 8) An Intra Correlation Coefficient-test using a two-way random test with absolute agreement was calculated in SPSS (IBM SPSS Statistics) to determine whether the different evaluators agreed with each other and whether their data to be used, is reliable.[27,28,49,50] Appendix 9 provides further details regarding the methodology of the ICC-test. The magnitude of the correlation coefficient can be interpreted as follows[59]:

- **0.00 – 0.30** Little or no correlation
- **0.30 – 0.50** Low or weak correlation
- **0.50 – 0.70** Medium correlation
- **0.70 – 0.90** High or strong correlation
- **0.90 – 1.00** Extremely high or extraordinarily strong correlation

Also, a paired t-test was performed to determine if there was a statistically significant difference between two groups (original prints versus enhanced prints).[21,22, 27,28,49,50]

Forensic Light Source and Lifting abilities

Besides that Hungarian Red can be photographed in daylight, it is also possible to photograph it under green light with a wavelength of 515-560 nm with a red filter of 600 nm.[1,4,9,23,24] In order to check whether it also works with the modified Hungarian Red, this was investigated in the form of a pilot experiment. This was not included in the main study, because the complete traces could not be observed at a glance under green light with a red filter. The results were shown under 'Recommendations,' because they need further research before statements can be made.

Furthermore, traces treated with Hungarian Red and Iron Oxide can be lifted with a white gelatin foil[4,9,13,15,44], which was therefore tested as well. This was also not included in the main study, because the quality of the foil turned out to be insufficient. It is important to mention that the white gelatin foil was not stored in the freezer, because it is not possible in practice and because it is not possible to work with a foil that was frozen, since it loses its flexibility. The lifted results were photographed immediately after lifting as the lifted traces may begin to fade as it soaks into the gelatin foil over time.[43] Again, the results were shown under 'Recommendations', because they need further research before statements can be made.

VIII Results & Discussion

In order to achieve the answer to the main question of the research, some small tests, preliminary experiments and the main research were executed. It is important to keep in mind that this study regards a pilot study, since this is assumably the first time that the Visual Blood Enhancement Techniques is researched at temperatures below zero degrees Celsius. Because of this, many variables were not included, but they can be included in follow-up optimization studies. However, in some ways this study already focuses on optimization, like multiple VBETs, ethanol percentages, dilutions and the comparison between before and after enhancement.[29] Recommendations about variables that needs optimization, are made in Chapter 9 'Recommendations'.

The results of the executed research are presented in this chapter. Additional results, images and raw data are presented in Appendices 6, 8 and 9.

Preliminary experiment: Waterbased Visual Blood Enhancement Techniques

In order to ensure that the waterbased VBETs freezes at temperatures of minus twenty degrees Celsius, those techniques were made, according to the proportions that were described in Appendix 5. Of those solutions, 25 millilitres were poured in centrifuge tubes and put in the freezer. Because the 25 milliliters in the tubes contain more liquid than when it is sprayed with micro-small droplets on a surface, the freezing time turned out to be much longer. Results showed that the waterbased VBETs were frozen after approximately one hour.

To show that the liquids in the fluids were frozen, the tubes were laid down in the freezer horizontally. When they were taken out the freezer vertically, the frozen VBETs stayed in the same place as that they were in the freezer (against gravity). An image of every VBET is shown in figures 13a-d. These results prove that the waterbased VBETs are not applicable on blood traces at temperatures below zero degrees Celsius. Therefore the VBETs need a change in the base fluid to make them applicable and to make the blood traces (more) visible.

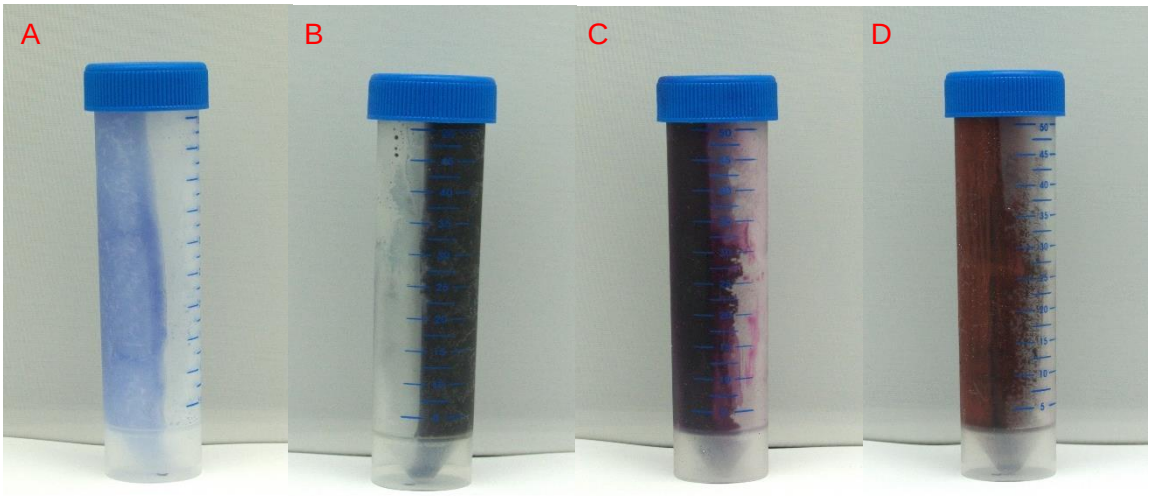


Figure 13: Frozen waterbased Visual Blood Enhancement Techniques: A) (A)LCV; B) Amido Black; C) Hungarian Red; D) Iron oxide

Preliminary experiment: Determination percentage ethanol

To determine what the most suitable and safest percentage ethanol in the ethanol/demineralised water solution was, to work with in the further experiments, four percentages were chosen: 20%, 30%, 40%, 50%. Again, 25 millilitres of the solutions were poured in a centrifuge tubes and put in the freezer. Just like the experiment with the waterbased VBETs, these tubes were laid down horizontally in the freezer for one hour. In this way, it is easier to see when a solution is frozen or not when they were taken out the freezer vertically. The results of the four solution is shown in figure 14, where the solutions with different ethanol proportions were placed next to each other.

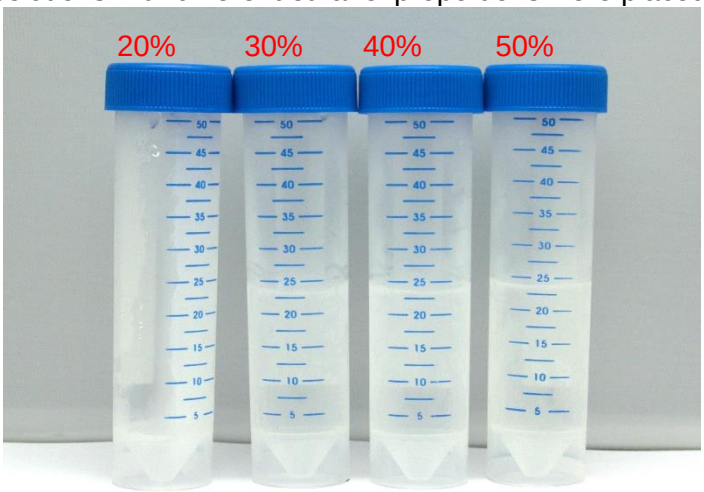


Figure 14: Results of the four solutions with different ethanol proportions

As can be seen on figure 14, the solution with 20% ethanol came out frozen, so this proportion was not suitable to work with. The other three solutions remained liquid, so a choice had to be made with which percentage of ethanol was used in the further experiments. The choice fell on the 40% ethanol solution. The reason there had not been chosen for the 30% ethanol was, that it was possibly too close to the frozen 20% ethanol as a result of which there was a chance that it would not fix the traces sufficiently in combination with the 2,5%-5-sulfosalicylic acid. The 50% ethanol had not been chosen, because the amount of ethanol was more, which results in a less safe solution due to its flammability. Based on these considerations, the choice fell on the 40% ethanol solution to work with.

Subsequently, it was investigated to what extent the active VBET substances and the 2,5%-5-sulfosalicylic acid had an influence on the ethanol/water solution at temperatures below zero degrees Celsius. So, the solutions were made according to the recipes in Appendix 3 and 5. To lower down the number of variables, the modified VBETs with the 40% ethanol in the solution were, just like the previous experiments, poured in the centrifuge tubes with the same amounts and laid down in the freezer for one hour, because the amount of liquid is larger than micro-small droplets and therefore needs more time to freeze. After that, they were taken out the freezer vertically and photographed. The results of the four techniques are shown in figure 15a-d, directly compared to the waterbased VBETs.

As can be seen in figure 15a-d, the waterbased VBETs of all the four techniques (left tubes) were frozen in contrary to all the right tubes of the four different VBETs, which were the VBETs with the 40% ethanol in the solution. Those modified VBETs also remained liquid, just like the 40% ethanol/water solution without active VBET substances and the 2,5%-5-sulfosalicylic acid. Therefore, it was decided to continue with the 40% ethanol/water solution as the base liquid for the further experiments.

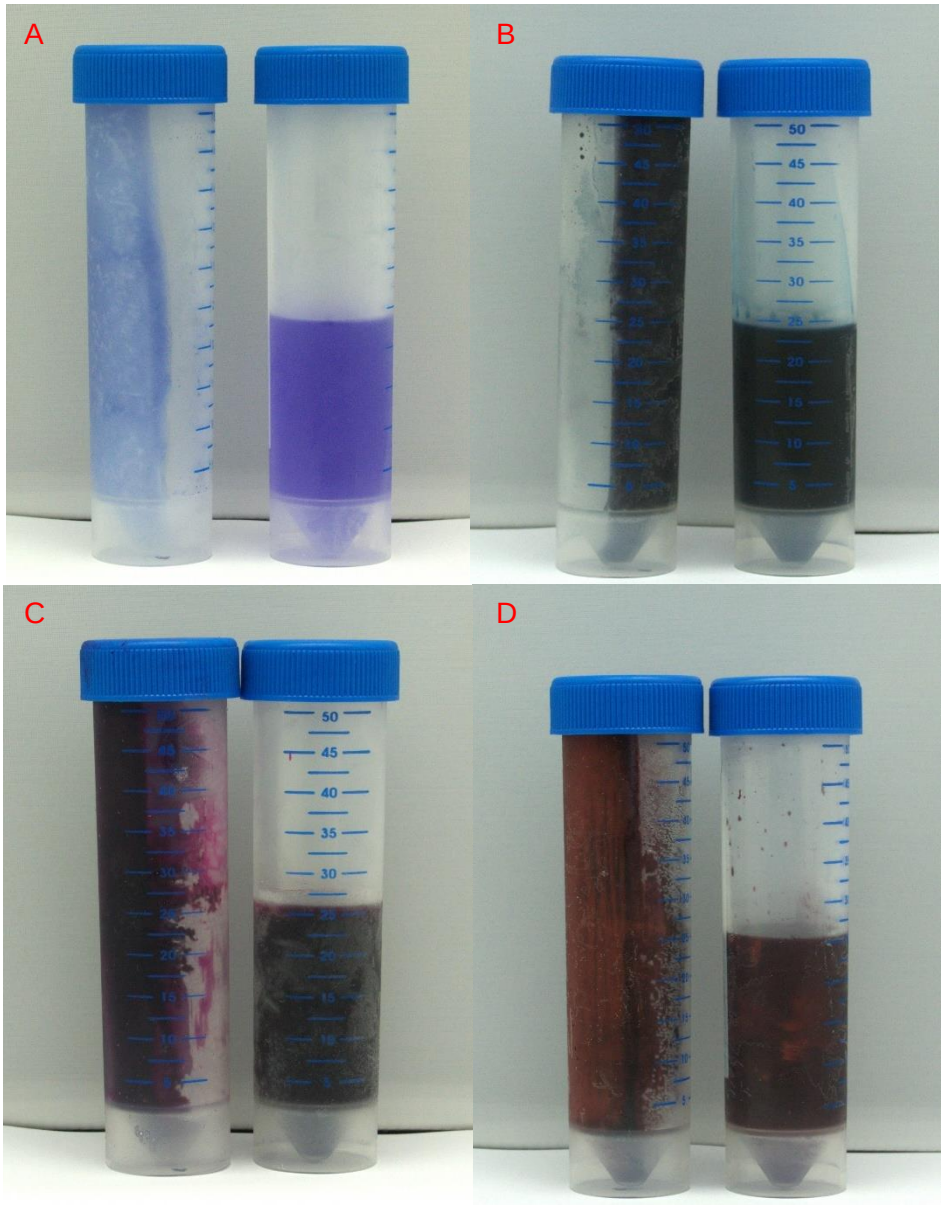


Figure 15: Comparison between the frozen waterbased VBET (left) and the liquid VBET (right) with a 40% ethanol solution; A) (A)LCV; B) Amido Black; C) Hungarian Red; D) Iron oxide

Optimal conditions

Optimal conditions of blood

In order to investigate the optimal conditions of blood, some variables were investigated, just like the maximum freezing time. The blood that was stamped on the tiles, which were laid down in the freezer, appeared to freeze within the five minutes, so that could be taken into account for the further experiments of the study. But it is important to report that these tiles were not in the freezer before the blood was applied on it, which means that they had not had the possibility to assume the same temperature as the freezer of minus twenty degrees Celsius. On the other hand, during the further experiments, the surfaces were already in the freezer for 24 hours, after which the blood was applied to the surfaces in the freezer. This results in that the blood froze faster than the maximum determined freezing time of five minutes.

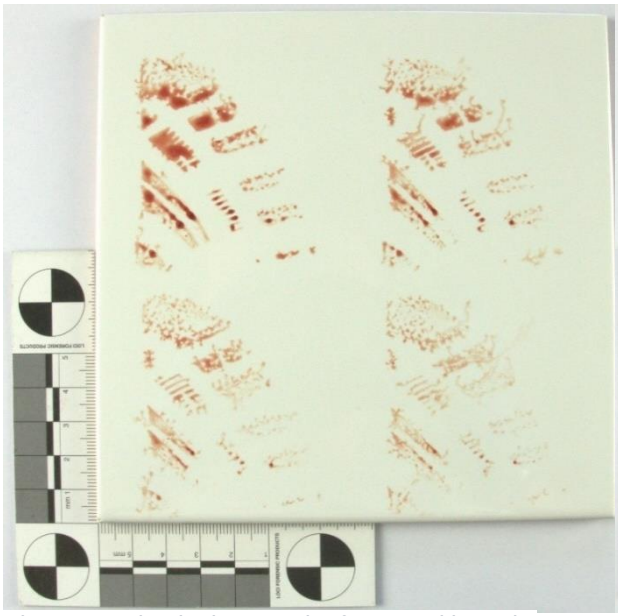


Figure 16: Blood taken out the freezer without the treatment of 2,5%-5-sulfosalicylic acid

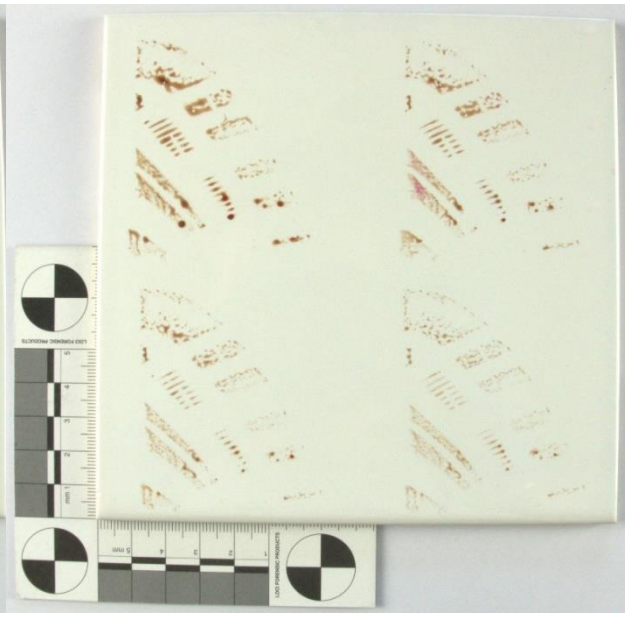


Figure 17: Blood taken out the freezer with the treatment of 2,5%-5-sulfosalicylic acid

Furthermore, there was investigated whether untreated blood defrosts and runs out after the surfaces had been taken out the freezer. The results can be seen in figure 16. As can be seen on the picture, the blood did defrost and ran out, resulting in the loss of the traces and its characteristics. This proves that confiscation of an object with a blood trace on it is not possible, because the pattern and characteristics will be lost.

This experiment was repeated, but now it was treated with a 2,5%-5-sulfosalicylic acid in an 40% ethanol/water solution, before it was taken out of the freezer. The result of this experiment can be seen in figure 17. As can be seen on the picture, the blood did not run out, with the preservation of the traces and characteristics as a result. This means that fixation with the 40% ethanol in the solution works and that the traces can be fixed on the crime scene itself with the application of 40% ethanol in the solutions.

Optimal conditions of the rinsing solution

Since the 0,2% of acetic acid in a water solution of the rinsing solution is not enough to decrease the freezing temperature of this solution extremely, the post-treatment solution had to be modified with 40% ethanol as well to figure out if this adjustment was able to remove only the excess dyes. Results showed that this adjustment was enough to remove the excess dye and leave the fixed and enhanced prints behind (figure 19). This means that the rinsing step is also possible at temperatures below zero degrees Celsius.

Optimal conditions of applying blood

To optimize the condition of applying the blood on the surfaces by stamping with a partial shoe print, two ways were tested; the rolling motion and the entire surface perpendicular to the tiles. The results are shown in figure 18a-b, where 18a is the perpendicular stamp and 18b

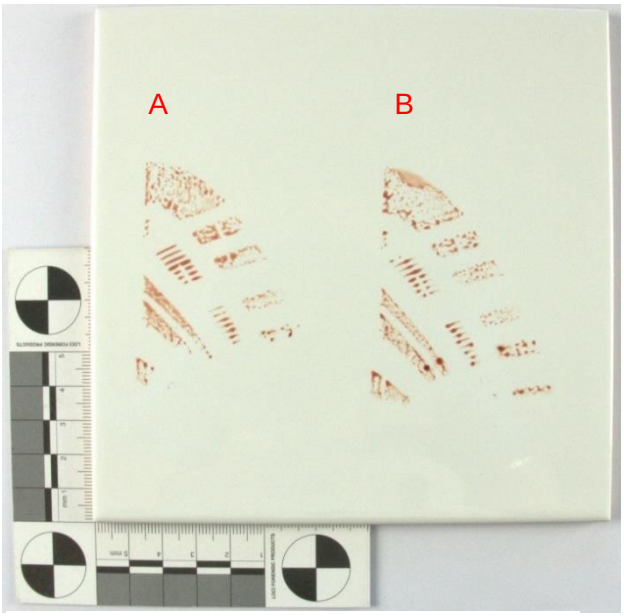


Figure 18: Two ways of applying the stamps; A) Perpendicular, B) Rolling motion

is the rolling motion. As can be seen on picture 18b, at the top of the stamps a fading of the blood trace can be seen. This arose when the sole was removed from the surface. As a result, the pattern of the sole is no longer visible in that part, unlike the stamps which are placed perpendicularly, see 18a. That is the reason there was chosen to use the perpendicular placing of the prints.

Besides, since the occasion of this study is a print, set with blood, on a car, it can also be the case that the print was set on the glass under a layer of ice. To cope with this issue, it was investigated if the modified VBETs still work. The results with the Hungarian Red showed that one layer of Hungarian Red was not enough to defrost the layer of ice and to fix the blood prints underneath it at the same time. So, after approximately one minute, a second layer was applied to see if it worked. With this second layer, the results showed that the prints were fixed with enough detail in them. See figure 19. It is plausible that the first layer removed the ice layer and the second layer fixed and enhanced the prints underneath it. However, this is still an assumption, which needs to be investigated further in a follow-up study.

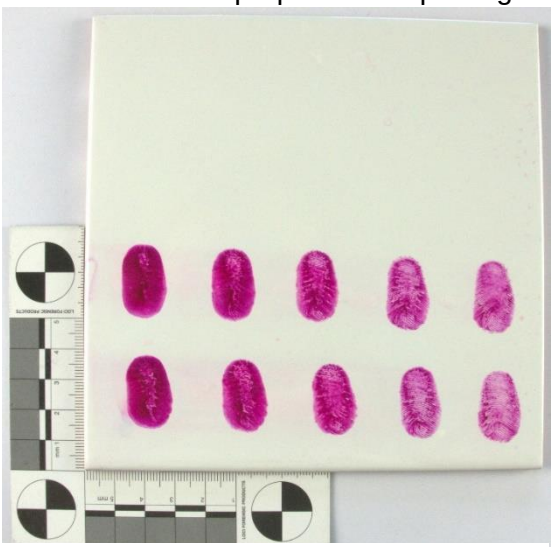


Figure 19: Result of enhanced fingerprint series with Hungarian Red, placed under a layer of ice

Comparison modified VBETs below and above zero degrees Celsius

In order to be able to compare the influence of the VBETs with the 40% ethanol/water solution compared to the waterbased VBETs on the effectiveness and applicability, they were also investigated at room temperature, otherwise it cannot be determined whether a possible result was caused by the addition of ethanol or by freezing. Results showed that the prints were just as well fixed and treated at room temperature as they were at freezing temperature, since there were no unexplainable differences visible. See figure 20a-b, where 20a is the result at room temperature and 20b is the result at minus twenty degrees Celsius. This means that the 40% ethanol addition to the techniques had no influence on the effectiveness of the results. Since this is known, more reliable statements can be made in the further experiments of this study.

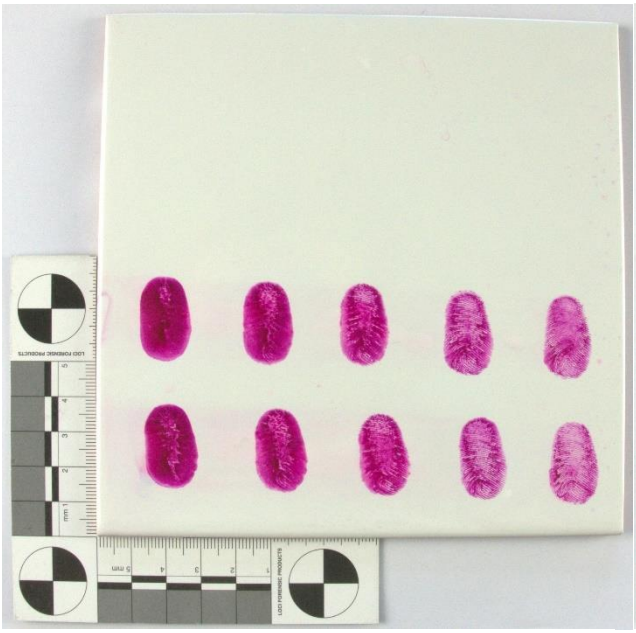


Figure 20a: Results of enhanced fingerprint series with Hungarian Red with 40% ethanol at room temperature

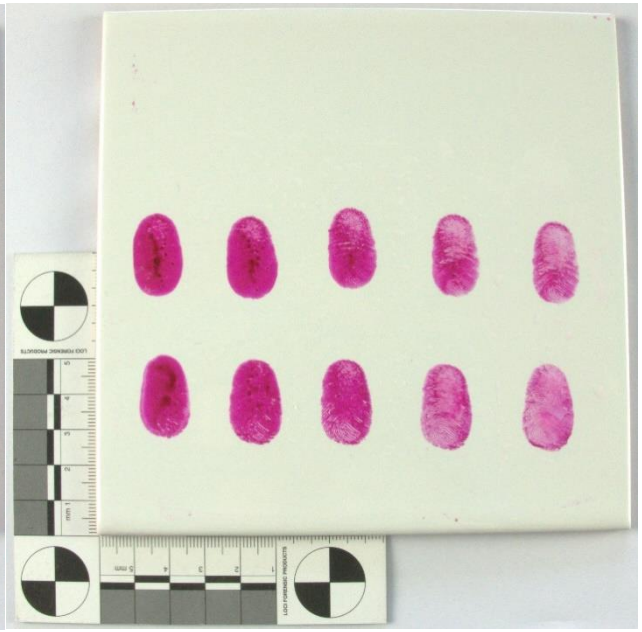


Figure 20b: Results of enhanced fingerprint series with Hungarian Red with 40% ethanol minus twenty degrees Celsius

This can be interpreted as if the VBETs with the 40% ethanol can replace the waterbased VBET, since it is applicable at temperatures below and above the zero degrees Celsius. However, when it will be applied at temperatures above the plus twenty degrees Celsius, the ethanol starts to decompose. If than a picture with a flash will be made, an explosion will occur because of its flammability. Therefore, the complete replacement of the waterbased VBET is not possible.[57]

Preliminary experiment: Dilutions

To detect what the detection limit of these modified VBETs is, a dilution series was investigated and enhanced. The dilutions used were 1/50, 1/100, 1/250, 1/500. Larger dilutions were not investigated, since that would not be realistic for practice. An example of every technique is shown in figure 21a-d.

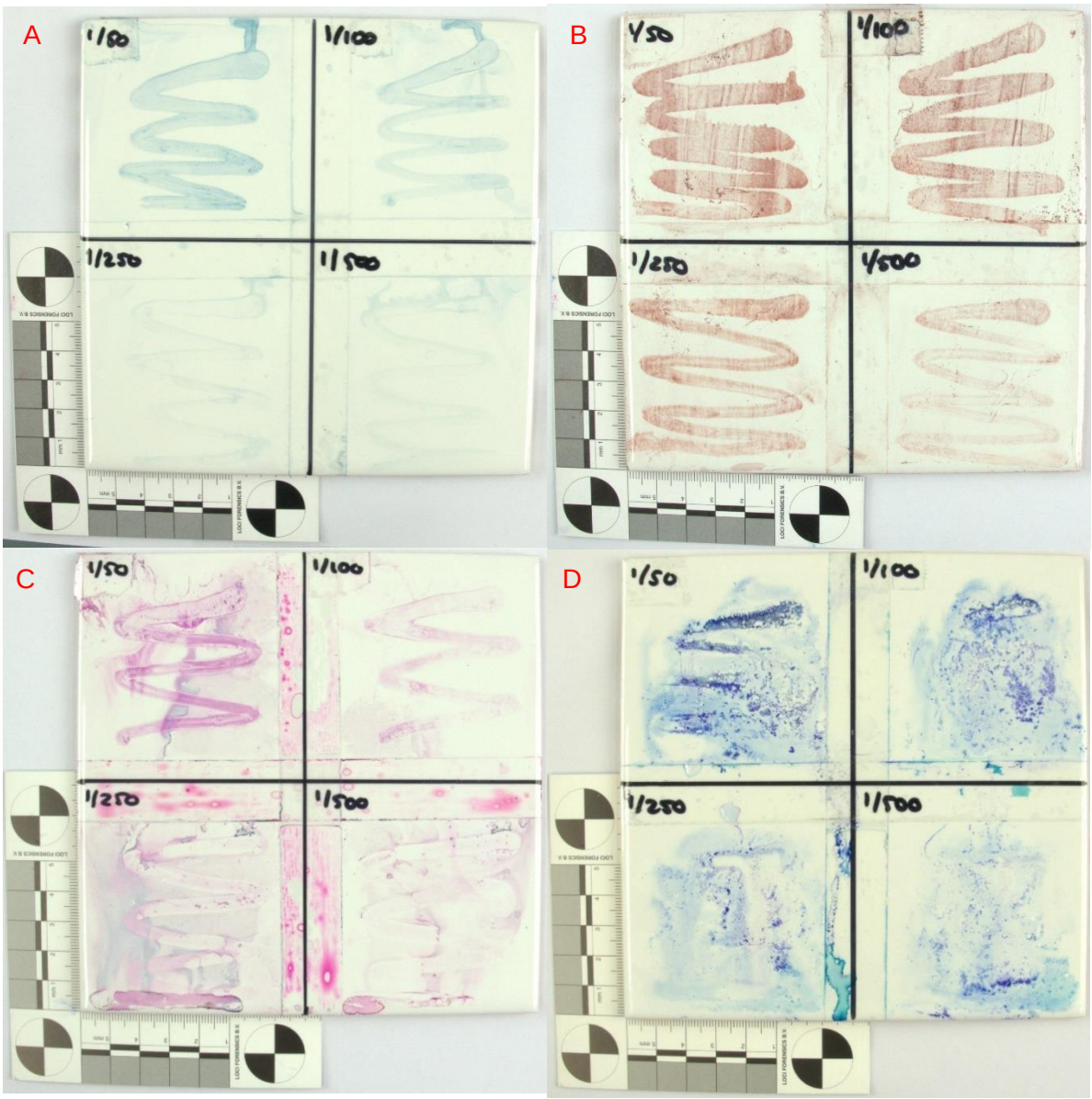


Figure 21: Results of enhanced dilution series with the modified VBETs with 40% ethanol on the white ceramic tiles; A) Amido Black; B) Iron oxide; C) Hungarian Red; D) (A)LCV

The results showed that Amido Black and Iron oxide gave remarkable results up to the highest dilution of 1/500. This corresponds to the study of A.B. Theeuwen et al.[1], which means that far diluted blood can still be enhanced with these techniques.

Reliable statements about Hungarian Red and (A)LCV on tiles could not quite be made, because (A)LCV rinsed away almost every diluted blood trace on the tiles and Hungarian Red stained a lot over the traces after it was dry. The cause of the latter is that a part of the Hungarian Red got under the adhesive tape, which could not be washed away with the rinsing solution. During drying, that unrinsed Hungarian Red came out from underneath, because liquids always want to assume its smallest shape. Subsequently, the liquid in Hungarian Red evaporated and the coloring agent remained on the applied dilutions, so that the traces were too much stained and no reliable statements could be made.The results of A.B. Theeuwen et al.[1] showed that Hungarian Red enhanced blood traces up till 16000 times diluted and (A)LCV 4000 diluted on non-porous surfaces (opaque glass). Those dilutions were not investigated, but they do show that these techniques should be very sensitive and need to be able to enhance the dilutions that were used. Presumably, when another surface or other way to divide the surface was used, better and more reliable results could be obtained about which statements could be made.

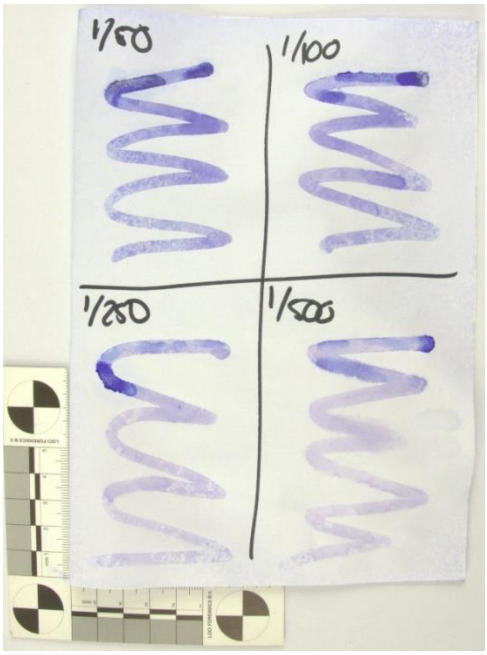


Figure 22: Results of enhanced dilution series with (A)LCV with 40% ethanol on paper

On the contrary, since (A)LCV is also applicable on porous surfaces, this was investigated as well. (A)LCV gave remarkable results on paper up to 1/500, which corresponds with A.B. Theeuwen et al.[1], where a good enhancement was seen up to a dilution of 16000 times. See figure 22. This, in combination with the observations of the results on the tiles, is why it was decided to use (A)LCV only on paper for this study.

Furthermore, it must be mentioned that the blood traces could not be applied evenly on the surfaces with the swabs. Hence why some traces are clearer and more visible at the top than at the bottom. See the 1/250 and 1/500 dilution in figure 22, for instance. However, the results still showed an enhanced result, which means that the VBETs still react with the components in the blood.

Overall discussion of the preliminary experiments

The preliminary research was focused on determining the optimal conditions of the blood, ethanol and the Visual Blood Enhancement Techniques. With those results, the main research was narrowed down in variables and obstacles were tackled. Therefore, these had to be done in advance to design a good research. Including too many variables may lead to ambiguity in results or deviant results. Also, the preset optimal conditions ensure the focus lies solely on determining to what extent the four VBETs with the 40% ethanol in the solution are applicable and yields useful and visible results with sufficient detail on prints, at temperatures below zero degrees Celsius, which is the aim of this study.

The first preliminary experiments concerned if the expectations in advance were correct. These expectations included, firstly, if unfixed and unenhanced blood indeed defrost and ran out when it was taken out of the freezer and, secondly, if the waterbased Visual Blood Enhancement

Techniques had the possibility to freeze when they were kept at temperatures below zero degrees Celsius. Results showed that the untreated blood thawed and ran out, with the loss of the pattern and characteristics as a result.[16,38] This proves that confiscation to a warmer place is not possible. Besides, the waterbased VBETs consist of a lot of water, so they were frozen and solid.[38] This results in that they were not applicable anymore. To make these techniques applicable, the base fluid had to be modified.

Subsequently, some preliminary experiments and tests were carried out to optimize the conditions for the main research. This includes the way the blood was applied, the optimum percentage ethanol, the determined percentage ethanol in the VBETs and the rinsing solution and the detection limit. Due to the outcome of the results, the best way to apply the blood was by perpendicular placing the stamps because of the number of characteristics and the 40% ethanol was chosen. This percentage was investigated to check whether it would freeze when the active VBET/rinsing substances were added. They appeared to stay liquid at minus twenty degrees Celsius. Also, the modified techniques and rinsing agent were tested in different circumstances and dilutions, which proved that the prints were generally enhanced and the excess dyes were rinsed away. Therefore, it was decided to work with the same percentage in the main research.

Main research: Depletion series treated with modified VBETs

Based on the results of the preliminary research, the main research was executed to reach the aim of this study. The enhancing abilities of the Visual Blood Enhancement Techniques with a 40% ethanol ratio were tested on depletion shoeprint series with blood on the white ceramic tiles and paper in larger sample sizes. In this paragraph, a selection of all the surfaces with the series is shown. This selection is made out of the other samples that were sent to the evaluators to assess, see Appendix 6. The samples in Appendix 6 were already a selection (ten per VBET) of all the samples that were tested during the conduction of this research. The figures of the samples included in this chapter are representative for all the obtained results during this research.

A total of 50 surfaces per VBET were used during this part of the study. These surfaces were initially washed and disinfected with ethanol and placed for 24 hours in the freezer, before the blood prints were placed. On every surface, a series of four stamps were placed perpendicular in the freezer without moistening the partial shoe sole in between. The first print had sometimes a lot of blood with the loss of some characteristics as a result. These prints were left to freeze, after which the VBETs with 40% ethanol and the rinsing solution were applied. Results of the enhanced prints show that for all the VBETs a good contrast and the pattern and characteristics can be observed unlike the unenhanced prints. The largest difference in contrast and number of characteristics can be observed between the bottom left and right prints on the surfaces.

The prints before enhancement were photographed to compare them with the same prints after enhancement. Of each VBET a result of the before and after treatment is shown in figure 23a-d.

Figure 23: Example of the comparisons between the unenhanced blood prints (left) and the enhanced blood print series (right) with the four Visual Blood Enhancement Techniques: A) Amido Black; B) Hungarian Red; C) (A)LCV; D) Iron oxide > Appendix 6

These results can be substantiated with the data obtained from the grading with a score from 0-4 by the sixteen evaluators, based on the amount of characteristics[29]. Since each print in a series had another amount of blood, they were graded separately. In this way, a comparison could be made by the same print positions between different surfaces.

The grades from the evaluators were inverted in Excel and the frequencies of how many times a score was granted, were determined. The frequencies of the results in figure 23a-d are shown in table 5. The complete data set of all the assessed surfaces are presented in Appendix 6 and 8.

Table 5: Frequency of granted scores for the prints in figure 23a-d; AB = Amido Black; HR = Hungarian Red; (A)LCV = (A)LCV; IO = Iron oxide. The bold numbers show the most common score for that specific print.

		Data shoe print grading (n = 16)		*Frequency table (number of times the score is granted per shoeprint)*			
VBET	Before/after	Position	Score 0	Score 1	Score 2	Score 3	Score 4
AB	Before	Upper left	0	4	**9**	1	2
AB	Before	Upper right	1	**11**	3	1	0
AB	Before	Bottom left	**8**	**8**	0	0	0
AB	Before	Bottom right	**10**	6	0	0	0
AB	After	Upper left	1	3	**6**	5	1
AB	After	Upper right	0	2	**7**	6	1
AB	After	Bottom left	0	5	**9**	1	1
AB	After	Bottom right	1	**7**	6	1	1
HR	Before	Upper left	0	2	**11**	3	0
HR	Before	Upper right	1	**13**	2	0	0
HR	Before	Bottom left	5	**11**	0	0	0
HR	Before	Bottom right	**9**	7	0	0	0
HR	After	Upper left	0	1	**11**	3	1
HR	After	Upper right	0	4	**9**	3	0
HR	After	Bottom left	0	**10**	6	0	0
HR	After	Bottom right	0	**11**	5	0	0
(A)LCV	Before	Upper left	0	1	**11**	2	2
(A)LCV	Before	Upper right	0	2	**11**	1	2
(A)LCV	Before	Bottom left	0	**10**	5	1	0
(A)LCV	Before	Bottom right	1	**12**	3	0	0
(A)LCV	After	Upper left	0	0	4	**10**	2
(A)LCV	After	Upper right	0	1	**10**	2	3
(A)LCV	After	Bottom left	0	4	**10**	1	1
(A)LCV	After	Bottom right	0	7	**8**	0	1
IO	Before	Upper left	0	**6**	**6**	4	0
IO	Before	Upper right	0	**10**	5	1	0
IO	Before	Bottom left	0	**12**	4	0	0
IO	Before	Bottom right	1	**10**	5	0	0
IO	After	Upper left	0	4	**5**	**5**	2
IO	After	Upper right	0	5	**8**	2	1
IO	After	Bottom left	0	2	**9**	4	1
IO	After	Bottom right	0	2	**11**	2	1

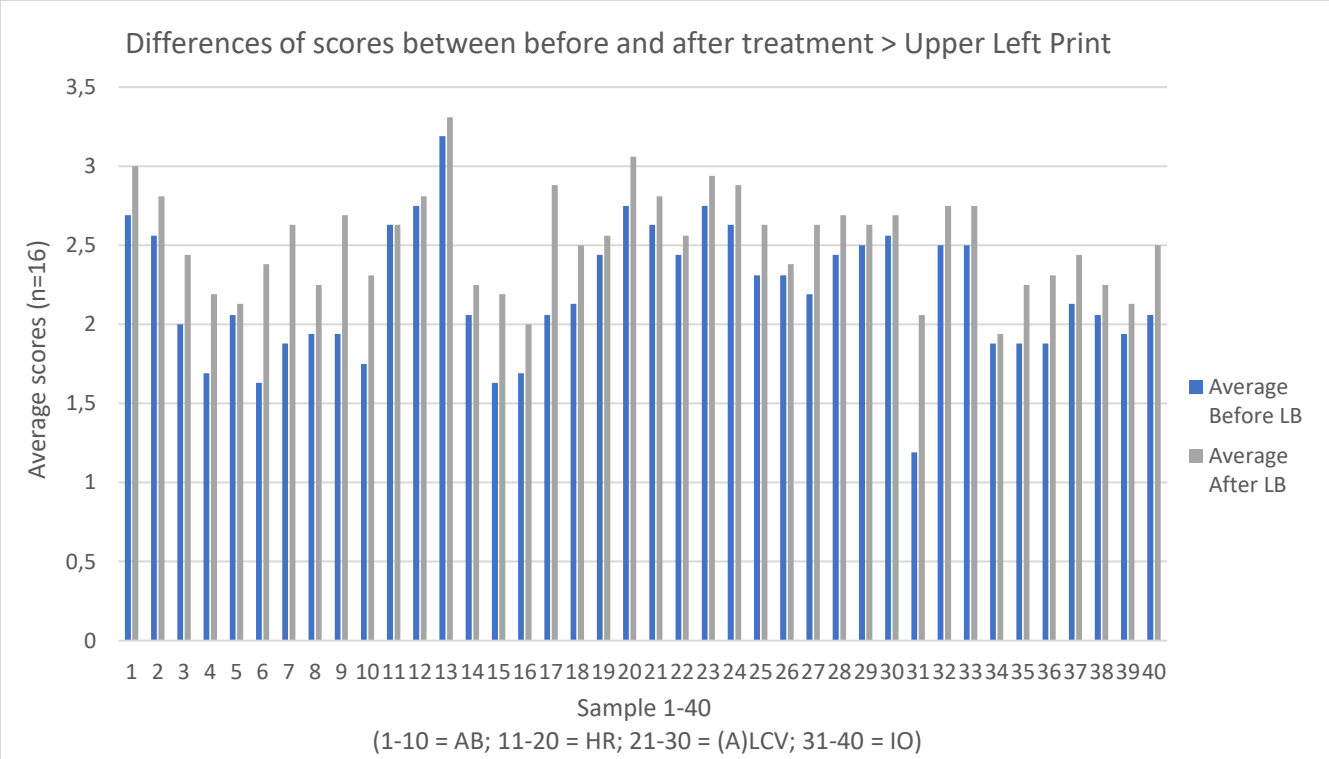
The upper left prints for all the VBETs in table 5 are mostly graded with a score 2 or 3, both before as after enhancement. This indicates that the prints are recognizable, with 1/3 to 2/3 of the characteristics is visible and had adequate contrast.[29] The upper right prints before enhancement were mostly graded with a 1, which means that the prints are recognizable, but less than 1/3 of the prints contains the characteristics and/or the contrast is poor.[29] The same prints post enhancement showed mostly a score 2. The bottom left prints prior enhancement showed mainly a score 1, while after enhancement the prints were most graded with a 2, except for Hungarian Red, where the main score of 1 remained. However, all the scores 0 (n = 5) were gone and now

there were more scores 2 (n = 6). So, a positive shift was made. Lastly, the bottom right prints were prior enhancement mainly scored with a 0 or 1, which indicates no development or the prints were recognizable, but less than 1/3 of the characteristics were visible.[29] Post enhancement, the main scores were a 1 or 2. According to these results, it appears to be an improvement between the original prints and the enhanced prints. To determine whether there is a significant difference between the original and the enhanced prints, a paired t-test was performed.[58] This allows possible negative, positive or neutral effects of the enhanced prints to be statistically detected.

Main research: Scoring the results

A total of 40 depletion series before and 40 series after treatment were graded by a group of sixteen evaluators. Each series consist of four partial shoeprints and they were individually graded with a score from 0 to 4[29], based on the amount of manually applied characteristics. Of every print, the frequencies of the granted scores were determined, after which the averages of the scores were calculated, Appendix 8.[58] Also, these averages are shown in graphs 1-4, where the before treatment prints are the blue stacks and the after-treatment prints are the grey stacks. The abbreviations LB, RB, LO and RO correspond to the positions of the prints on the surfaces:

LB = print upper left
RB = print upper right
LO = print bottom left
RO = print bottom right

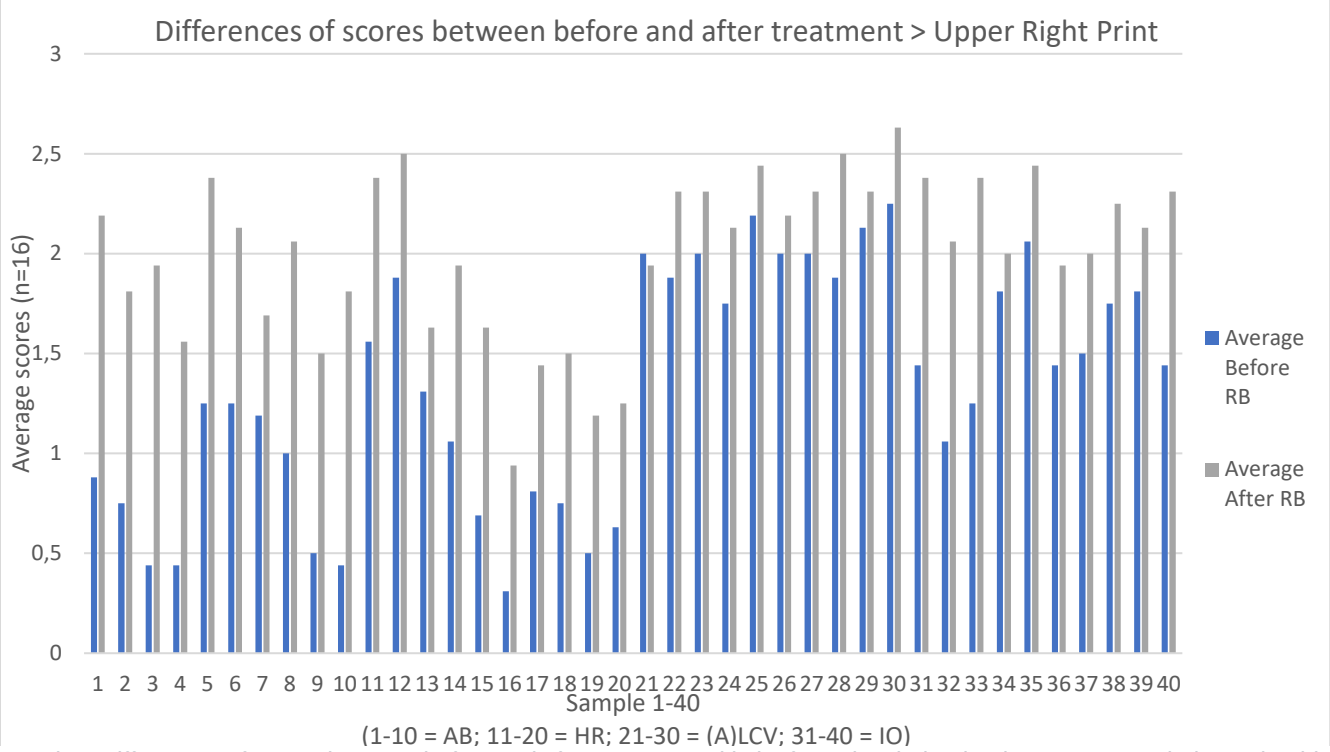


Graph 1: Differences of scores between before and after treatment with the four Visual Blood Enhancement Techniques (Amido Black, Hungarian Red, (A)LCV and Iron oxide) for the upper left prints > Appendix 8, Table 8,9,10,11

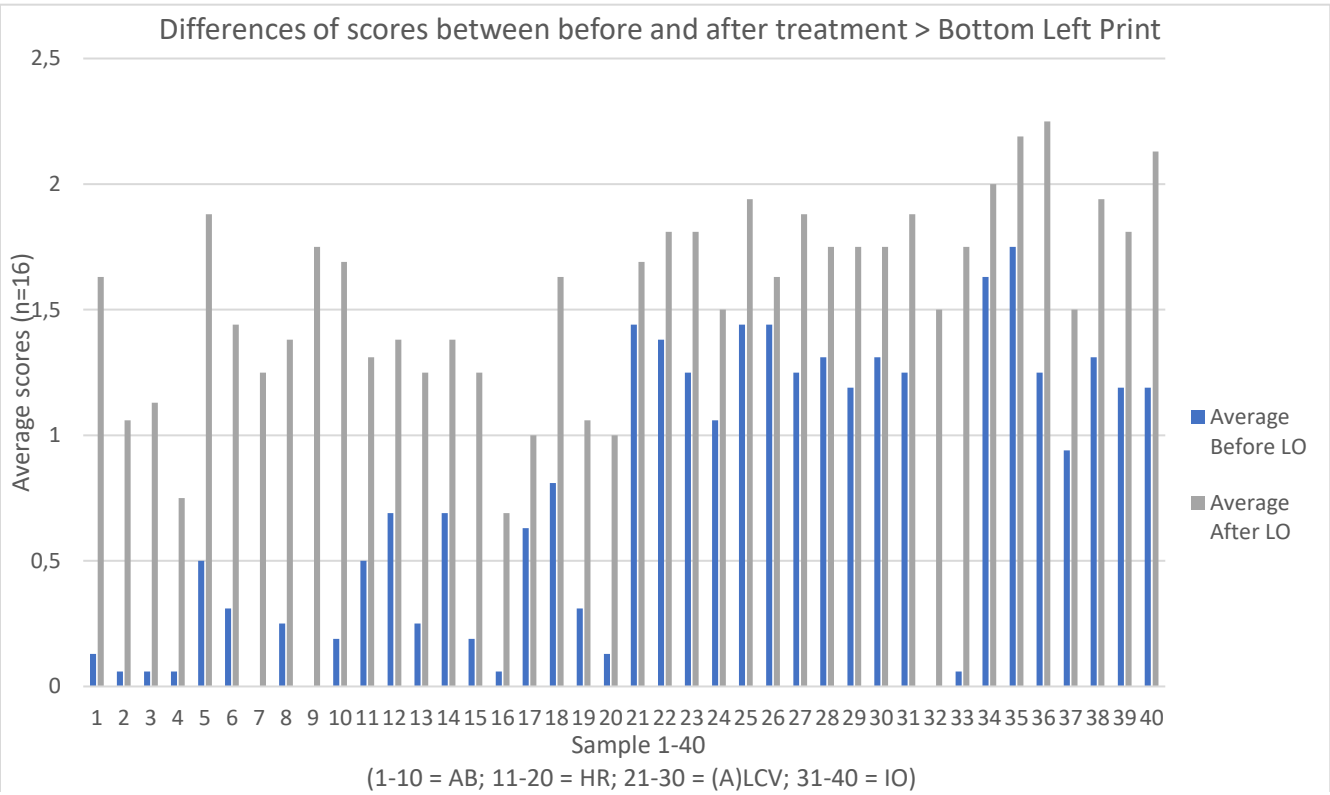
The graph (1) for the upper left prints of the depletion series show before enhancement average scores ranging from 1,19 to 3,19. On the contrary, the range of average scores after treatment for

the same prints vary between 1,94 to 3,31. This shows an improvement in the average scores after enhancement.

The graph (2) for the upper right prints of the depletion series show before enhancement average scores ranging from 0,31 to 2,25. On the contrary, the range of average scores after treatment for the same prints vary between 0,94 to 2,63. Again, an improvement in the average scores after enhancement can be seen.



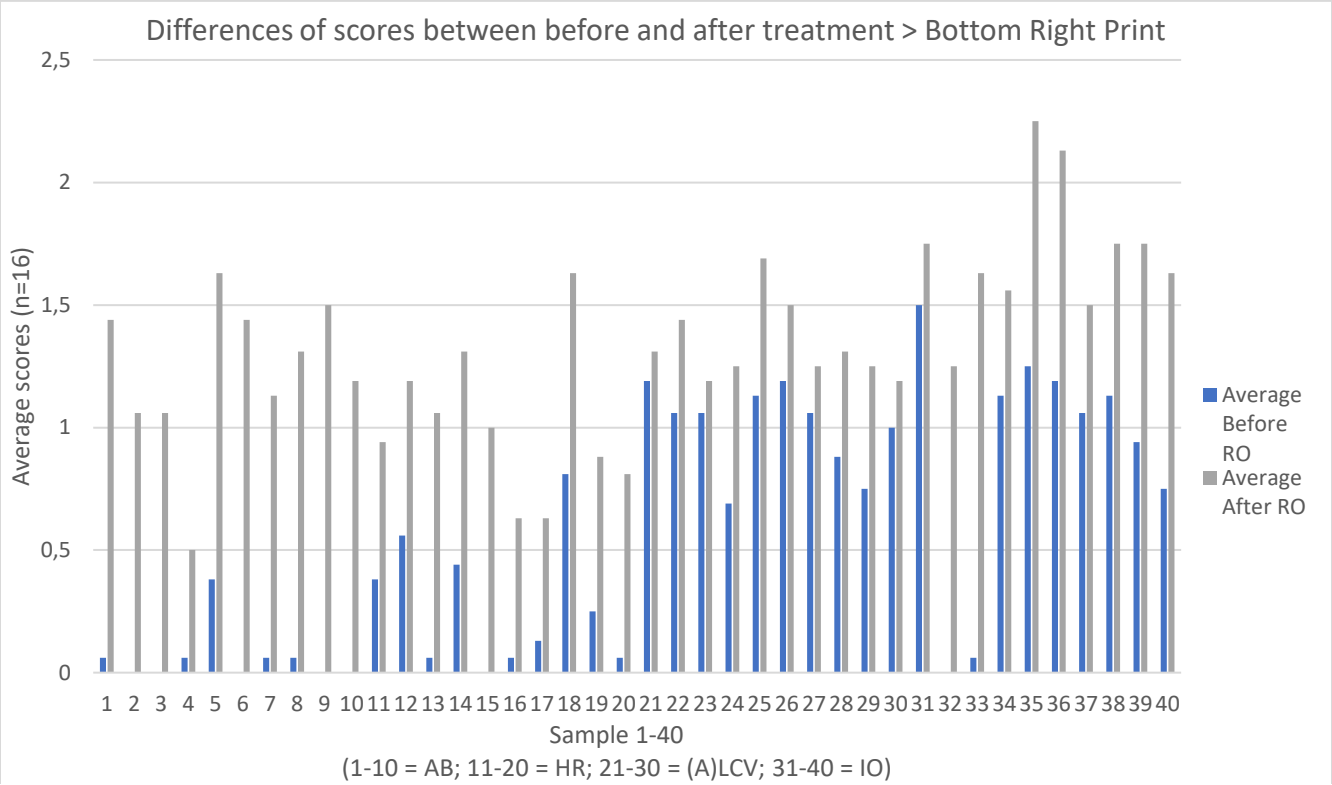
Graph 2: Differences of scores between before and after treatment with the four Visual Blood Enhancement Techniques (Amido Black, Hungarian Red, (A)LV and Iron oxide) for the upper right prints > Appendix 8, Table 13,14,15,16



Graph 3: Differences of scores between before and after treatment with the four Visual Blood Enhancement Techniques (Amido Black, Hungarian Red, (A)LCV and Iron oxide) for the bottom left prints > Appendix 8, Table 18,19,20,21

The graph (3) for the bottom left prints of the depletion series show before enhancement average scores ranging from 0 to 1,75. On the contrary, the range of average scores after enhancement for the same prints vary between 0,69 to 2,25. Also, this shows an improvement in the average scores after enhancement.

The graph (4) for the bottom right prints of the depletion series show before enhancement average scores ranging from 0 to 1,50. On the contrary, the range of average scores after treatment for the same prints vary between 0,50 to 2,25. Again, this shows an improvement in the average scores after enhancement.



Graph 4: Differences of scores between before and after treatment with the four Visual Blood Enhancement Techniques (Amido Black, Hungarian Red, (A)LCV and Iron oxide) for the bottom right prints > Appendix 8, Table 23,24,25,26

For all these prints in the series, an indication of a positive effect after enhancement is shown, when it is compared to the original, untreated prints. Especially, the before results of the upper right, bottom left en bottom right show that enhancing the prints is necessary to make characteristics (more) visible for identification and individualisation, because scores below 1 mean no development or some development with less than 1/3 of the characteristics visible. The upper left prints were generally already visible with good detail, since it was the first print with the most blood on it to transfer. Therefore, the enhancing was relatively less than the enhancement of the follow-up prints. Furthermore, the graphs show no significant difference between the four different techniques, since the peak hights are generally around the same hight, except for a few prints.

To determine whether there is a significant difference between the original untreated shoeprint series and the enhanced prints, a paired t-test was performed (Appendix 8)[58]. This allows statistically possible negative, positive of neutral effects of the enhanced prints in contrary to the original ones to be detected. This was done for every Visual Blood Enhancement Technique individually. The results of the paired t-test are shown in table 6. For this calculation, the

determined averages of every single print were used. According to the paired t-test values towards the Critical Value, significant difference can be found between the two groups. These results can be interpreted as that the Visual Blood Enhancement Techniques with 40% ethanol have a positive effect on the visualisation of the partial shoeprints, set with blood, in a depletion series.

Table 6: Paired t-test between the averages scores of the original partial shoe print versus the enhances prints with the four Visual Blood Enhancement Techniques individually, where the data was derived from the grading using the modified CAST-score system [29]. Paired t-test values > critical value shows that there is a significant difference between the average scores of group 1 and group 2. Confidence interval was 95% (alpha = 0,05 and n = 16) [58] > Table 12,17,22,27 Appendix 8

Group 1	Group 2	Paired t-test value	Critical Value[58]
Original print upper left	Amido Black	6,2629	2,262
Original print upper left	Hungarian Red	3,2515	2,262
Original print upper left	(A)LCV	5,9359	2,262
Original print upper left	Iron oxide	4,4255	2,262
Original print upper right	Amido Black	12,3986	2,262
Original print upper right	Hungarian Red	12,5285	2,262
Original print upper right	(A)LCV	5,2593	2,262
Original print upper right	Iron oxide	6,194	2,262
Original print bottom left	Amido Black	12,9264	2,262
Original print bottom left	Hungarian Red	12,4427	2,262
Original print bottom left	(A)LCV	10,3057	2,262
Original print bottom left	Iron oxide	5,9429	2,262
Original print bottom right	Amido Black	12,3012	2,262
Original print bottom right	Hungarian Red	12,6705	2,262
Original print bottom right	(A)LCV	6,0623	2,262
Original print bottom right	Iron oxide	6,4318	2,262

However, for this part of the study a sample size of 50 per Visual Blood Enhancement Technique was used, but a selection of ten samples was assessed by the evaluators. If this research will be repeated for validation and reliability, a greater sample size must be used and evaluated. After conducting a post-hoc power analysis, the calculated and desired sample size turned out to be 88 samples to achieve a power of 95% and a 5% error probability. The 50 samples per VBET showed a power of 79,89% and a 20,11% error probability, if they were all assessed. (Appendix 7) But, this was not done, because it was not feasible for the evaluators and for processing the results. However, based on the results that were visually perceived, it can be said with a 79,89% chance that the results are genuinely better visible after enhancement than before enhancement.

Main research: Validation using a statistical test

To determine if there is an overall agreement between the sixteen evaluators, an Intraclass Correlation Coefficient (ICC) test was executed with SPSS.[49,50,58] A high agreement ensures a highly reliability of the obtained results with a 95% confidence interval. The method used for calculating the ICC was a two-way random intraclass correlation with absolute agreement for multiple evaluators. The ICC was conducted for both before enhancement and after enhancement. Besides the scores for the upper left, upper right, bottom left and bottom right were calculated separately with an ICC-test. Results of the ICC-test are shown in table 7.

Table 7: Output of the SPSS reliability analysis based on the data, supplied by the evaluators (n=16). The table contains the corresponding two-way random intraclass correlation coefficient with absolute agreement for multiple evaluators, for single and average measures, with a 95% confidence interval. (Appendix 9)

Intraclass Correlation Coefficient			
Before enhancement – Upper left (figure 30)			
	Intraclass correlation	95% Confidence Interval	
		Lower bound	Upper bound
Single measures	0,195	0,112	0,316
Average measures	0,795	0,670	0,881
Before enhancement – Upper right (figure 31)			
	Intraclass correlation	95% Confidence Interval	
		Lower bound	Upper bound
Single measures	0,469	0,328	0,620
Average measures	0,934	0,887	0,963
Before enhancement – Bottom left (figure 32)			
	Intraclass correlation	95% Confidence Interval	
		Lower bound	Upper bound
Single measures	0,589	0,461	0,718
Average measures	0,958	0,932	0,976
Before enhancement – Bottom right (figure 33)			
	Intraclass correlation	95% Confidence Interval	
		Lower bound	Upper bound
Single measures	0,610	0,489	0,732
Average measures	0,962	0,939	0,978
After enhancement – Upper left (figure 34)			
	Intraclass correlation	95% Confidence Interval	
		Lower bound	Upper bound
Single measures	0,105	0,053	0,190
Average measures	0,653	0,473	0,790
After enhancement – Upper right (figure 35)			
	Intraclass correlation	95% Confidence Interval	
		Lower bound	Upper bound
Single measures	0,180	0,102	0,296
Average measures	0,778	0,644	0,871
After enhancement – Bottom left (figure 36)			
	Intraclass correlation	95% Confidence Interval	
		Lower bound	Upper bound
Single measures	0,215	0,124	0,343
Average measures	0,814	0,693	0,893
After enhancement – Bottom right (figure 37)			
	Intraclass correlation	95% Confidence Interval	
		Lower bound	Upper bound
Single measures	0,240	0,144	0,374
Average measures	0,835	0,728	0,905

The single measures obtained an ICC-value that varies between the 0,105 and the 0,610. Single measures indicate the comparison of one measure to another measure. These scores indicate a variation between no correlation to medium correlation between these values. In other words, no absolute agreement to medium absolute agreement. However, this is explainable, based on the feedback and comments of the evaluators. Some evaluators mentioned that it was hard to choose

between two scores, like a score 0 of 1. This was based on two reasons. First, there were 7 characteristics made in the shoe sole, which is a number that was not possible to evenly divide between the scores. Therefore, when some counted the number of characteristics visible and converted that to a score in the system, it was sometimes hard to choose whether to go for a lower of higher score. That is where the human judgment comes in, which is subjective. Secondly, it was sometimes hard to determine whether there were partially no signs of a print at all or just no development. That made the choice between two scores hard, as well.
However, the ICC-value that is important to determine the reliability between the evaluators is that of the average measures. There was found an ICC-value that varies between the 0,653 and the 0,962. According to the division of how strong the correlation is[59], the scores indicate a medium to extremely high/strong correlation. This means that there is a good absolute agreement between the evaluators. Since the evaluators agree with each other in a good agreement, it can be interpreted that the high correlation ensures a high reliability of the data that was obtained from the evaluators.

Overall discussion of the main experiments

In the main research, the difference between the blood prints before and after enhancement with the Visual Blood Enhancement Techniques, where the 40% ethanol was added to, were tested in larger sample sizes. Therefore, depletion series of four print per series were perpendicular placed on the ceramic tiles/paper by the same person with approximately the same pressure. There were four VBETs used in this research with 50 surfaces per VBET. This means 200 prints per VBET. The large sample sizes higher the reliability of this research. Although the post-hoc power analysis showed that 88 surfaces per VBET was desired. (Appendix 7) Besides, the consistency of the obtained results is high and there were no deviant results found within the series or variables that might lower the reliability and reproducibility.[42] The reason is that a lot of constant factors were considered and the VBETs used were already validated, except for the ethanol in the solutions. However, it was not feasible for the evaluators and for processing the results to assess all the 50 surfaces per VBETs. Therefore, a selection was made of ten surfaces per VBET. This selection was based on the highest quality of the prints, so the largest contrast, the most characteristics and no disruptive factors visible, although they were representative for the rest.

The positive control involved the 50 series placed with blood for each VBET. Negative controls were considered, but were believed to not be of any value to this research. However, since the prints were placed on clean surfaces and were photographed before enhancement, this was regarded as a negative control.

Statistically, significant differences with a 95% CI[58] were found between the scores of the original shoeprints and the enhanced ones for all the VBETs and for all the prints within the series, obtained from the sixteen evaluators. Consistency was obtained by letting the same evaluators score all the selected prints. This 95% CI indicates that the data is accurate.[58] Besides, the averages of the unenhanced prints were (much) lower than the averages of the same prints, but post enhancement. This includes for all the VBETs. Therefore, it is considered that the four VBETs have a positive effect on the frozen blood prints. However, it had to be investigated whether the evaluators agreed with each other, to check if a comparison could be made between prior and post enhancement. Therefore, a Intraclass Correlation Coefficient (ICC = 0.653-0.962) was calculated, which showed a good absolute agreement with a 95% confidence interval.[49,50,58,59] This indicates a high inter-rater reliability. So, it can be stated that the Visual Blood Enhancement Techniques with 40% ethanol are able to enhance the blood prints at temperatures below zero degrees Celsius with a significant difference towards the original blood prints. Therefore, this modification to the solutions is proven to be a good method for those temperature circumstances.

IX Conclusion

The case presented in the introduction, in which a car was found on the crime scene with a blood trace, at temperatures below zero degrees Celsius, is an example of a case that can be solved through the findings of this study. Up until this day, there is no known method for enhancing blood traces that are frozen, despite their high forensic relevance. The Visual Blood Enhancement Techniques (Amido Black, Hungarian Red, (A)LCV and Iron oxide including 2,5%-5-sulfosalicylic acid) with 40% ethanol in the solutions provides a method that can fix and enhance the blood prints.

The preliminary tests showed that the determined 40% ethanol in all the four visual blood enhancement techniques did not freeze at minus twenty degrees Celsius and they provided great fixed and enhanced prints at both temperatures below and above zero degrees Celsius. These enhancements provided a high number of visible characteristics and contrast between the surface and the prints. Also, the detection limit was investigated. Amido Black and Iron oxide showed great results on the tiles up to the highest dilution factor of 1/500 times. On the contrary, the tiles were a disruptive factor for Hungarian Red and (A)LCV, so no statements could be made. However, the application of (A)LCV on blood dilutions on paper showed excellent results up to 1/500 times diluted, as well. In the main research, depletion series with undiluted blood on the surfaces were placed. These series were compared between before and after enhancement and showed significant improvement. Also, based on the partial shoeprint grading data, provided by the sixteen evaluators, the enhanced prints yields higher averaged scores than the same prints before treatment. Furthermore, statistical analysis showed that there was a positively significant difference between the prints prior enhancement and post enhancement for all the prints and all the Visual Blood Enhancement Techniques.

In conclusion, the main question of the research can be answered. The main question is: ‘To what extent are four different Visual Blood Enhancement Techniques with an ethanol/water solution applicable and yields useful and visible results with sufficient detail on prints, set with blood, at temperatures below zero degrees Celsius?’. This study found that 40% ethanol in the solutions of the Visual Blood Enhancement Techniques is suitable to apply on frozen blood traces in environments with temperatures below zero degrees Celsius without any thawing and running out. Besides, these modified solutions showed that the blood prints were excellent fixed and enhanced after the excess dyes were rinsed away with the modified rinsing solution. So, the Visual Blood Enhancement Techniques are applicable, when 40% ethanol is added in the solutions, on frozen blood prints that are found on crime scenes with temperatures below zero degrees Celsius and they yields useful and visible results with sufficient detail for follow-up studies.

X Recommendations

However, the findings of this study regarding the modification of the Visual Blood Enhancement Techniques with 40% ethanol in the solutions to be able to apply and yields improved and visible results of blood prints at temperatures below zero degrees Celsius, looks very promising, they were mostly based on pilot experiments. As mentioned, to execute this research, it was important to start at the base, since no publications are known in the scientific literature about this problem. This means that the variables had to be very minimal. So, the experiments in this study showed great results, but it is unknown if that is still the case whether other variables are taken into account. Therefore, it is recommended to perform the same experiments (Appendix 5) in larger sample sizes and with other variables, like different materials and colours of surfaces and different temperatures to gain more insight. Besides, it is not possible to recommend which VBVT of these four techniques is preferred, based on the results, as that depends very much on several factors, like the colour, the material and the form of the surface and which type of print is present. If there is a red ceramic tile with a blood trace on it, it is not desirable to choose Hungarian Red, as the contrast is very low, in contrast to a white tile. But if there is a red curved surface with a bloody fingerprint on it, that needs to be lifted, then that is a reason to choose for Hungarian Red or Iron Oxide. But because Iron Oxide is a suspension agent, and thus forms a layer over the trace, it will also do the same on 'normal' finger tracks, See figure 24. In that case, it is not desirable to use Iron oxide on surfaces where many fingerprints are expected. So, the choice of which VBVT is preferable to use, depends on the circumstances. The only recommendation that can be made is to use (A)LCV on porous substrates.

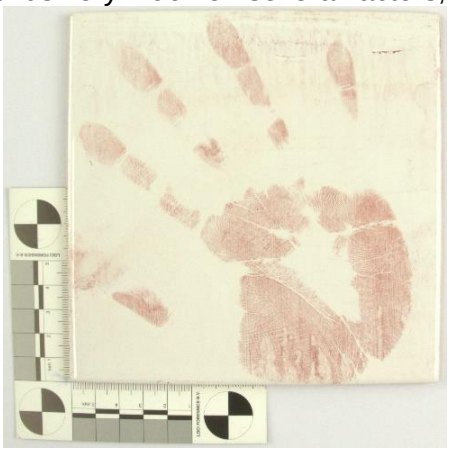


Figure 24: Handprint, without blood, treated with Iron Oxide by minus twenty degrees Celsius

Furthermore, for this study, fresh cows blood with 3% EDTA was used. For this type of blood was chosen, since it had the same components as human blood and it is much safer to work with. However, on crime scenes, the chance of finding human blood is much bigger. Therefore, it is recommended to conduct these experiments when using human blood of different donors. In this way, it can also be investigated whether some slight differences in blood between individuals have an influence on the reaction with the Visual Blood Enhancement Techniques with 40% ethanol and the results. Besides, it is unknown whether different antiquities of blood and different quantities of blood have an influence on the results. So, it is recommended to investigate it, as well, in a follow-up study to optimize the method.

Another recommendation is to investigate what the influence of the added EDTA is on the reaction between the VBETs and the blood. To gain insight about this, these experiments should be conducted with and without added EDTA.

Also, since ethanol is added to the solution, it should be investigated whether it has an influence on the DNA in the blood samples. Therefore, it is recommended to conduct experiments where enhanced blood with the 40% ethanol VBETs, is isolated and quantified for DNA-research, although ethanol is used in the isolation process for DNA-research.[55,57]

Besides, since VBETs are also used to enhance fingerprints, it should be investigated to what extent these prints can be enhanced with the modified VBETs and if they contain enough typica for identification. For this study, different donors can be used to place their fingerprints. After

enhancement, their fingerprints can be compared with their references by a dactyloscopic expert to see if an identification is possible.

In addition, as mentioned earlier, forensic investigators use the fixation step separately, prior to the enhancement technique. Therefore, it is recommended to investigate what the influence is of using a fixative with 40% ethanol and the VBETs with 40% separately on frozen blood prints. Besides, it can also be investigated what the influence is of using a fixative with 40% ethanol and then the water based VBETs. In this way, the most useful protocol can be made up.

Moreover, since there could not be made any statements of the dilution experiment with Hungarian Red and (A)LCV on the non-porous surface, it is recommended to repeat this experiment for these techniques on another non-porous surface or with another way of marking the surface. In that way, statements could be made about the detection limit for these two techniques on non-porous surfaces at temperatures below zero degrees Celsius.

Another recommendation is to investigate if the assumption of the two-layer application on blood prints underneath an ice-layer is correct. So, if it is the case that the first dye-layer removes the ice layer and the second dye-layer fixes and enhances the blood prints. This will also help to make up a good protocol for using these VBETs with 40% ethanol at temperatures below zero degrees Celsius.

Forensic Light Source and Lifting abilities

Lastly, as mentioned in the research design, it was investigated whether application of 40% ethanol to the Hungarian Red technique did not influence the ability of fluoresce under green light of 515-560 nm with a red goggle. The result is shown in figure 25. As can be seen, Hungarian Red with the 40% ethanol could still fluoresce under green light and it showed great detail in comparison with the print on the left side of the image. However, since this was only tested in a pilot experiment, it can only be concluded that the application of the 40% ethanol has a potential to fluoresce under green light with a red goggle. This needs further investigation in a follow-up study, which is not included in this one.

Besides, the ability of lifting the enhanced prints with Hungarian Red and Iron oxide was also tested. As can be seen in figure 26, where an example of lifted Hungarian Red from the tiles is shown, is it still possible to lift the prints from the surface with a white gelatin foil. This also applies to the prints that were enhanced with Iron oxide. But since the quality of the gelatin foils were insufficient, no hard statements can be made. However, the application of the 40% ethanol in the solution seemed to have no influence on the ability to lift the prints. This needs further investigation in a follow-up study, as well.

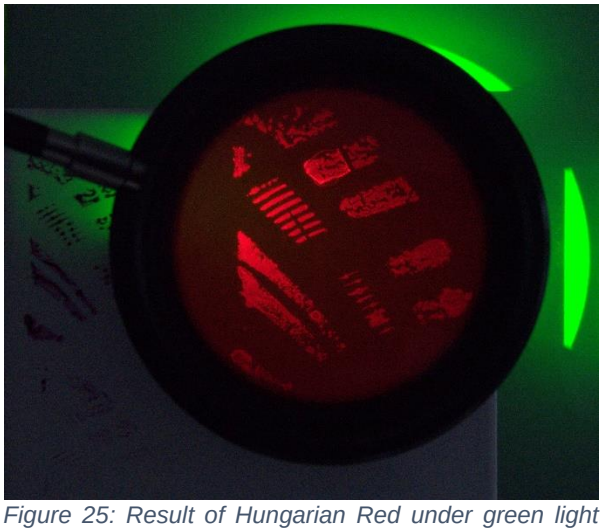


Figure 25: Result of Hungarian Red under green light of 515-560 nm with a red filter

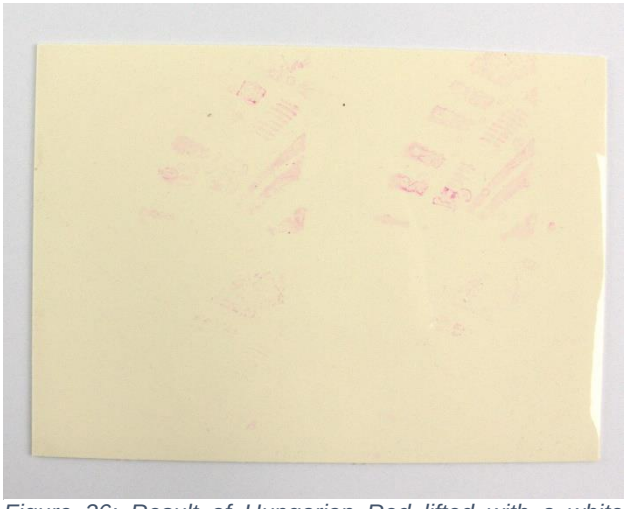


Figure 26: Result of Hungarian Red lifted with a white gelatin foil

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XII Appendices

Appendix 1: List of Materials

Table 8: Overview of where the materials and chemicals come from

- Scientific literature
- FO-guideline 02.70
- Protective gear
  - Gloves
  - Facemask
  - White suit
  - Hairnet
  - Safety goggles
- Sterile workplace
- Cow's blood with 3% EDTA
- Container for blood
- Surfaces
  - White ceramic tiles
  - White paper 250 g/m^2 (Mondi Color Copy)
- Swabs
- Permanent marker
- Adhesive tape
- Pen and paper for notes
- Amido Black with internal fixative of 2.5% solution of 5-sulfosalicylic acid in water
- (A)LCV with internal fixative of 2.5% solution of 5-sulfosalicylic acid in water
- 3%-hydrogen peroxide
- Hungarian Red with internal fixative of 2.5% solution of 5-sulfosalicylic acid in water
- Iron oxide with internal fixative of 2.5% solution of 5-sulfosalicylic acid in water
- Substances for (A)LCV
  - Sodium acetate 1 gr
  - Leucocrystalviolet
- Amido Black powder
- Hungarian Red powder
- Iron oxide powder
- Bioethanol ≥96%
- Demineralised water
- Measuring cups/Erlenmeyer flasks
- Centrifuge tubes of 50 mL
- Micro sprayers
- Acetic acid
- Drip container with drainer for chemicals
- Freezer with minus twenty degrees Celsius
- Partial shoe with sole
- Studio lights
- Scalpel
- Pipet of 2 millilitre
- Tork paper
- Camera with macro lens + tripod
- Forensische Lightsource
- Timer
- Red filter/Red goggles of 600 nm
- Dark room
- White fingerprint film
- Forensics students and investigators as evaluators
- Assessment forms (appendix 6)

	Manufacturer	**Cat. No.**	**Country**	**Comments**
Amido Black	Sigma	N3393-25G	Germany	-
Hungarian Red (Acid Fuchsin)	Sigma	F8129-25G	Germany	-
(A)LCV	Roth	7703.2	Germany	≥98%
Iron(III)oxide powder	Sigma	33,005-0	Germany	≥99% ≤ 5 micron
Demineralized water	Pearlpaint	-	The Netherlands	Bought at the Praxis
Bioethanol 95%	ESPAR	6095-40-01	The Netherlands	Bought at the Action
5-sulfosalycic acid	Sigma	S2130-500G	Germany	≥99%
Acetic acid	Sigma	A6283	Germany	≥99%
Hydrogen peroxide	Roth	7641.1	Germany	≥35%
White fingerprint film	BVDA	B-14000	The Netherlands	13x18 cm
Camera	Sony	DSC-RX10M3	Japan	-
Lens	Zeiss	RX10III	Germany	-
Tripod	Manfrotto	055	Italy	-
Forensic Lightsource	Lumatec	Superlite 400	Germany	-
Studio Lights	FalconEyes	LH-ESB5050	Hong Kong	-
Cow's blood	-	-	The Netherlands	3% EDTA added

Appendix 2: Camera settings

All samples are photographed with a Sony DSC-RX10M3 in a fixed setup.

The settings used are shown in the table below.

Table 9: *Camera settings*

	Aperture	Shutter speed	ISO	Focal length	Light (nm)	Filter
Blood tile	f/8.0	1/60	800	9 mm	-	-
Blood paper	f/8.0	1/60	800	9 mm	-	-
Amido Black tile	f/8.0	1/60	800	9 mm	-	-
Hungarian Red tile	f/8.0	1/60	800	9 mm	-	-
(A)LCV paper	f/8.0	1/60	800	9 mm	-	-
Iron oxide tile	f/8.0	1/60	800	9 mm	-	-
Hungarian Red ALS	f/3.2	1/60	800	18 mm	600 nm	Red

Figure 27: *Setup for photographing the results*

Appendix 3: Chemical recipes

At this appendix, the recipes of all the chemicals are described for 500 millilitres. These recipes have an internal fixative.

Amido Black

Amido Black dye solution with fixative for blood
11 grams of 5-sulfosalicylic acid
11 mL Citric acid solution
1,5 grams of Naphthol Blue Black ≤85%
293 mL Demineralised water
196 mL Bio-ethanol 95%

- Add and dissolve the 196 mL ethanol to the 293 mL demineralised water in a clean 500 mL volumetric flask
- Add 11 mL of Citric acid solution to the flask and dissolve it
- Add 11 grams of 5-sulfosalicylic acid to the flask and dissolve it
- Weigh out 1,5 grams of Naphthol Blue Black ≤85% on a balance and add to the flask and dissolve it
- Stir the solution with a magnetic stirring device for ≥ 10 minutes
- Pour the solution, with the use of a clean funnel into a dark micro sprayer and store it

Hungarian Red

Hungarian Red dye solution with fixative for blood
11 grams of 5-sulfosalicylic acid
11 mL Citric acid solution
1,5 grams of Acid Fuchsin
293 mL Demineralised water
196 mL Bio-ethanol 95%

- Add and dissolve the 196 mL ethanol to the 293 mL demineralised water in a clean 500 mL volumetric flask
- Add 11 mL of Citric acid solution to the flask and dissolve it
- Add 11 grams of 5-sulfosalicylic acid to the flask and dissolve it
- Weigh out 1,5 grams of Acid Fuchsin on a balance and add to the flask and dissolve it
- Stir the solution with a magnetic stirring device for ≥ 5 minutes
- Pour the solution, with the use of a clean funnel into a dark micro sprayer and store it

(A)LCV

(A)LCV solution with fixative for blood
11 grams of 5-sulfosalicylic acid
1 grams of Leucocrystal Violet
3,7 grams sodium acetate
300 mL Demineralised water
200 mL Bio-ethanol 95%
50 tablets of 0.3% hydrogen peroxide

- Add and dissolve the 200 mL ethanol to the 300 mL demineralised water in a clean 500 mL volumetric flask
- Weigh out 3.7 grams of sodium acetate on a balance and add to the flask and dissolve it
- Add 11 grams of 5-sulfosalicylic acid to the flask and dissolve it
- Stir the solution with a magnetic stirring device for ≥ 5 minutes
- Weigh out 1 gram of Leucocrystal Violet on a balance and add to the flask and dissolve it
- Stir the solution with a magnetic stirring device for ≥ 20 minutes
- Add the tablets hydrogen peroxide to the solution for a 3% hydrogen peroxide solution

- Stir the solution with a magnetic stirring device for ≥ 20 minutes to dissolve the tablets
- Pour the solution, when all the tablets are dissolved, with the use of a clean funnel, into a dark micro sprayer and store it in a refrigerator.

Iron oxide

Iron oxide Suspension dye with fixative for blood

11 grams of 5-sulfosalicylic acid
8 grams of Iron(III)oxide
300 mL Demineralised water
200 mL Bio-ethanol 95%

- Add and dissolve the 200 mL ethanol to the 300 mL demineralised water in a clean 500 mL volumetric flask
- Add 11 grams of 5-sulfosalicylic acid to the flask and dissolve it
- Weigh out 8 grams of Iron(III)oxide on a balance and add to the flask and dissolve it
- Stir the solution with a magnetic stirring device for ≥ 10 minutes
- Pour the solution, with the use of a clean funnel into a dark micro sprayer and store it

Rinse solution

0,5% v/v aqueous rinse solution

2,5 mL Glacial acetic acid
298,5 mL Demineralised water
199 mL Bio-ethanol 95%

- Add the ethanol to the demineralised water in a clean 500 mL volumetric flask
- Add the 2.5 mL of acetic acid to the demineralised/ethanol solution
- Stir the solution with a magnetic stirring device for ≥ 15 minutes
- Pour the solution, with the use of a clean funnel into a dark micro sprayer and store it

Appendix 4: MSDS Chemicals

1. Ethanol
    - Safety ratings
        - Health: 2 - Moderate
        - Flammability: 3 - High
        - Reactivity: 1 – Slight

    Asih Miniarti – MSDS Ethanol – Material Safety Data Sheet – SCRIBD - Date: unknown – Accessed on April 28th, 2022 - https://www.scribd.com/document/96820890/Msds-Ethanol

2. Acetic Acid 100%
    - Safety ratings
        - Health: 3 – High
        - Flammability: 2 - Moderate
        - Reactivity: 2 – Moderate
        - Contact: 4 – Extreme

    Parchem – Safety Data Sheet – (Acetic Acid Glacial) – Date: January 13th, 2012 – Accessed on April 28th, 2022 - https://www.google.com/search?q=safety+ratings+acetic+acid+100&client=firefox-b-d&sxsrf=ALiCzsbCNu-5TMyhSrgyqHBt2hO7Y6DmdA%3A1651145837680&ei=bXxqYvSQKYeNkwXJ9JSIAg&oq=safety+ratings+acetic+&gs_lcp=Cgdnd3Mtd2l6EAMYATIFCCEQoAEyBQghEKABOgcIABBHELADOgQIIxAnOgYIABAWEB46BwgjEOoCECc6DgguELEDEIMBEMcBENEDOgQIABBDOg4ILhCABBCxAxDHARDRAzoICAAQsQMQgwE6CAgAEIAEELEDOgsIABCABBCxAxCDATоFCC4QgAQ6CwguEIAEEMcBEKMCOgUIABCABDoLCC4QgAQQxwEQrwE6CAguEIAEELEDOhEILhCABBCxAxCDARDHARCvATоHCAAQsQMQQzoECC4QQzoECAAQAzoFCAAQywFKBAhBGABKBAhGGABQyQNYwkdglFFoAnABeAOAAX-IAaEXkgEEMzUuNJgBAKABAbABCsgBCMABAQ&sclient=gws-wiz – (Second link)

3. 5-sulfosalicylic acid
    - Safety ratings
        - Health: 1 – Slight
        - Flammability: 0 – Insignificant
        - Reactivity: 0 – Insignificant

    LabChem – 5-sulfosalicyclic acid, dihydrate – Safety Data Sheet – Date: September 2nd, 2012 – Version 1,1 – Accessed on April 28th, 2022 - https://www.google.com/search?q=safety+ratings+5-sulfosalicylic+acid&client=firefox-b-d&sxsrf=ALiCzsZUOqReIGj7u6mf6atZB1l1qVelfg%3A1651146030050&ei=Ln1qYtzcApWakgW5k4eIBw&oq=safety+ratings+5-sulfosalic&gs_lcp=Cgdnd3Mtd2l6EAMYADIHCCEQChCgATоHCAAQRxCwAzoECCMQJzoFCAAQywE6BggAEBYQHjoICAAQFhAKEB46BQghEKABSgQIQRgASgQIRhgAUIABWK4yYJk-aAJwAXgAgAGCAYgB_hGSAQQyMy41mAEAoAEByAEIwAEB&sclient=gws-wiz – (First link)

4. Hydrogen peroxide
    - Safety ratings
        - Health: 0 – Insignificant
        - Flammability: 0 – Insignificant
        - Reactivity: 0 – Insignificant

    29CFR1910/1200 & GHS Rev. 3 – Hydrogen Peroxide 3% - Safety Data Sheet – Date: December 16th, 2014 – Accessed on April 28th 2022 - https://www.google.com/search?q=safety+ratings+hydrogen+peroxide&client=firefox-b-d&sxsrf=ALiCzsaJmDLF8m6w_5p_m1QWELHqU7vtZw%3A1651146760242&ei=CIBqYu

yrDpPakwX6hajQBA&oq=safety+ratings+hydrogen+per&gs_lcp=Cgdnd3Mtd2l6EAMYADIFCCEQoAEyCAghEBYQHRAeMggIIRAWEB0QHjoECCMQJzoFCAAQywE6BggAEBYQHjoICAAQFhAKEB46BAghEBU6BwghEAoQoAFKBAhBGAFKBAhGGABQdljaKmDJO2gDcAB4AYABygGIAf8QkgEGMTUuNy4xmAEAoAEBwAEB&sclient=gws-wiz – (First link)

5. Amido Black
    - Safety ratings
        - Health: 0 – Insignificant
        - Flammability: 0 – Insignificant
        - Reactivity: 0 – Insignificant

    1907/2006/EC (REACH), 1272/2008/EC (CLP) & OSHA GHS – Amido Black – Safety Data Sheet – Date: December 12th, 2014 – Accessed on April 28th 2022 - https://www.google.com/search?q=safety+ratings+amido+black&client=firefox-b-d&sxsrf=ALiCzsafJCltxJ1Sm4euFf_0M5ie5Ukmgg%3A1651146968749&ei=2IBqYsGtLYXBlAa2uZaoCA&ved=0ahUKEwjB-azk2bb3AhWFIMUKHbacBYUQ4dUDCA0&uact=5&oq=safety+ratings+amido+black&gs_lcp=Cgdnd3Mtd2l6EANKBAhBGAFKBAhGGABQAFgAYLwBaAFwAHgAgAEAiAEAkgEAmAEAwAEB&sclient=gws-wiz – (First link)

6. Hungarian Red
    a. Safety ratings
        i. Health: 2 – Moderate
        ii. Flammability: 1 – Slight
        iii. Reactivity: 0 – Insignificant

    Sirchie – LV503 Hungarian Red – Safety Data Sheet – Date: March 26th, 2012 – Accessed on April 28th, 2022 - https://www.google.com/search?q=safety+data+sheet+hungarian+red&client=firefox-b-d&sxsrf=ALiCzsb0YFEbs6gk6Dde_owpub2ySr4byA%3A1651147915597&ei=i4RqYtiKJMzWkgWBmpSQCQ&ved=0ahUKEwiY_Oun3bb3AhVMq6QKHQENBZIQ4dUDCA0&uact=5&oq=safety+data+sheet+hungarian+red&gs_lcp=Cgdnd3Mtd2l6EAMyBQghEKABMgUIIRCgATIICCEQFhAdEB4yCAghEBYQHRAeMggIIRAWEB0QHjIICCEQFhAdEB4yCAghEBYQHRAeMggIIRAWEB0QHjIICCEQFhAdEB4yCAghEBYQHRAeOgQIIxAnSgQIQRgBSgQIRhgAUIIBWKkCYOIHaAFwAHgAgAGAAYgBxAGSAQMxLjGYAQCgAQHAAQE&sclient=gws-wiz – (First link)

7. Aqua Leuco Crystal Violet
    a. Safety ratings
        i. Health: 2 – Moderate
        ii. Flammability: 0 – Insignificant
        iii. Reactivity: 0 – Insignificant

    ThermoFisher Scientific – Leuco Crystal Violet – Safety Data Sheet – Date: December 24th, 2021 – Accessed on April 28th, 2022 - https://www.google.com/search?q=safety+data+sheet+aqua+leuco+crystal+violet&client=firefox-b-d&sxsrf=ALiCzsZbL3aH5GYeSra9YqTkmQvqV_LkdA%3A1651147512444&ei=-IJqYsLiGpbtkgXslYnQBQ&ved=0ahUKEwjCvs3n27b3AhWWtqQKHexKAloQ4dUDCA0&uact=5&oq=safety+data+sheet+aqua+leuco+crystal+violet&gs_lcp=Cgdnd3Mtd2l6EAMyBwgAEEcQsAMyBwgAEEcQsAMyBwgAEEcQsANKBAhBGABKBAhGGABQAFgAYMUBaAFwAXgAgAEAiAEAkgEAmAEAyAEDwAEB&sclient=gws-wiz – (Second link)

8. Iron Oxide
    a. Safety ratings
        i. Health: 1 – Slight
        ii. Flammability: 0 – Insignificant
        iii. Reactivity: 0 – Insignificant

    Fisher Scientific – Iron Oxide – Material Safety Data Sheet – Date: November 08th, 2007 – Accessed on April 28th, 2022 - https://fscimage.fishersci.com/msds/09765.htm

Appendix 5: Protocols conducting experiments

Constant factors

Constant variables	Description
Working place	Wear protective gear Collect the necessary materials Brown paper on the table
Temperature freezer	~ minus twenty degrees Celsius (freezer fluctuates a bit in temperature (opening and closing doors))
Blood	One cow with 3% EDTA Room temperature (± twenty degrees Celsius)
Cleaning tiles before placed in freezer	1. Washed in dishwasher 2. Disinfected with ethanol 3. Placed in the freezer ≥ two hours later
Time surfaces in freezer	24 hours
Apply blood on the surfaces	In the freezer
Time of freezing blood	10 minutes
Placing shoeprints	Same person Medium pressure Order of placing prints Directly stamped with entire surface of sole – no rolling motion
Preparation enhancement techniques	Same flasks and glassware, so the same flaws Balance with two decimal places
Time between enhancing and rinsing	4 minutes
Photographing the samples	Before enhancing After rinsing and drying for one hour
Cleaning the drip container (To prevent contamination)	Between every chemical Between placing new surfaces for 24 hours
Grading results	General system The same results + instructions to the evaluators

Determination percentage ethanol

1. Wear protective gear and create a clean working place
2. Set the freezer to minus twenty degrees Celsius
3. Prepare 100 mL of the ethanol solutions with different percentages (20%, 30%, 40% and 50%), according to the scheme below:

Solution	20%	30%	40%	50%
Demi-water	80 mL	70 mL	60 mL	50 mL
Ethanol 95%	20 mL	30 mL	40 mL	50 mL
Total (+)	100 mL	100 mL	100 mL	100 mL

4. Pour 25 mL of each modified VBET in a single centrifuge tube
5. Put the centrifuge tubes in the freezer for ≥24 hours
6. Photograph the four different solutions

Current Visual Blood Enhancement Techniques

1. Wear protective gear and create a clean working place
2. Set the freezer to minus twenty degrees Celsius
3. Prepare 100 mL of all the Visual Blood Enhancement Techniques in a measuring cup, in the way they are currently used, so waterbased. (See appendix 3 for the protocols)

For Amido Black use the following proportions:

Amido Black dye solution with fixative for blood (500 mL)
11 grams of 5-sulfosalicylic acid
11 mL Citric acid solution
1,5 grams of Naphthol Blue Black ≤85%
489 mL Demineralised water

For Hungarian Red use the following proportions:

Hungarian Red dye solution with fixative for blood (500 mL)
11 grams of 5-sulfosalicylic acid
11 mL Citric acid solution
1,5 grams of Acid Fuchsin
489 mL Demineralised water

For (A)LCV use the following proportions:

(A)LCV solution with fixative for blood (500 mL)
11 grams of 5-sulfosalicylic acid
1 grams of Leucocrystal Violet
3,7 grams sodium acetate
500 mL Demineralised water
50 tablets of 0.3% hydrogen peroxide

For Iron oxide use the following proportions:

Iron oxide Suspension dye with fixative for blood (500 mL)
11 grams of 5-sulfosalicylic acid
8 grams of Iron(III)oxide
500 mL Demineralised water

IMPORTANT: THOSE PROPORTIONS ARE FOR 500 mL, SO EVERYTHING HAS TO BE DIVIDED BY 5 TO GET AN AMOUNT OF 100 mL

4. Pour 25 mL of each VBET in a single centrifuge tube
5. Put the centrifuge tubes in the freezer for ≥24 hours
6. Also prepare 100 mL of all the modified Visual Blood Enhancement Techniques with the determined percentage ethanol (40%) according to the protocols of Appendix 3 in a measuring cup. THOSE PROPORTIONS ARE FOR 500 mL, SO THESE HAS TO BE DIVIDED BY 5 AS WELL TO GET AN AMOUNT OF 100 mL
7. Pour 25 mL of each modified VBET in a single centrifuge tube
8. Put the centrifuge tubes in the freezer for ≥24 hours
9. Photograph the results by comparing the waterbased VBET with the modified VBET next to each other. Do this for all types of the Visual Blood Enhancement Techniques

Dilutions

1. Wear protective gear and create a clean working place
2. Set the freezer to minus twenty degrees Celsius
3. Clean the tiles by washing them in the dishwasher
4. Let them dry for ≥ two hours
5. Divide every tile in four even squares by marking them with a permanent marker.
6. Every square is for one blood dilution. So, write every dilution in the upper left corner of the square with the permanent marker as well.
7. Stick some adhesive tape over the marked lines and dilution indications (see figure 12)

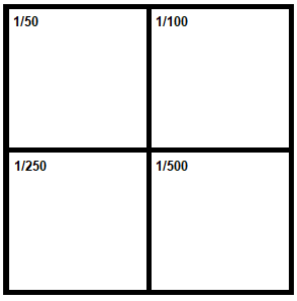


Figure 12: Division of areas for dilutions

8. Disinfect the squares with ethanol 95%
9. Let the ethanol evaporate and dry for ≥ two hours
10. After drying, lay ten tiles in the freezer by putting them in the drip container with the drainer and let it freeze for ≥24 hours
11. Prepare the modified VBETs and the rinse solution with the 40 percent ethanol according to Appendix 3 and fill the micro sprayers with the chemicals
12. Write down on every micro sprayer, which chemical is in it.
13. Make the blood dilutions, according to the scheme below:

Dilution	1/50	1/100	1/250	1/500
Blood (mL)	1	0,5	0,2	0,1
Demi-water (mL)	49	49,5	49,8	49,9
Final volume (mL)	50	50	50	50

14. Use for every dilution a swab to apply the diluted blood on the squares with the same dilution indication, with a zigzag movement
15. Let the diluted blood samples freeze for at least 10 minutes
16. Apply one VBET on the ten surfaces by spraying and let it react for 4 minutes with closed doors. Do it one surface by one to make it as easy as possible.
17. Rinse off the excess liquid with the rinse solution and let it dry for at least one hour
18. In the meantime, clean the drip container between the different VBETs to provide contamination.
19. Photograph the results on a white background with a ruler next to it
20. Import the photos on the computer for further use
21. Prepare the surfaces again for the next ten and lay them in the freezer for the next day IMPORTANT: FOR (A)LCV IT IS NEEDED TO USE PAPER, INSTEAD OF TILES. FOR THE PREPERATION, IT IS ONLY NECESSARY TO DIVIDE IT INTO FOUR SQUARES AND FREEZE IT FOR 24 HOURS.
22. Repeat the application and the enhancement of the blood samples for the other VBETs. IMPORTANT: FOR (A)LCV, THE APPLICATION OF THE DILUTED BLOOD IS THE SAME. FOR THE ENHANCEMENT OF THE BLOOD SAMPLES, ONLY THE APPLICATION OF (A)LCV IS NECESSARY, NO RINSING STEP.

Main experiments: stamping

1. Wear protective gear and create a clean working place
2. Set the freezer to minus twenty degrees Celsius
3. Clean the tiles by washing them in the dishwasher
4. Let them dry for ≥ two hours
5. Number them by writing down a number from 1 to 50 on the back of the tiles
6. Disinfect the squares with ethanol 95%
7. Let the ethanol evaporate and dry for ≥ two hours
8. After drying, lay 25 tiles in the freezer by putting them in the drip container with the drainer and let it freeze for ≥24 hours
9. Prepare the modified VBETs and the rinse solution with 40 percent ethanol, according to Appendix 3 and fill the micro sprayers with the chemicals
10. Write down on every micro sprayer, which chemical is in it
11. Take a container and lay some Tork paper in it to simulate an ink pad
12. Pour some blood on the Tork paper and let it soak in it until everything is soaked
13. Get the partial shoe and make some characteristics with a scalpel in the sole
14. Clean the sole with some ethanol 95% in advance
15. Use the partial shoe and moisten it with blood by pushing the sole on the soaked blood
16. Stamp a series of four blood prints on the surface (tile or paper), without moistening it with blood in between, in the same order every time: Upper left > Upper right > Bottom left > Bottom right. IMPORTANT: DO THIS IN THE FREEZER ITSELF
17. Let the prints freeze for 10 minutes

18. Photograph the surfaces by putting them out the freezer for a very short time
19. Lay it as soon as possible back in the freezer and let it freeze again for 4 minutes
20. Apply one VBET on the 25 surfaces by spraying and let it react for 4 minutes with closed doors. Do it one surface by one to make it as easy as possible.
21. Rinse off the excess liquid with the rinse solution
22. Repeat it for all the 25 surfaces in the freezer
23. Let the surfaces dry for at least one hour
24. In the meantime, clean the drip container between the different VBETs/days to provide contamination.
25. Photograph the results on a white background with a ruler next to it
26. Import the photos on the computer for further use
27. Prepare the surfaces again for the next 25 and lay them in the freezer for the next day
    IMPORTANT: FOR (A)LCV IT IS NEEDED TO USE PAPER, INSTEAD OF TILES. FOR THE PREPERATION, IT IS ONLY NECESSARY NUMBER THEM FROM 1 TO 50 AND TO FREEZE THEM FOR 24 HOURS.
28. Repeat the application and the enhancement of the blood samples for the other VBETs.
    IMPORTANT: FOR (A)LCV, THE APPLICATION OF THE BLOOD IS THE SAME. FOR THE ENHANCEMENT OF THE BLOOD SAMPLES, ONLY THE APPLICATION OF (A)LCV IS NECESSARY, NO RINSING STEP.

29. *OPTIONAL:*
    For Iron oxide and Hungarian Red, it is possible to lift the prints with a white gelatin film.
    In the case of Iron oxide:
    - Remove the cover from the white gelatin film
    - Place the film on the surface
    - AVOID AIR BUBBLES
    - Remove the film from the surface and replace the cover on the film
    - AGAIN, AVOID AIR BUBBLES
    - Photograph the film

    In the case of Hungarian Red:

    - Investigate the prints with a forensic light source at a wavelength of approximately 600 nm and red goggles
    - Photograph those results
    - Remove the cover from the white gelatin film
    - Place the film on the surface
    - AVOID AIR BUBBLES
    - Leave the film for 45 minutes on the surface, so the Hungarian Red prints can pass into the film
    - Remove the film from the surface
    - Photograph the results as soon as possible, because after some time, Hungarian Red soaks into the foil, resulting in the loss of the prints
    - Investigate the prints with a forensic light source at a wavelength of approximately 600 nm and red goggles
    - Photograph those results as well
    - Replace the cover on the film
    - AGAIN, AVOID AIR BUBBLES

Appendix 6: Results for the evaluators incl. reference used shoe sole

Instructions

1. To rate the results, you will be presented with a selection of photos of ten stamp series per Visual Blood Enhancement Technique. There are four different techniques (Amido Black, Hungarian Red, (A)LCV and Iron Oxide). This means forty stamp series in total. You will see the photo of each stamp series before and after the treatment.
2. To show to you which colours the four different techniques have, they are listed here below:
    a. Amido Black = dark blue
    b. Hungarian Red = red purple
    c. (A)LCV = blue purple
    d. Iron oxide = rust coloured
3. Each photo has one stamp set, consisting of four prints. You are requested to rate all prints separately of both photos with a 0-4, on the basis of table 3. Please write the scores in the correct row in the table for the relevant photos, as shown in an example.

    1. LB = print upper left
    2. RB = print upper right
    3. LO = print bottom left
    4. RO = print bottom right

Print	Score
LB	
RB	
LO	
RO	

    This order has been determined, because this has also been the order of stamping.

Table 3: Scoring table for rating the results

Score	Details
0	No development
1	Signs of contact but <1/3 of mark with characteristic aspects
2	1/3-2/3 of mark with characteristic aspects
3	>2/3 of mark with characteristic aspects, but not quite a perfect mark
4	Full development – whole mark clear with characteristic aspects

4. The emphasis of scoring the results is on the extent to which characteristics are visible in the prints. For the execution, therefore, characteristics have been applied manually in the shoe sole, see figure ?.
5. Scoring all results will probably take +/- 45 minutes of your time.
6. If you have any comments or remarks during or afterwards rating the results, you can write them down and they will be taken into account when processing the results.

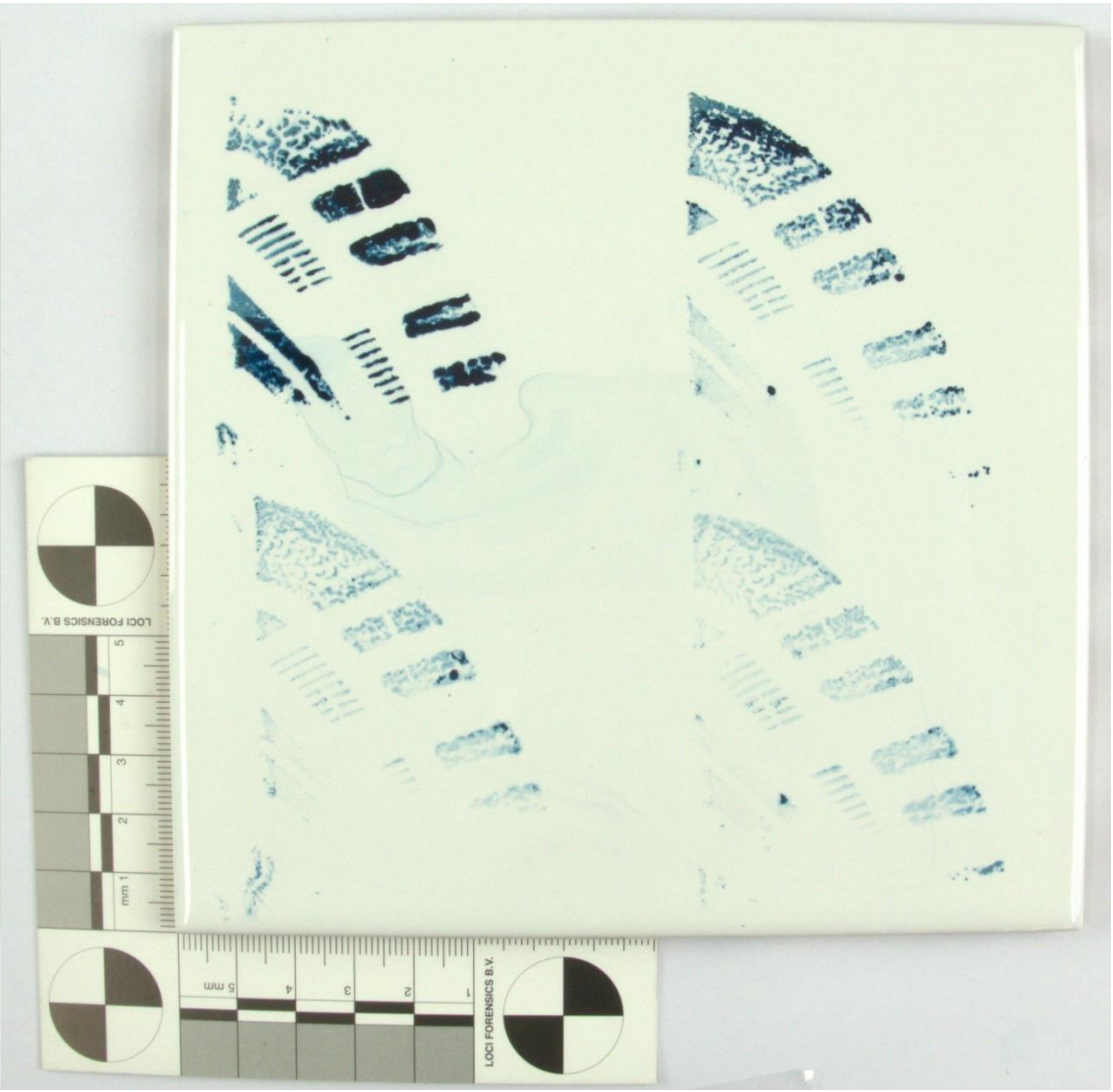
I would like to thank you for your time and for rating the results, because that helps a lot with my research.

Image of the shoe sole used with the markings of the manually applied characteristics

Figure 11: Image of the shoe sole used with the markings of the manually applied characteristics

Amido Black 1

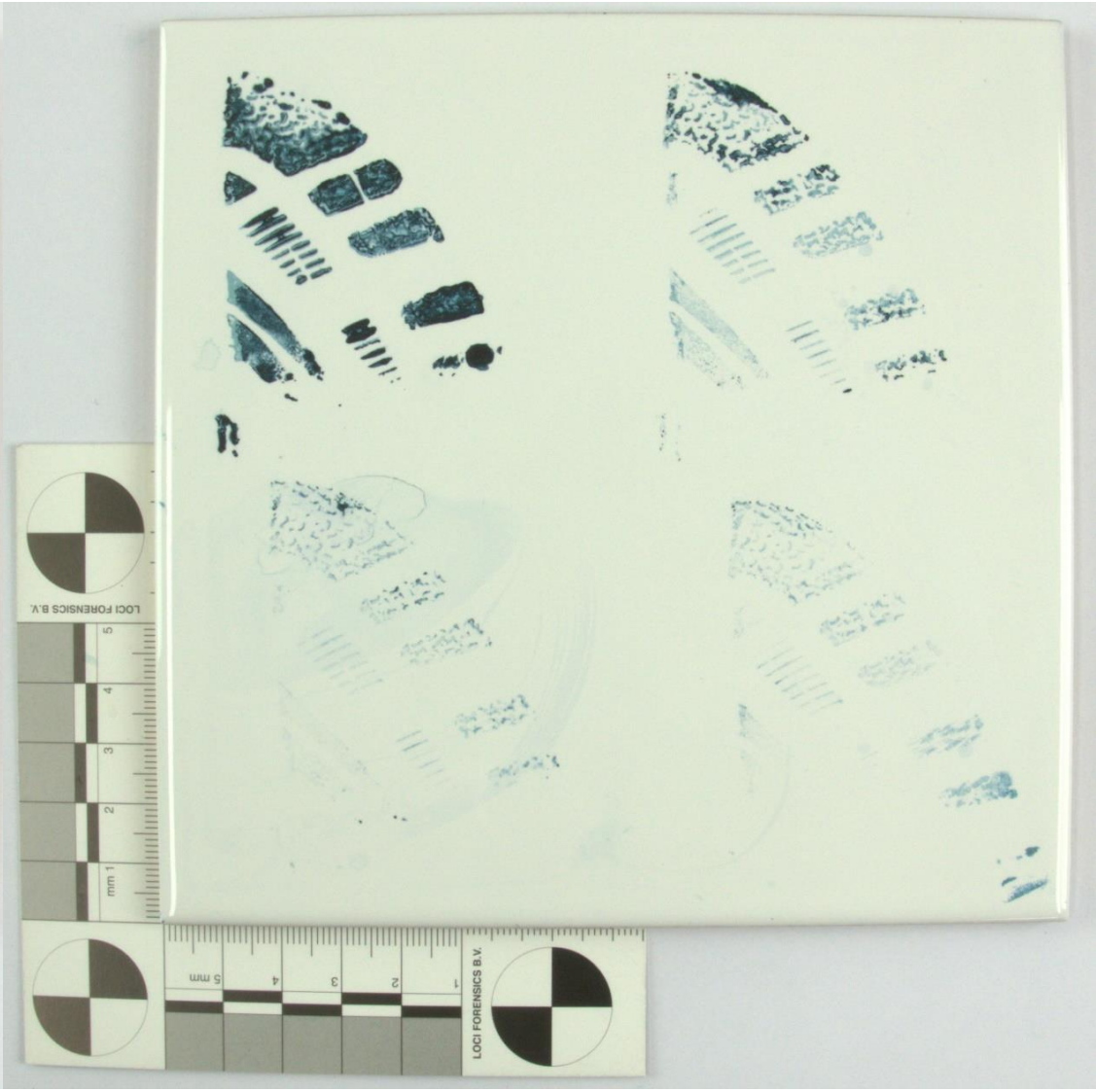
Print	Score
LB	
RB	
LO	
RO	



Print	Score
LB	
RB	
LO	
RO	

Amido Black 2

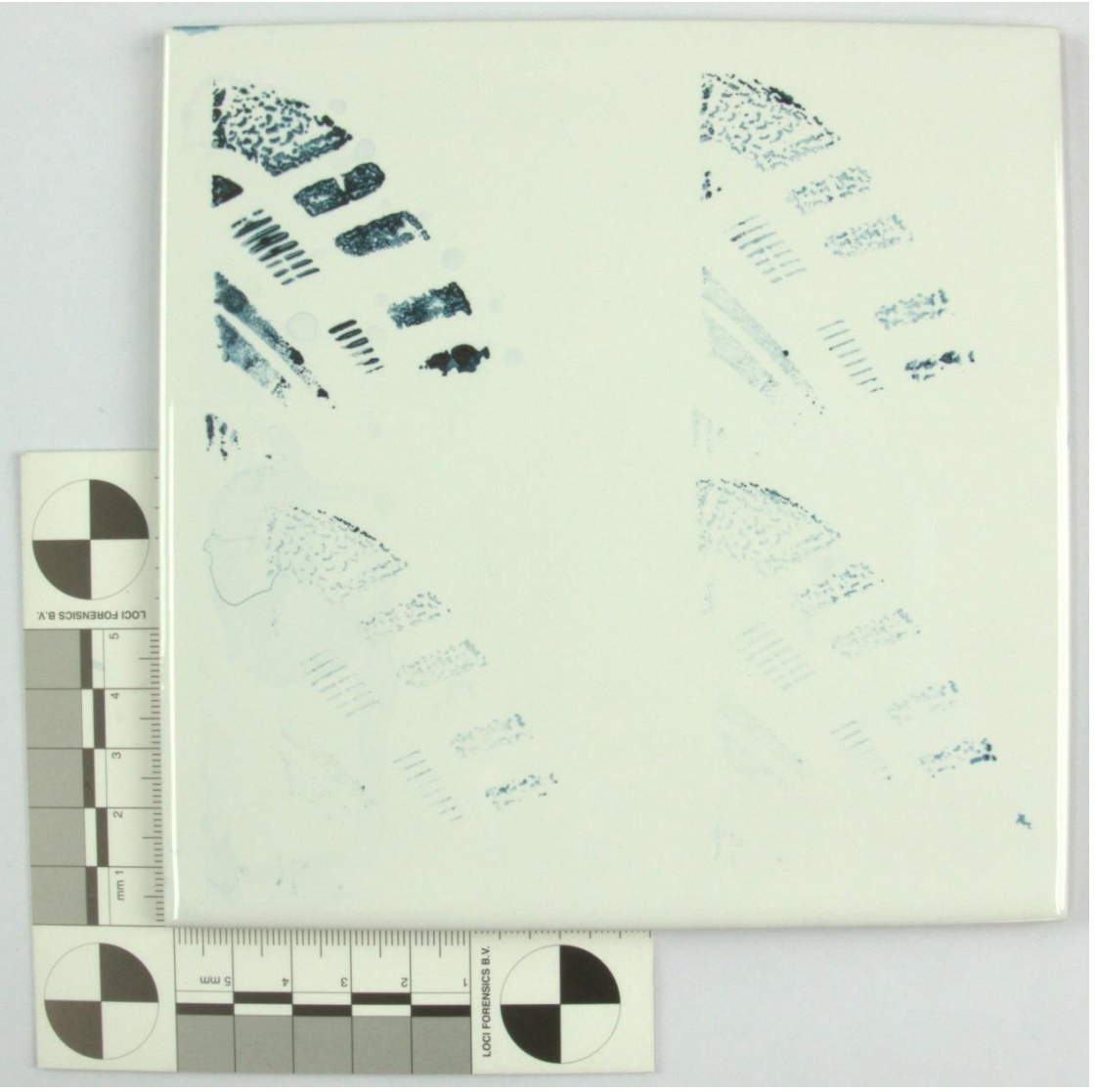
Print	Score
LB	
RB	
LO	
RO	



Print	Score
LB	
RB	
LO	
RO	

Amido Black 3

Print	Score
LB	
RB	
LO	
RO	



Print	Score
LB	
RB	
LO	
RO	

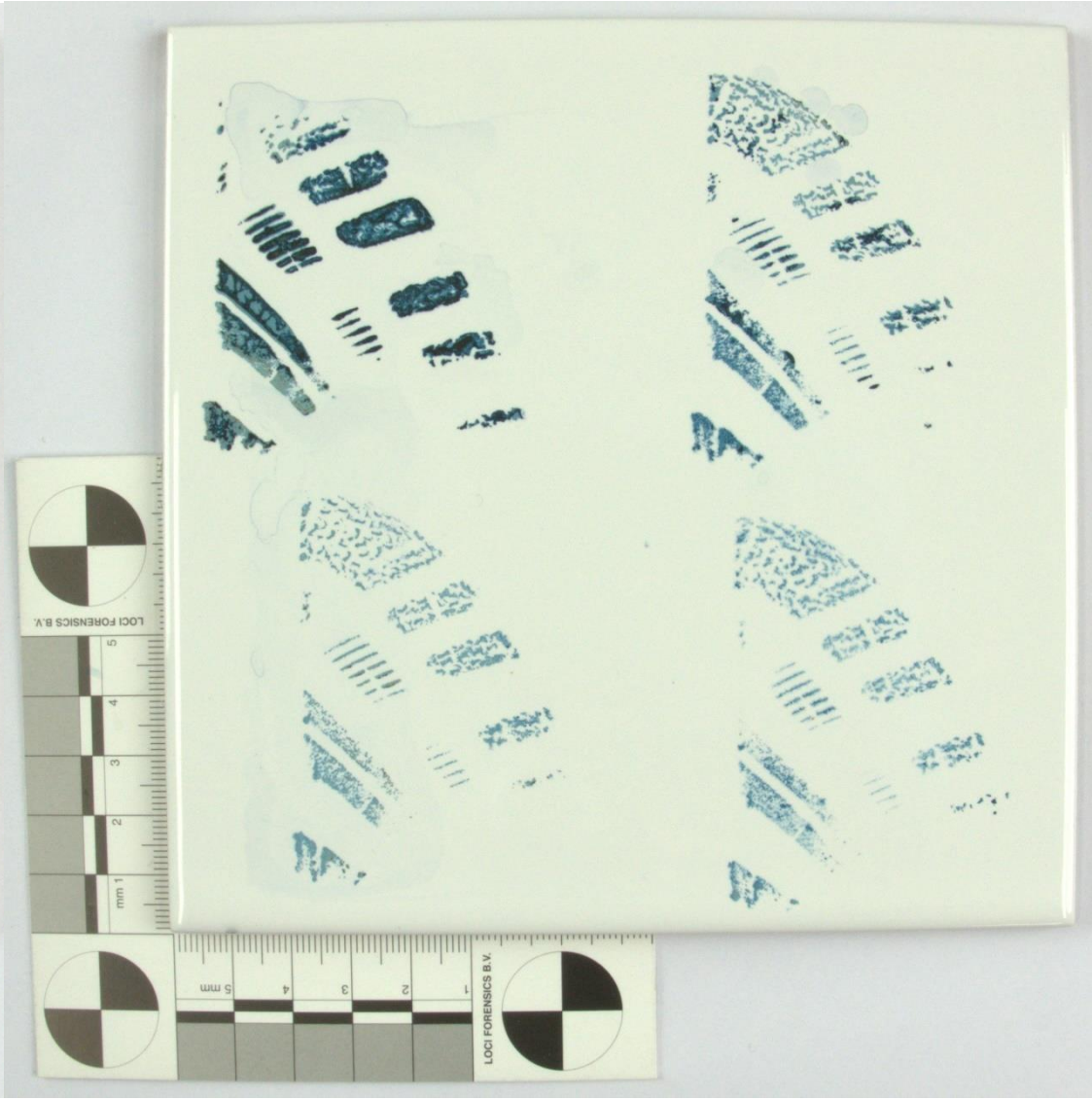
Amido Black 4

Print	Score
LB	
RB	
LO	
RO	

Print	Score
LB	
RB	
LO	
RO	

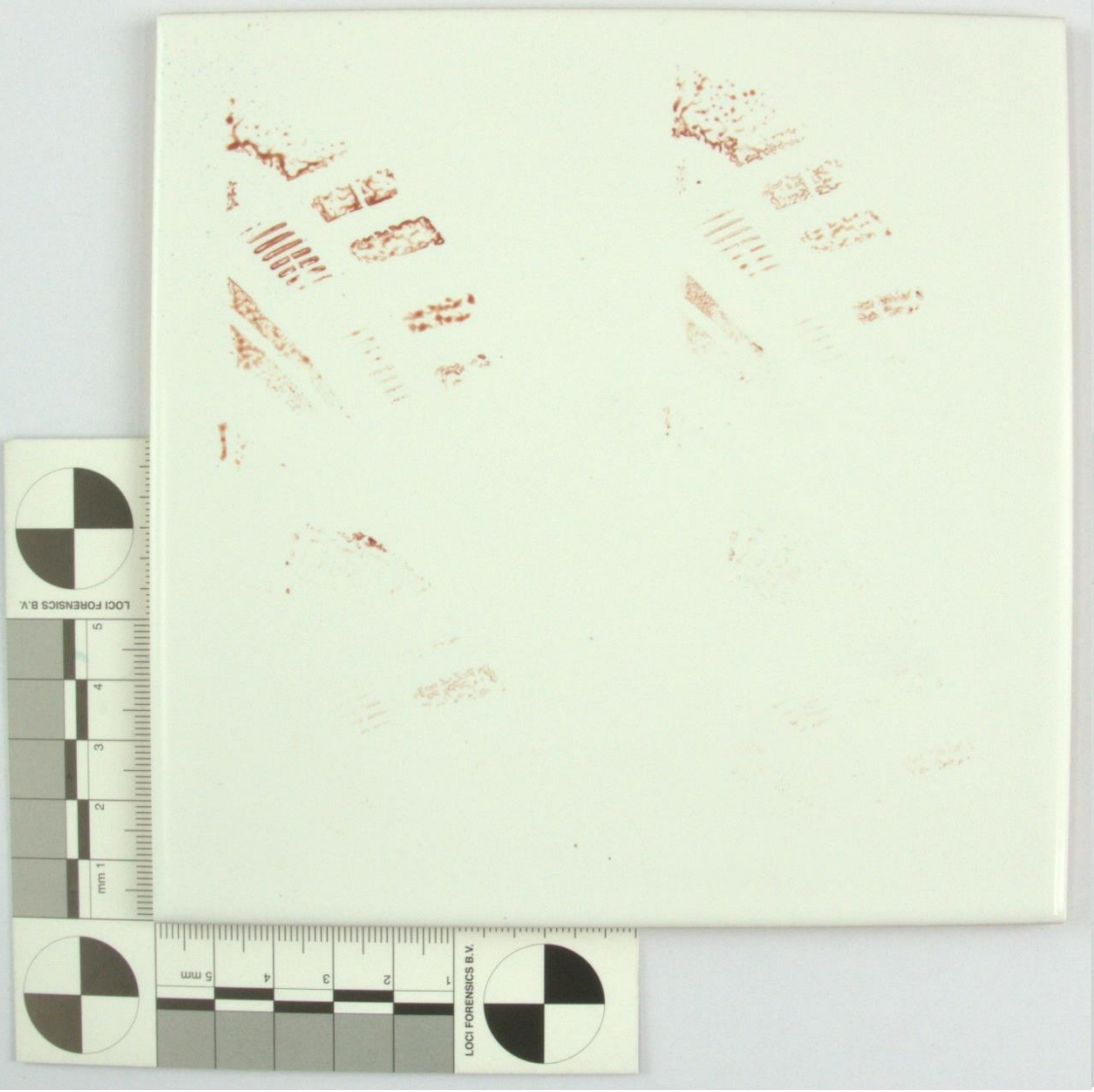
Amido Black 5

Print	Score
LB	
RB	
LO	
RO	

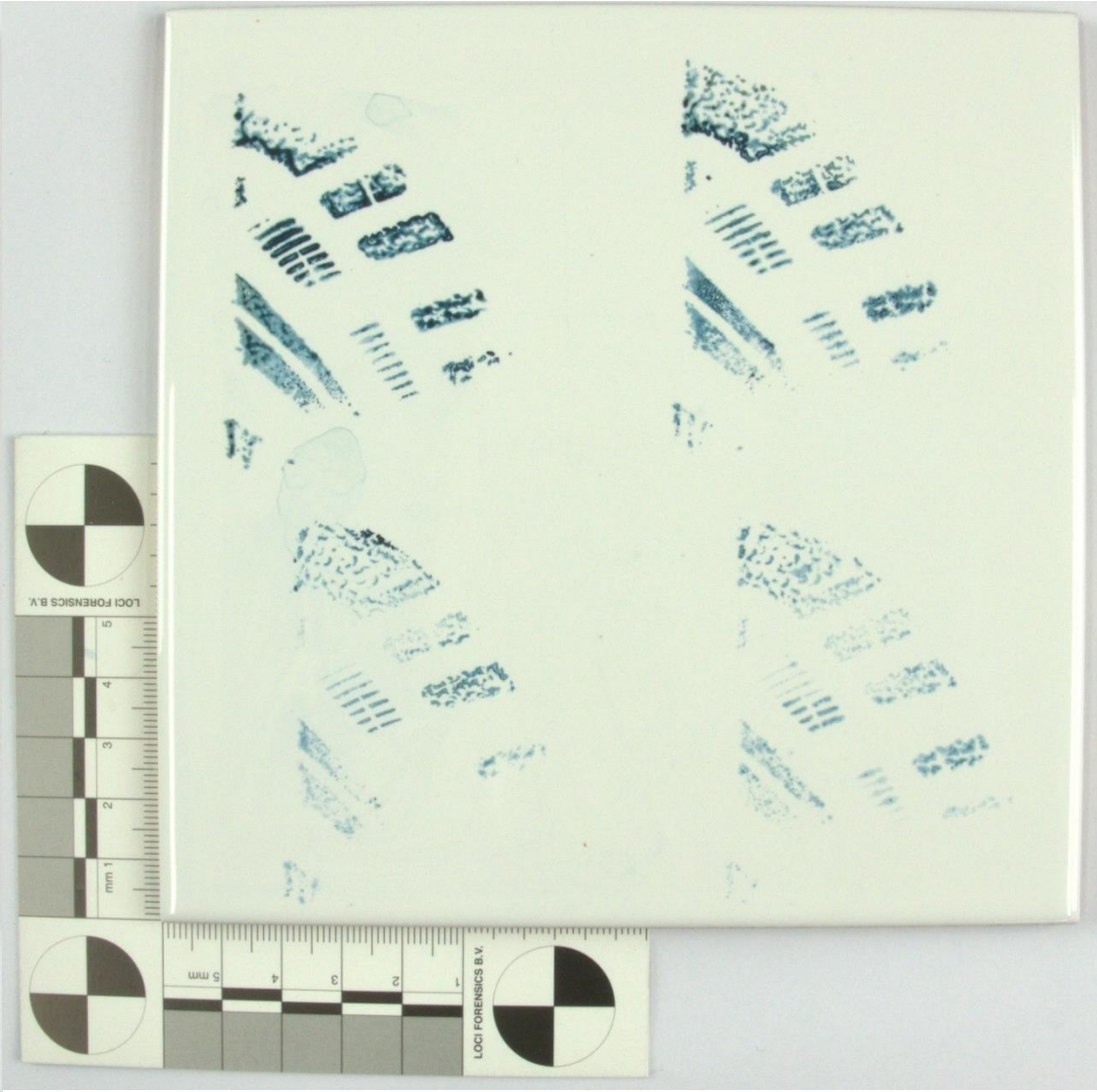


Print	Score
LB	
RB	
LO	
RO	

Amido Black 6

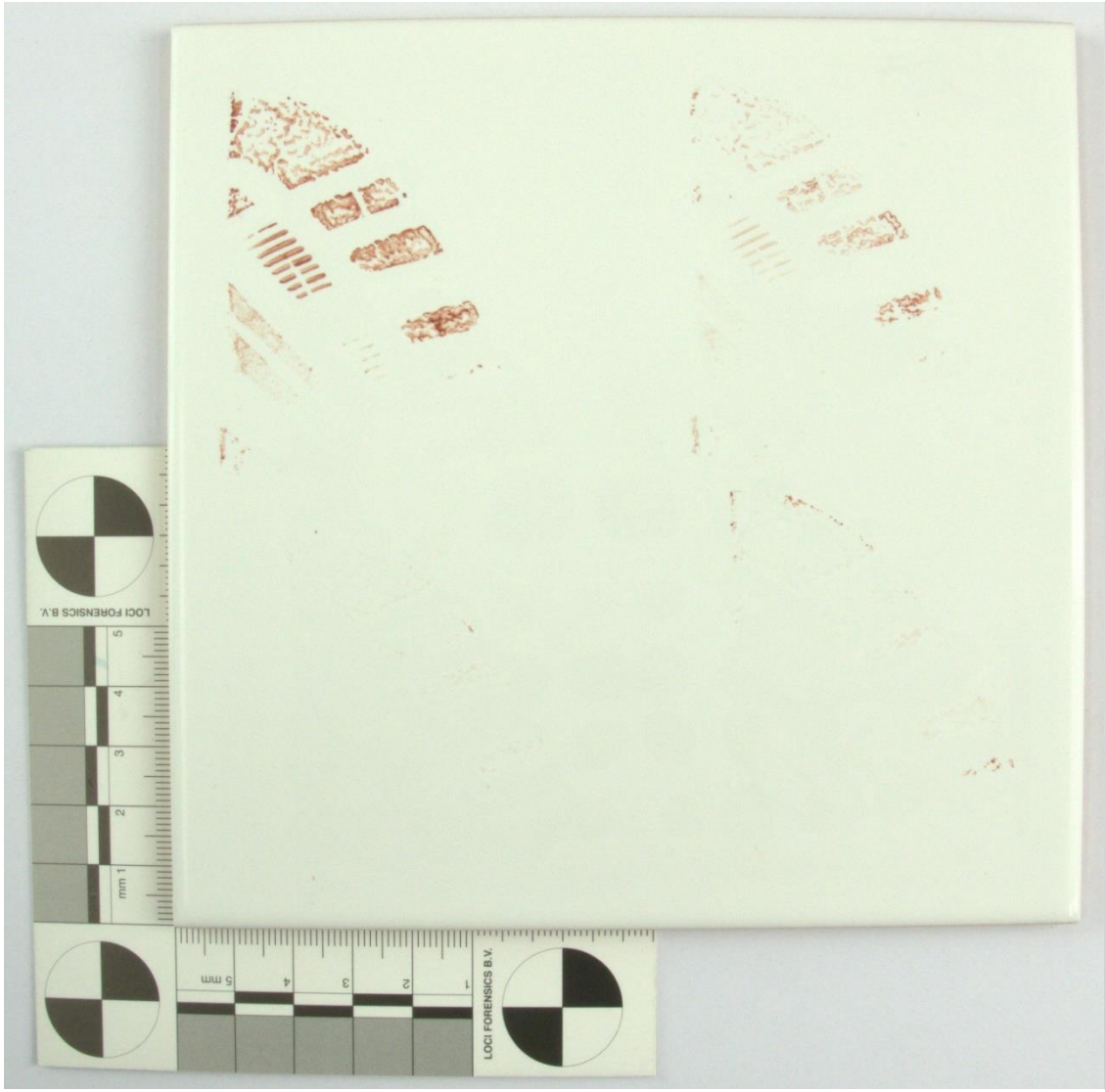


Print	Score
LB	
RB	
LO	
RO	

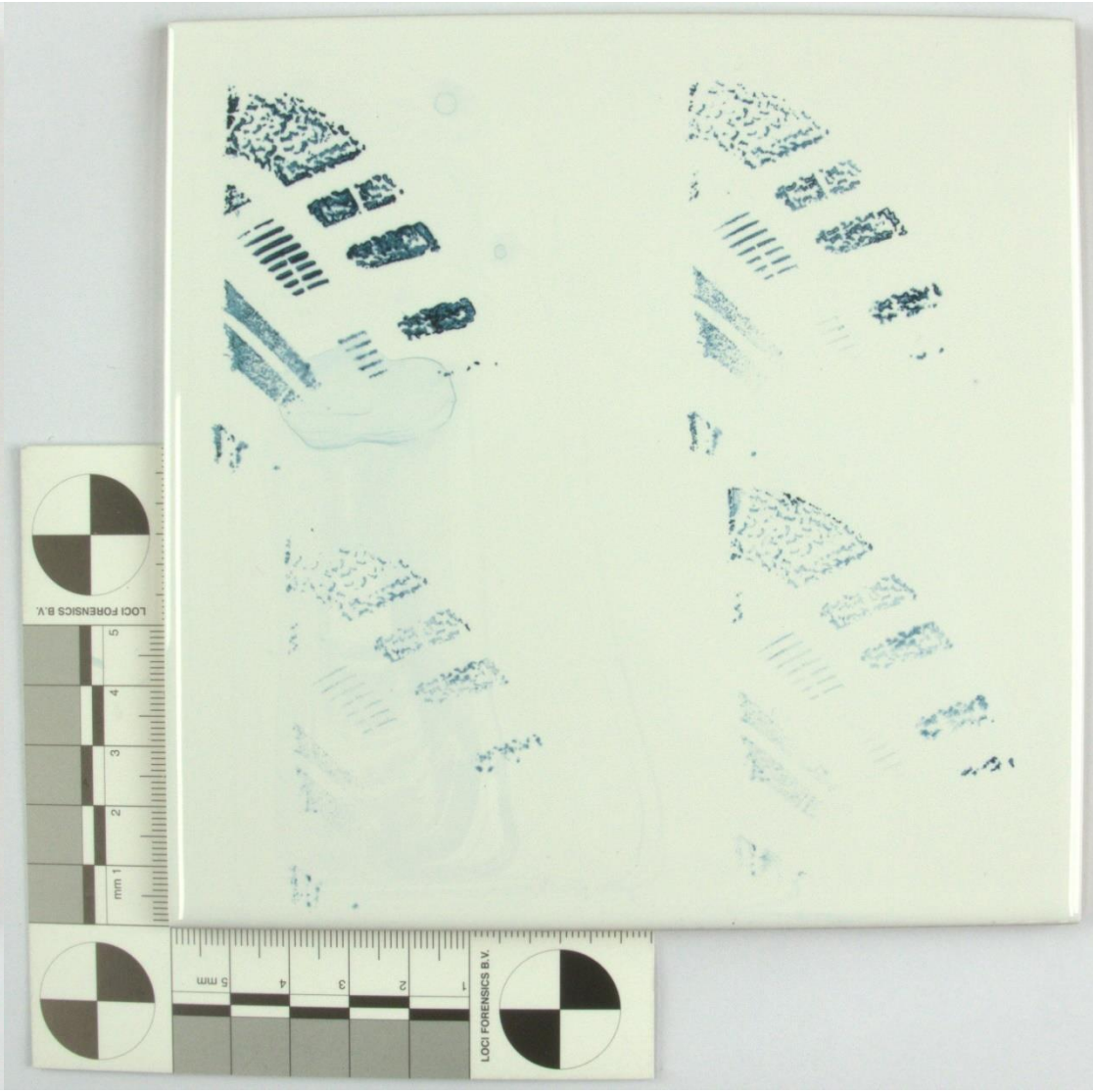


Print	Score
LB	
RB	
LO	
RO	

Amido Black 7



Print	Score
LB	
RB	
LO	
RO	



Print	Score
LB	
RB	
LO	
RO	

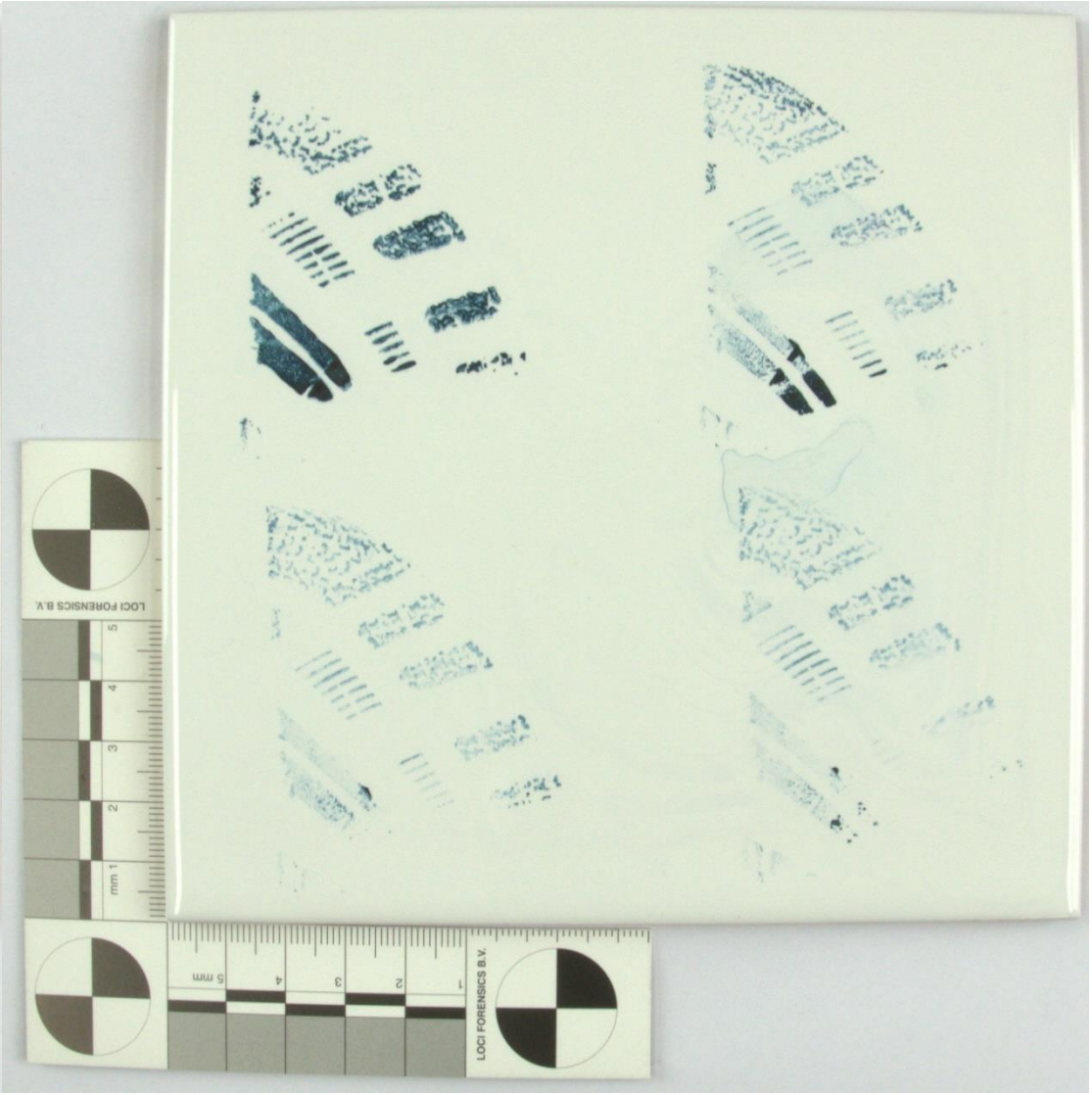
Amido Black 8

Print	Score
LB	
RB	
LO	
RO	

Print	Score
LB	
RB	
LO	
RO	

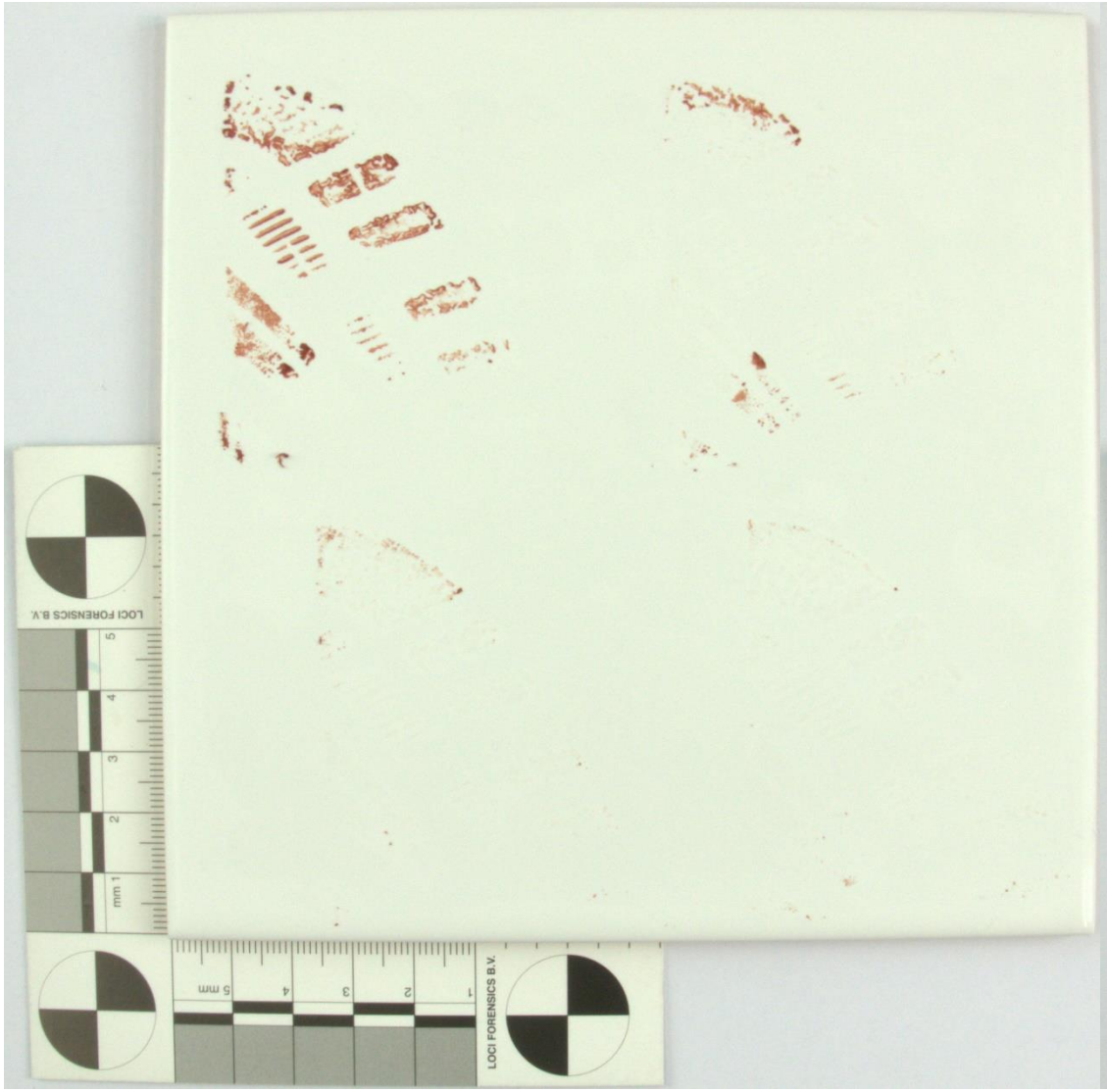
Amido Black 9

Print	Score
LB	
RB	
LO	
RO	

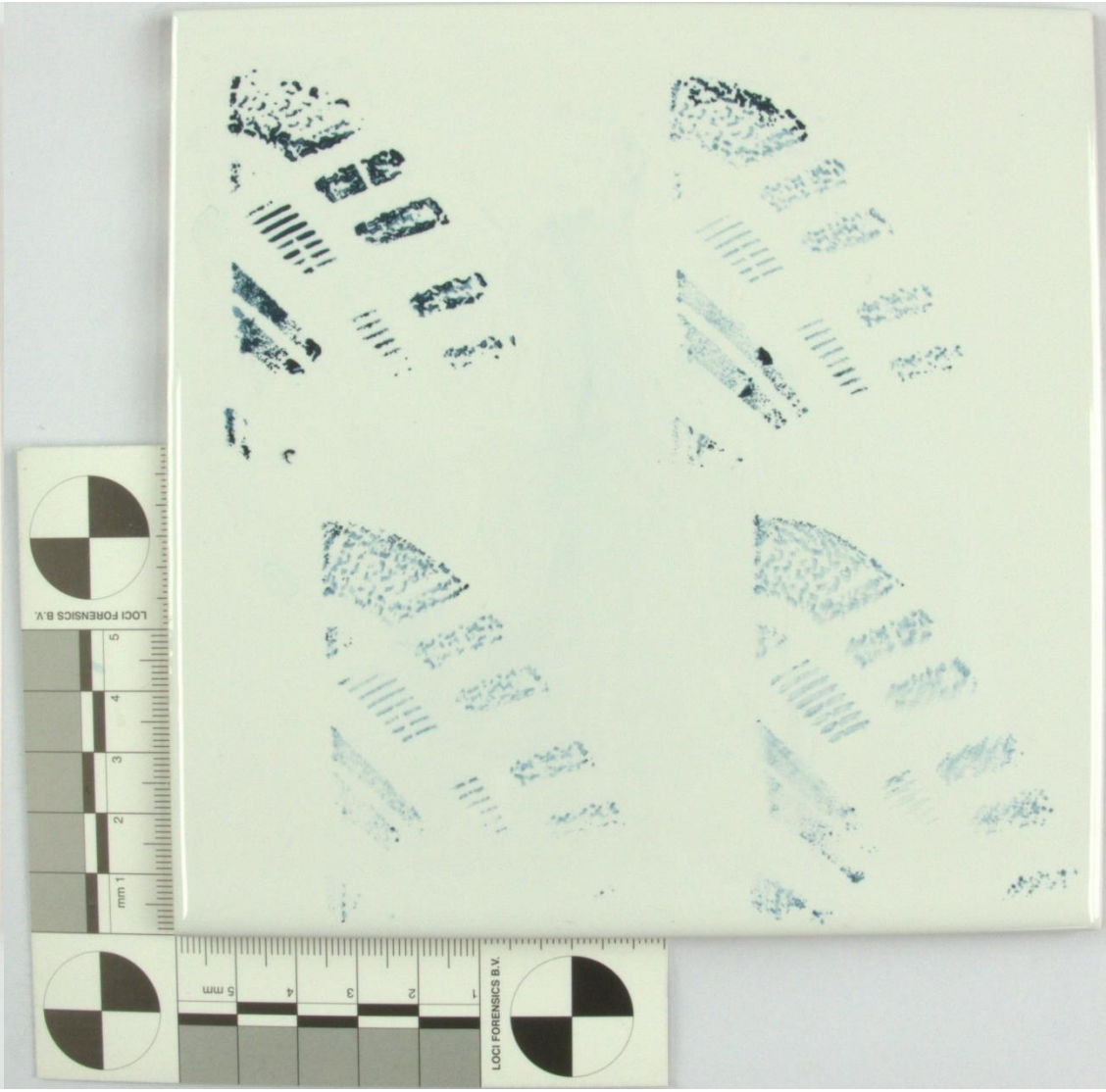


Print	Score
LB	
RB	
LO	
RO	

Amido Black 10

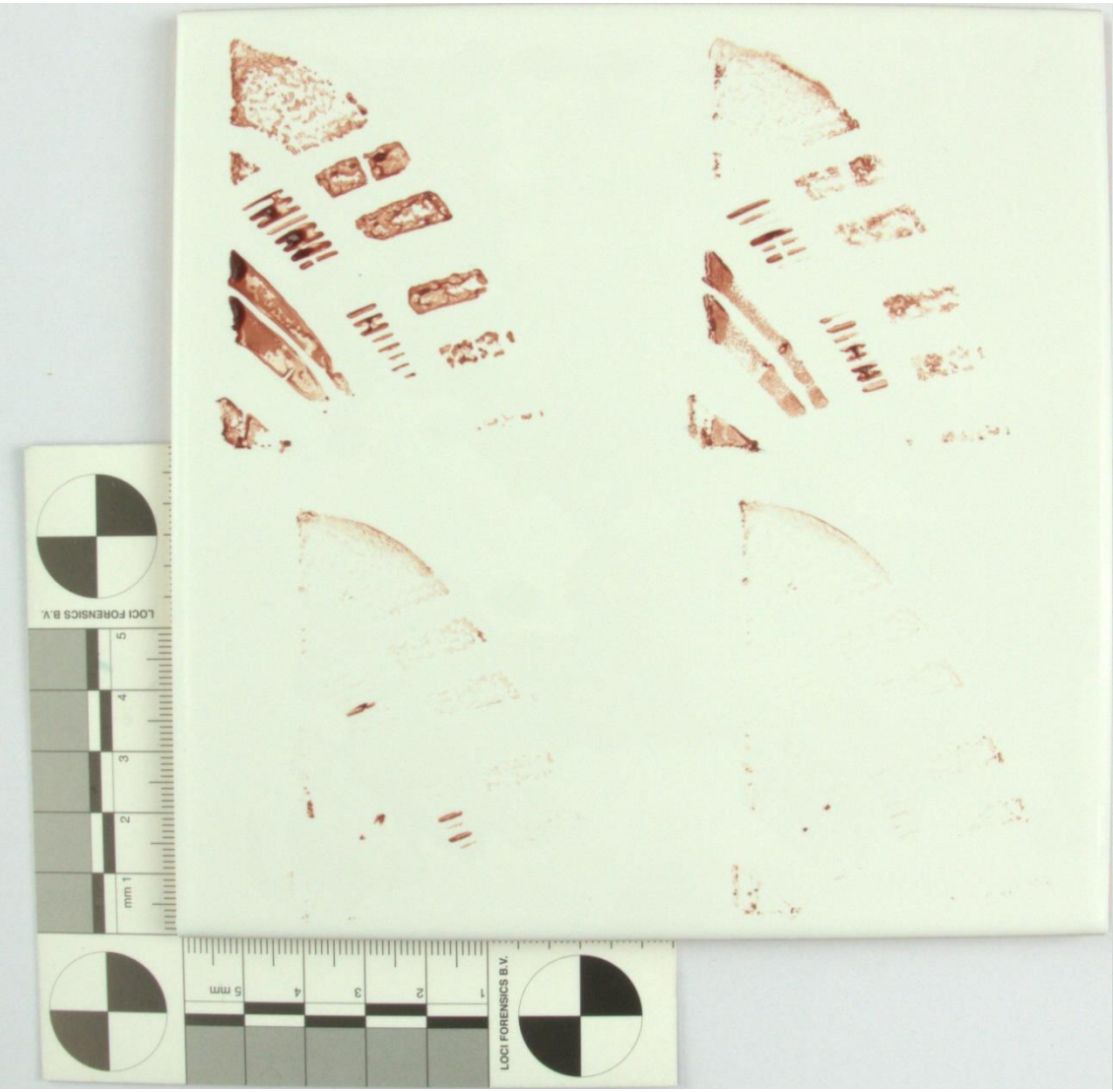


Print	Score
LB	
RB	
LO	
RO	



Print	Score
LB	
RB	
LO	
RO	

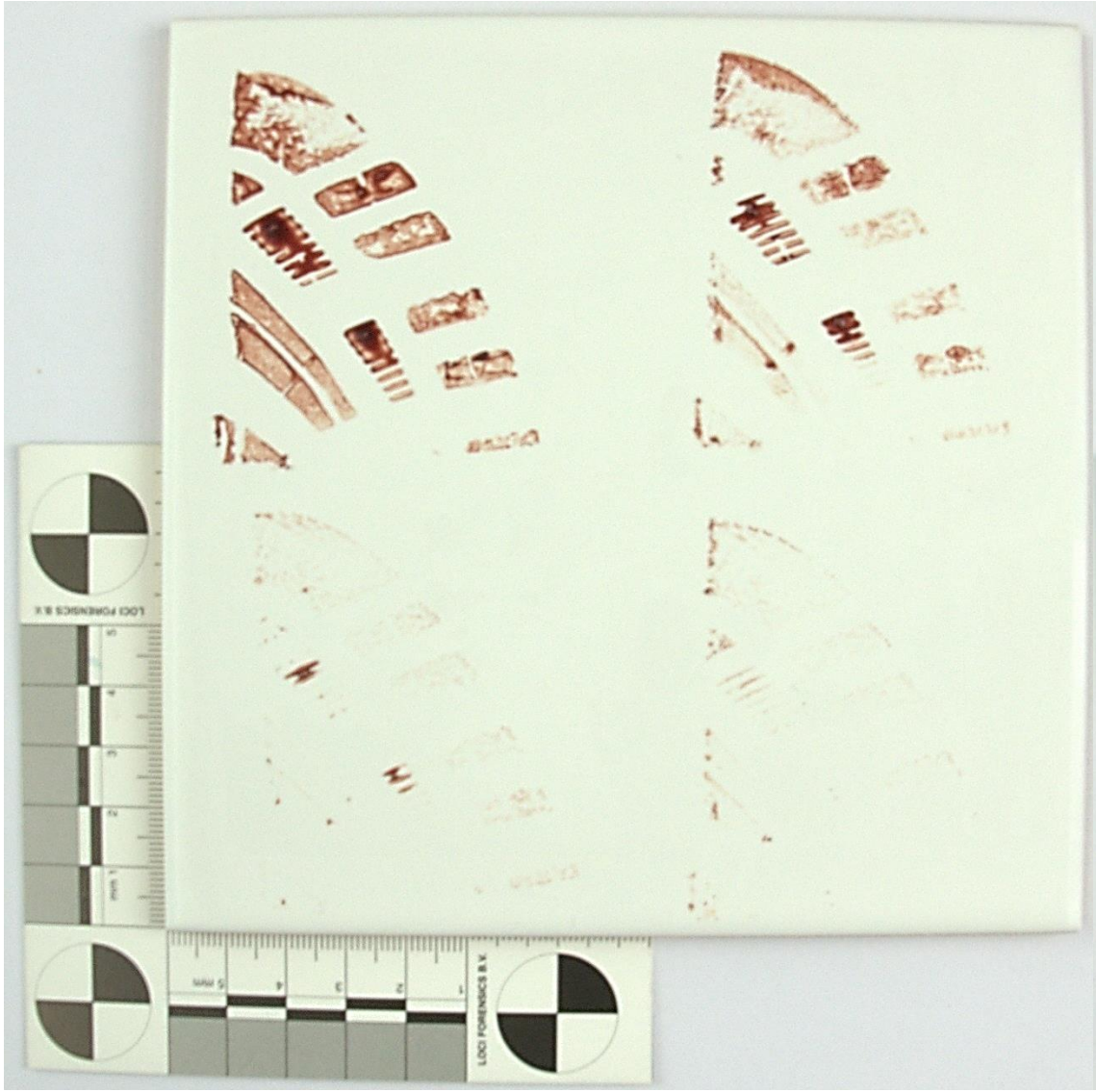
Hungarian Red 1



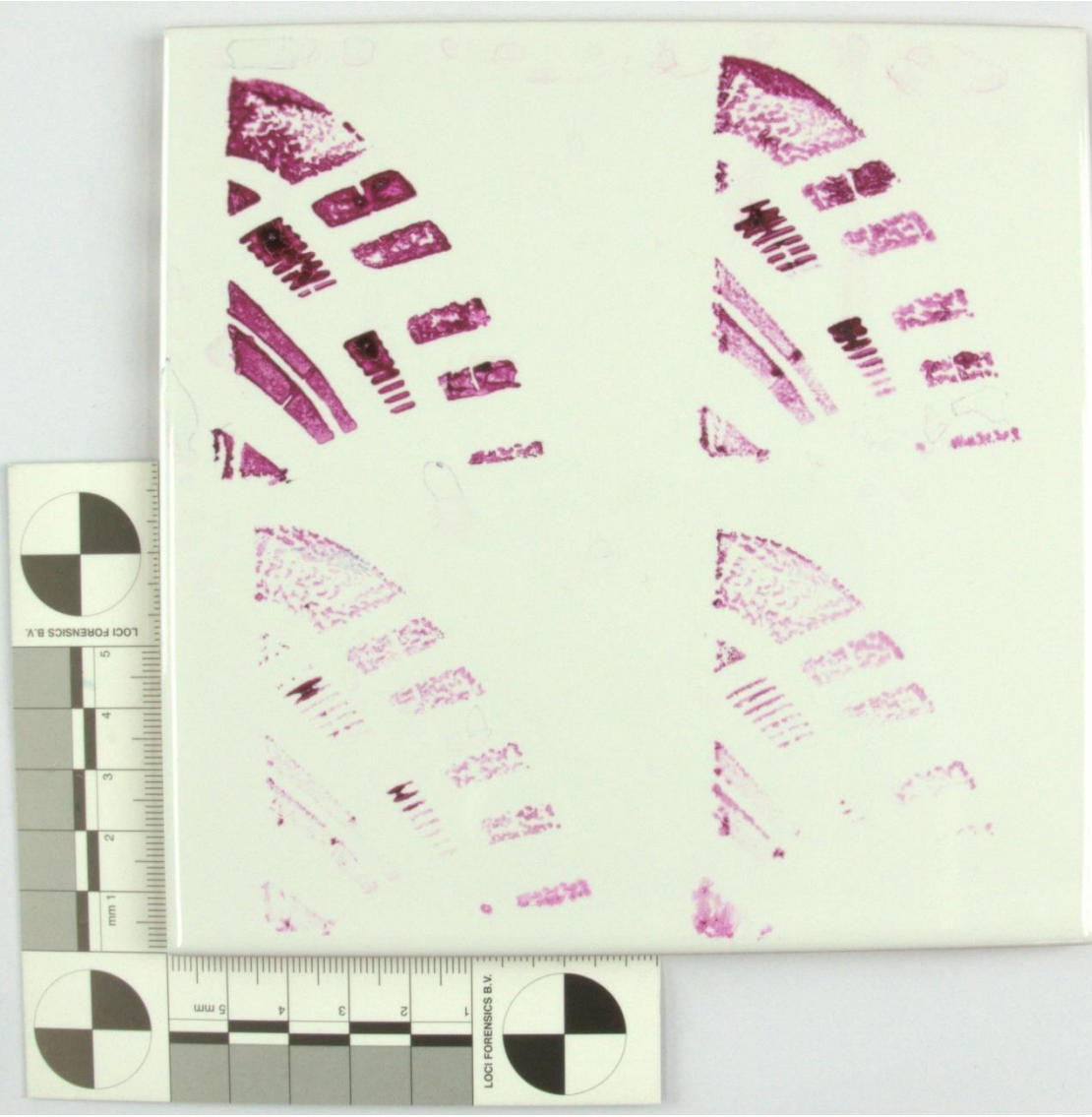
Print	Score
LB	
RB	
LO	
RO	

Print	Score
LB	
RB	
LO	
RO	

Hungarian Red 2



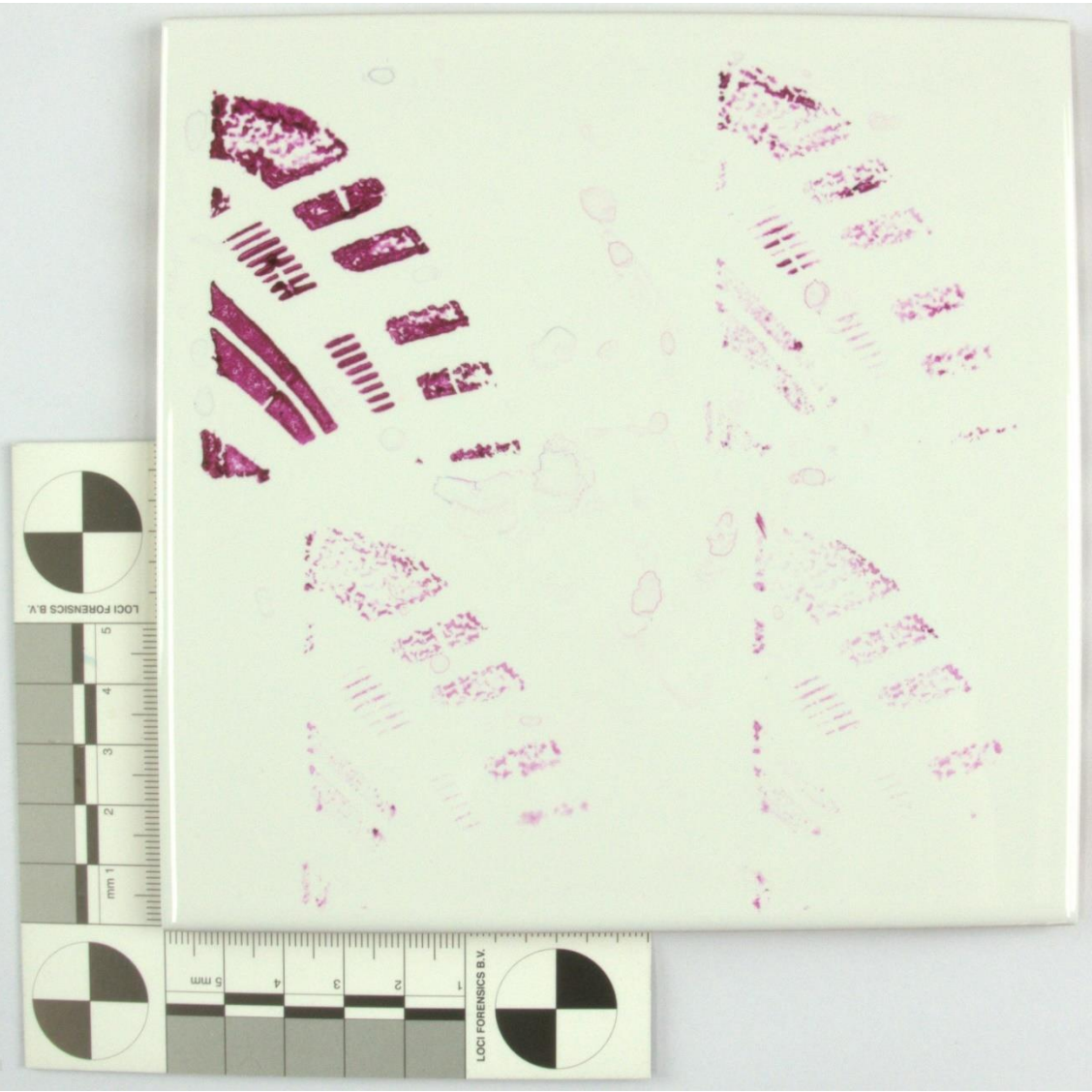
Print	Score
LB	
RB	
LO	
RO	



Print	Score
LB	
RB	
LO	
RO	

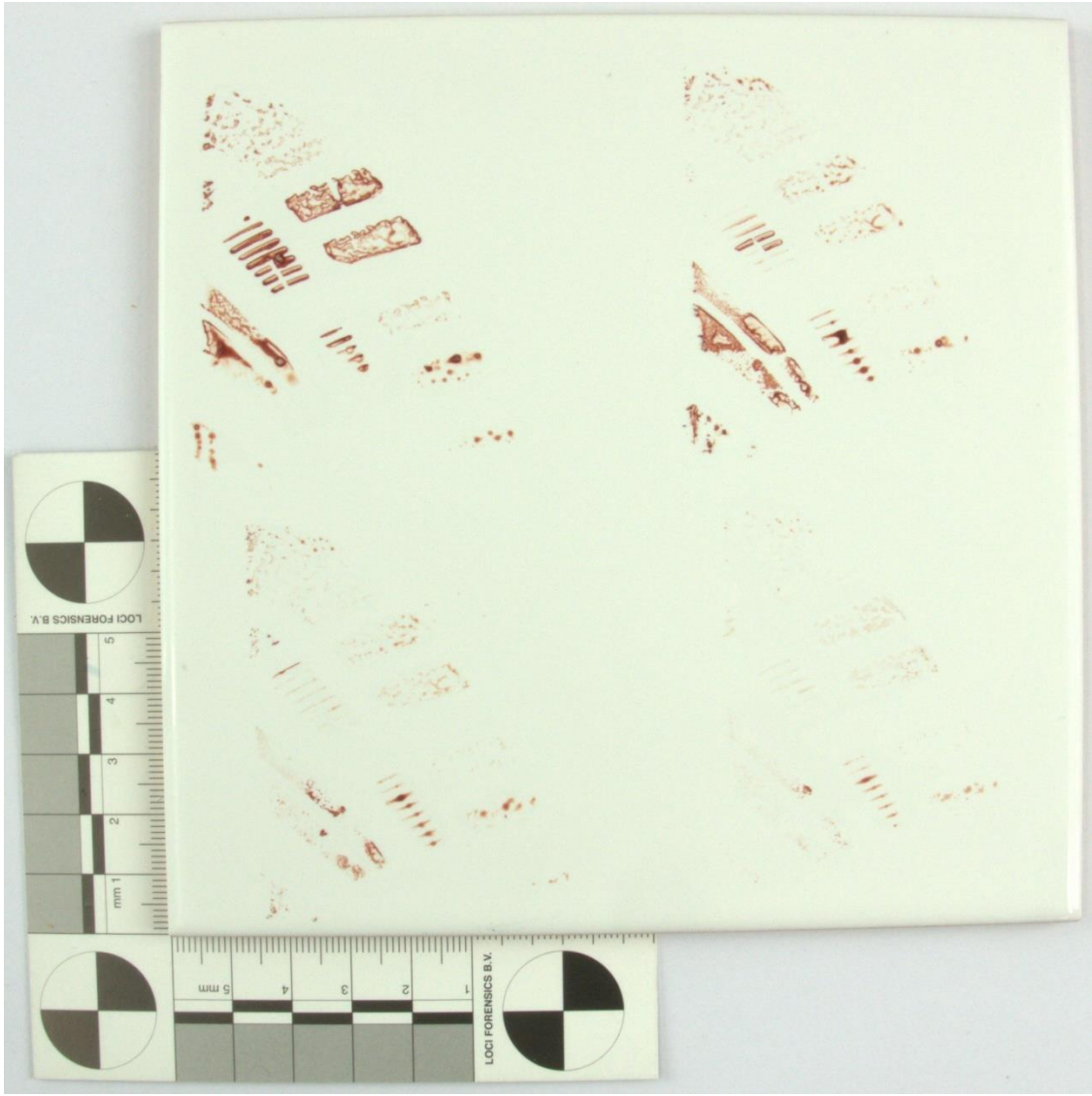
Hungarian Red 3

Print	Score
LB	
RB	
LO	
RO	



Print	Score
LB	
RB	
LO	
RO	

Hungarian Red 4

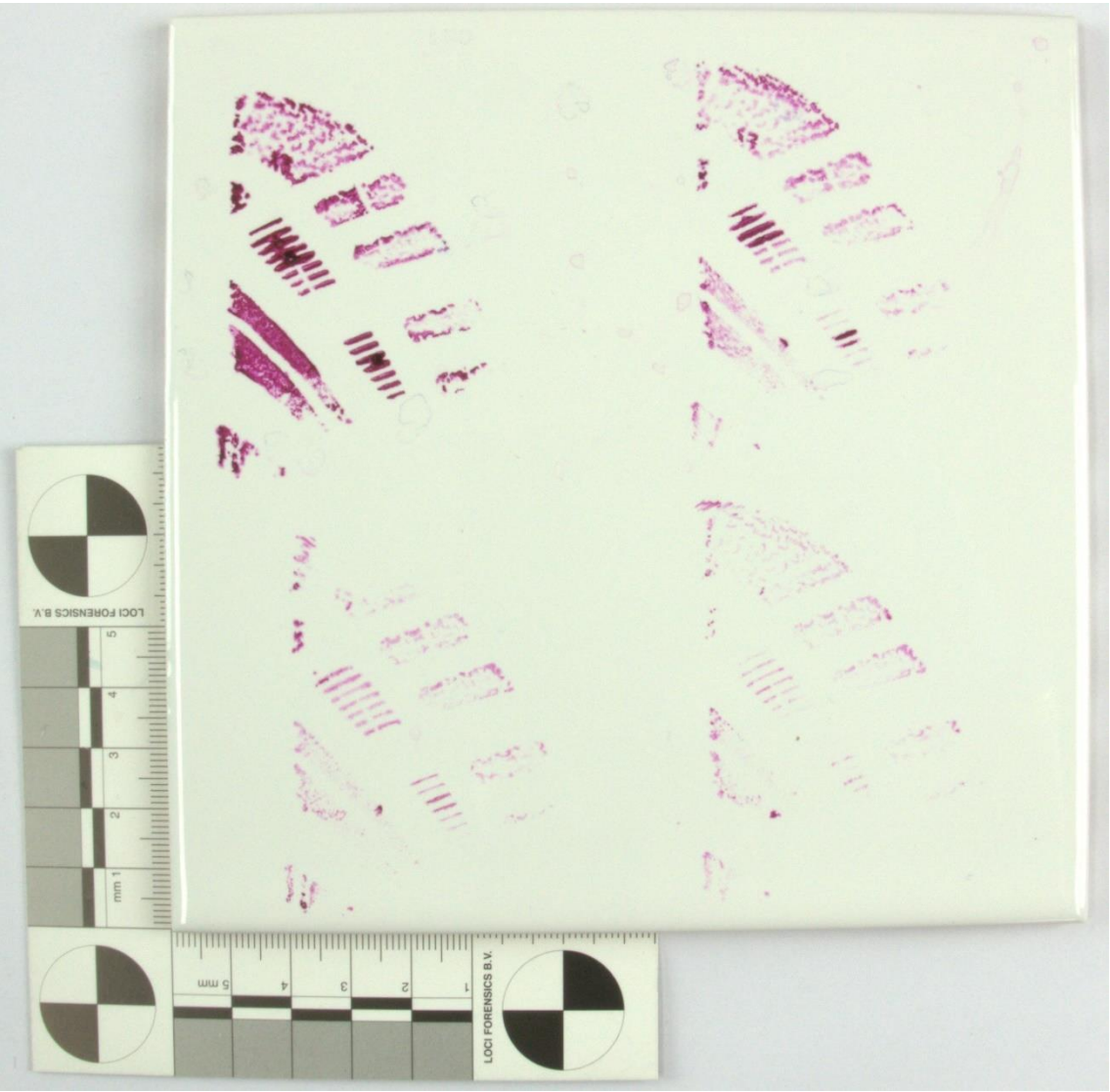


Print	Score
LB	
RB	
LO	
RO	

Print	Score
LB	
RB	
LO	
RO	

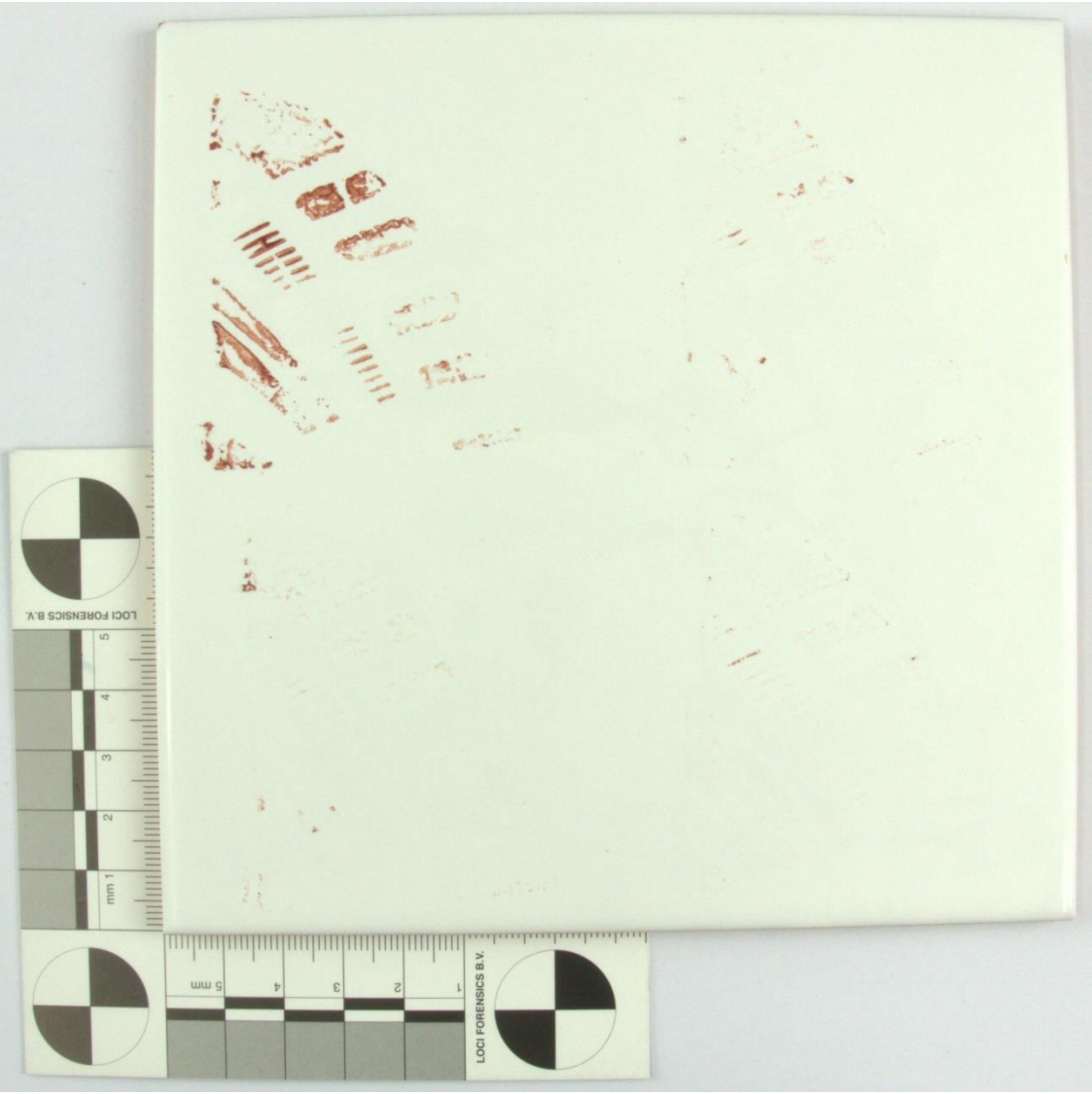
Hungarian Red 5

Print	Score
LB	
RB	
LO	
RO	

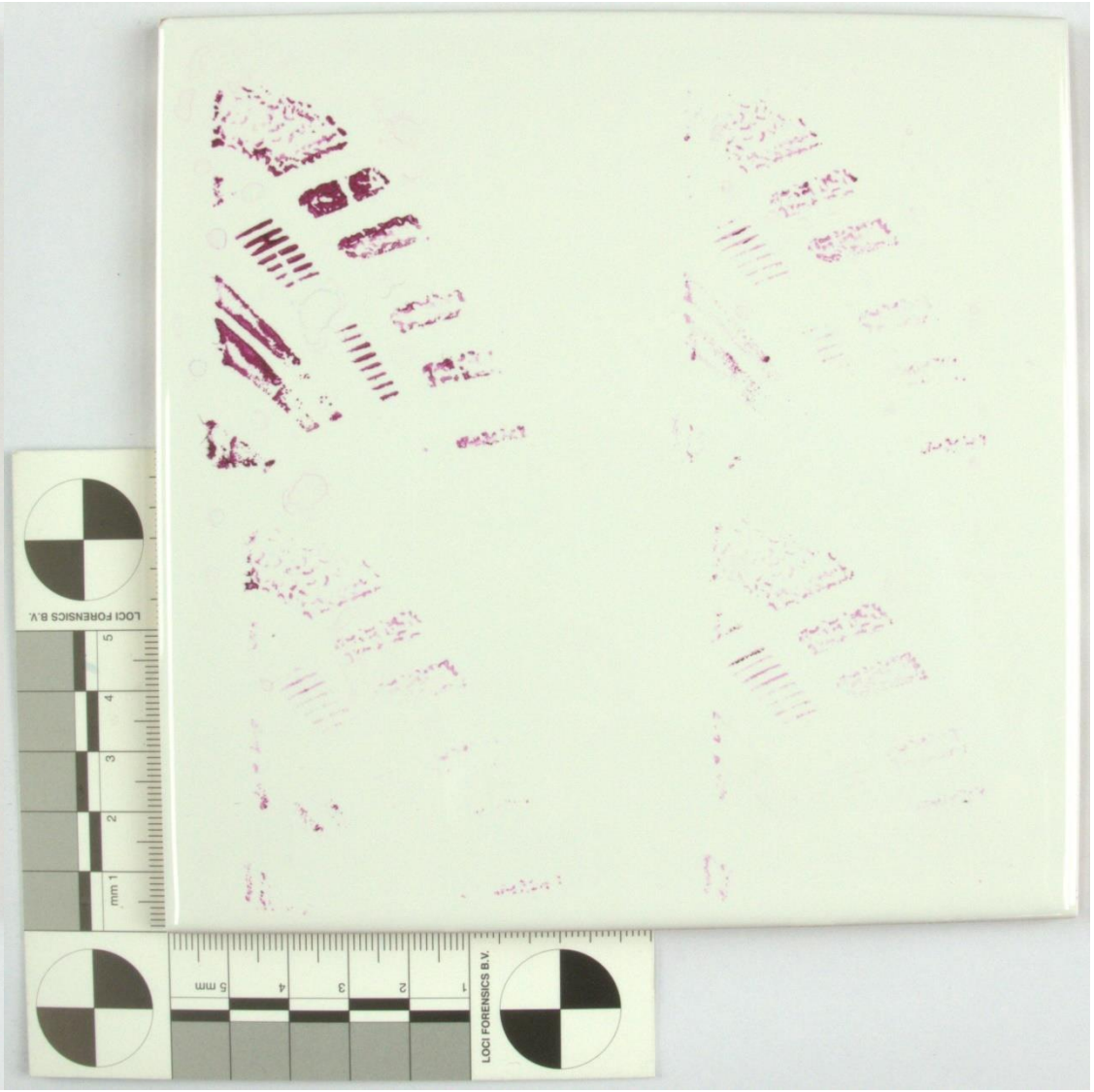


Print	Score
LB	
RB	
LO	
RO	

Hungarian Red 6



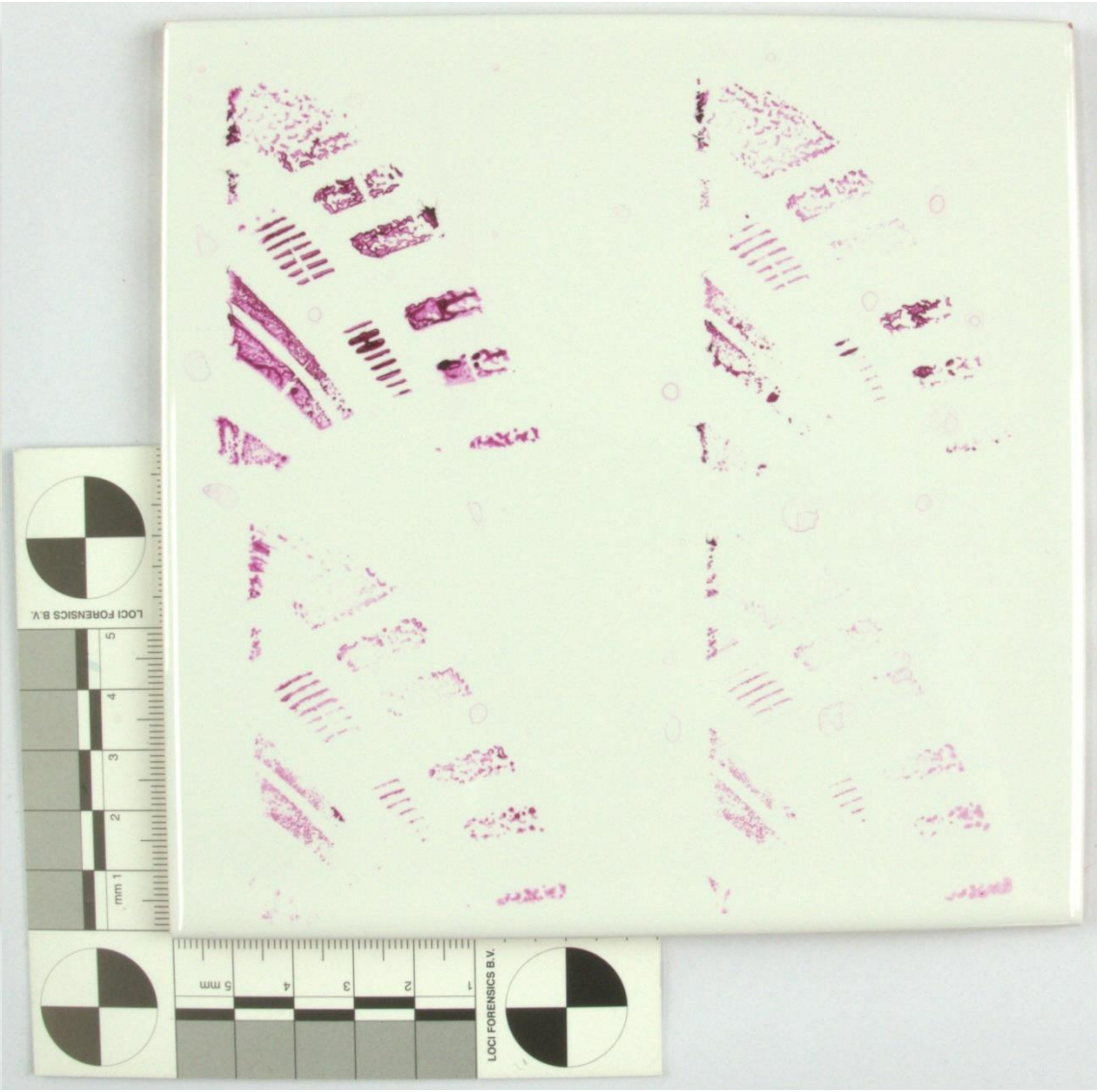
Print	Score
LB	
RB	
LO	
RO	



Print	Score
LB	
RB	
LO	
RO	

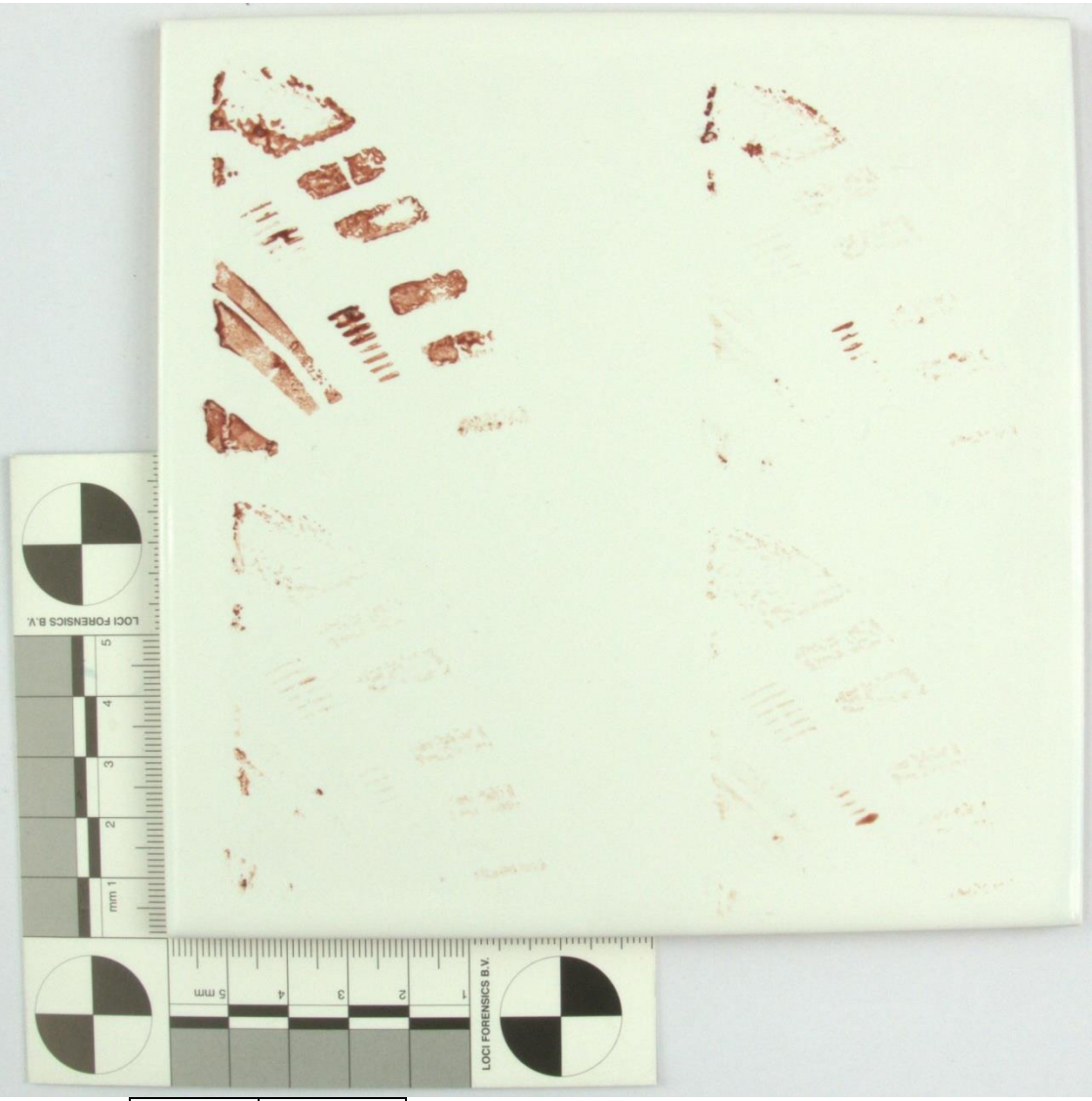
Hungarian Red 7

Print	Score
LB	
RB	
LO	
RO	

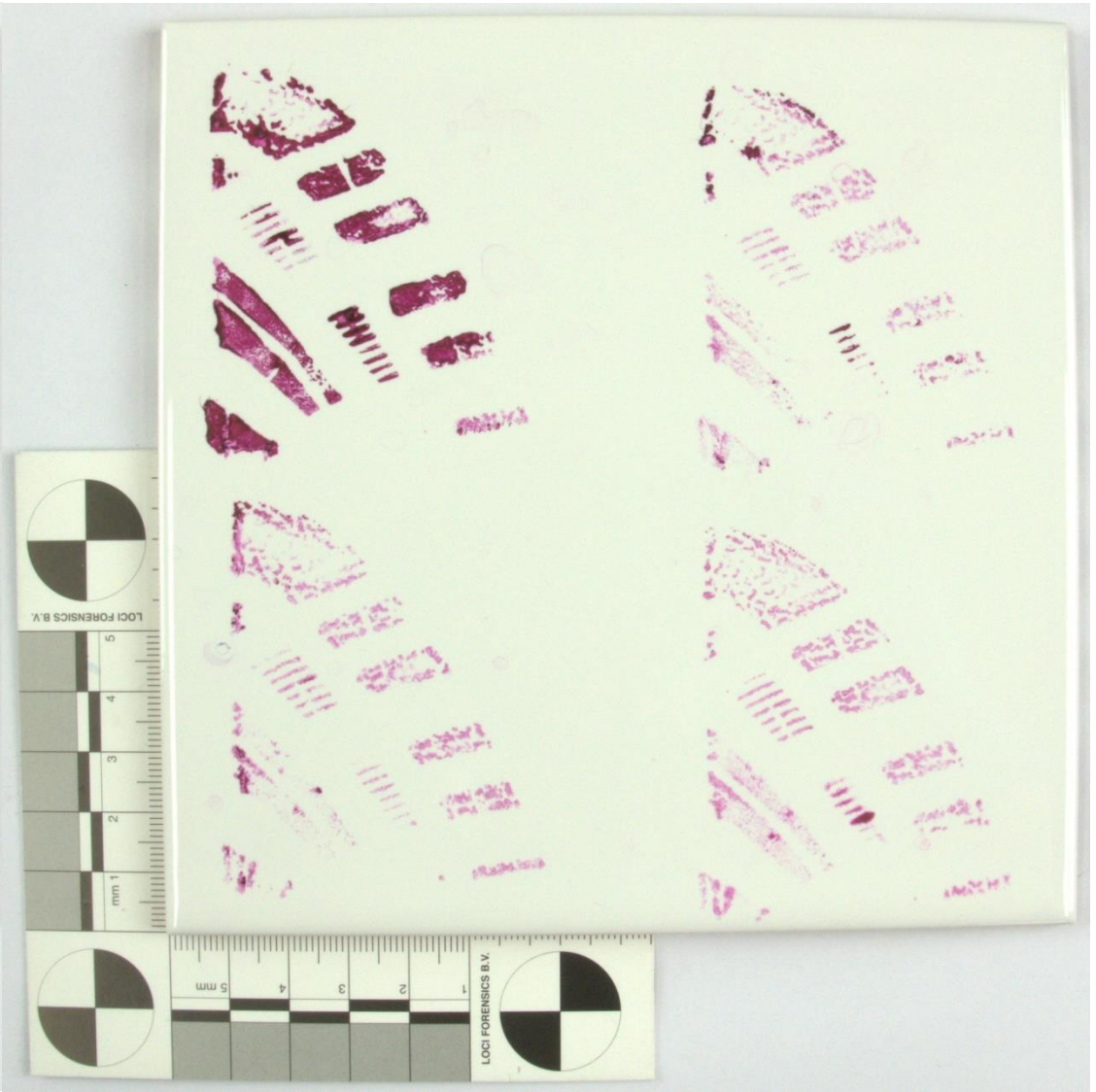


Print	Score
LB	
RB	
LO	
RO	

Hungarian Red 8



Print	Score
LB	
RB	
LO	
RO	



Print	Score
LB	
RB	
LO	
RO	

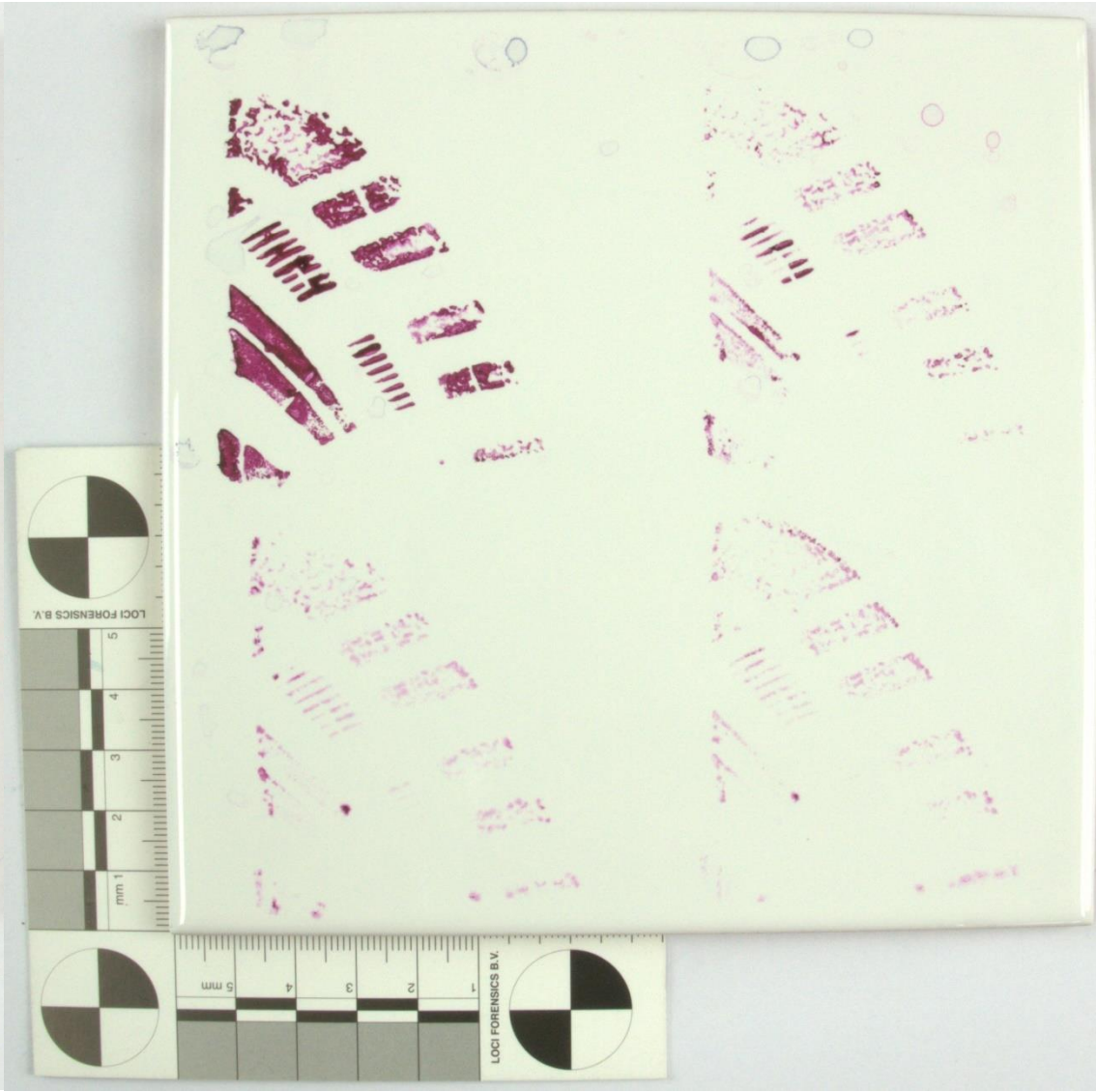
Hungarian Red 9

Print	Score
LB	
RB	
LO	
RO	

Print	Score
LB	
RB	
LO	
RO	

Hungarian Red 10

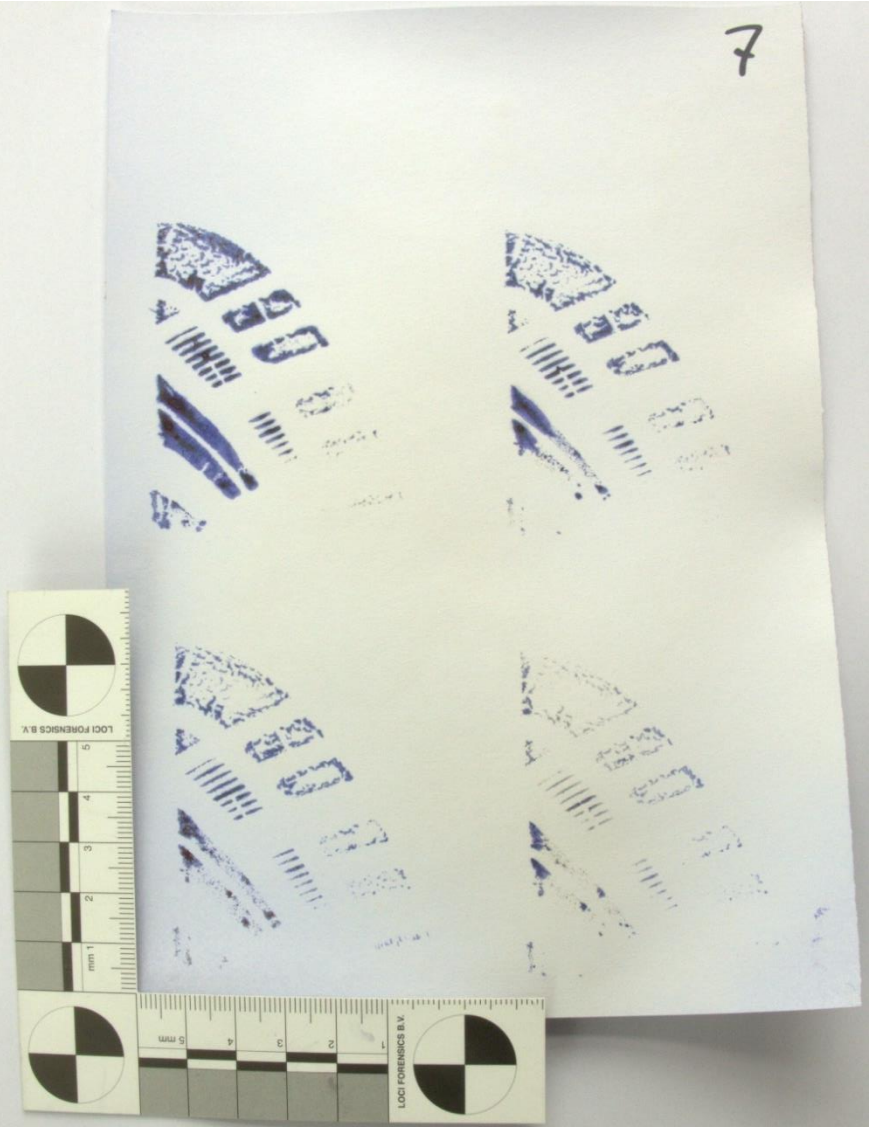
Print	Score
LB	
RB	
LO	
RO	



Print	Score
LB	
RB	
LO	
RO	

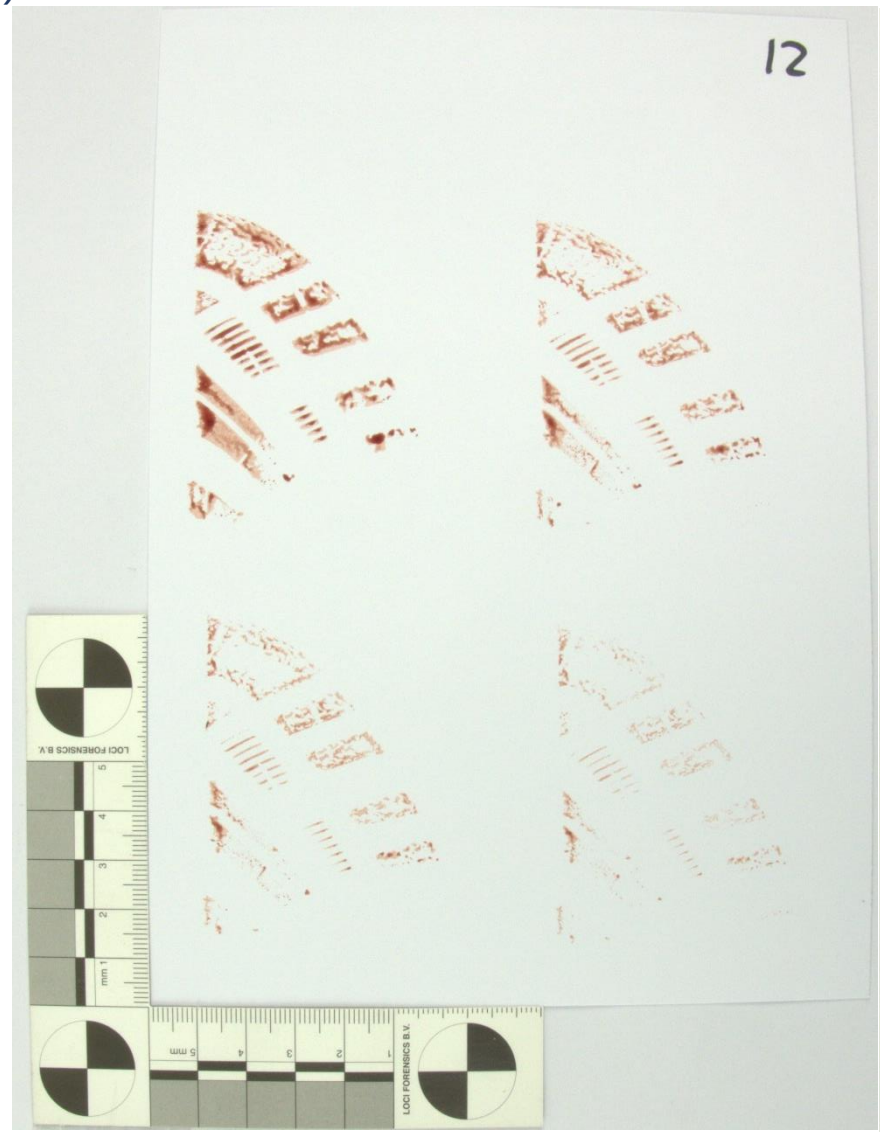
(A)LCV 1

Print	Score
LB	
RB	
LO	
RO	

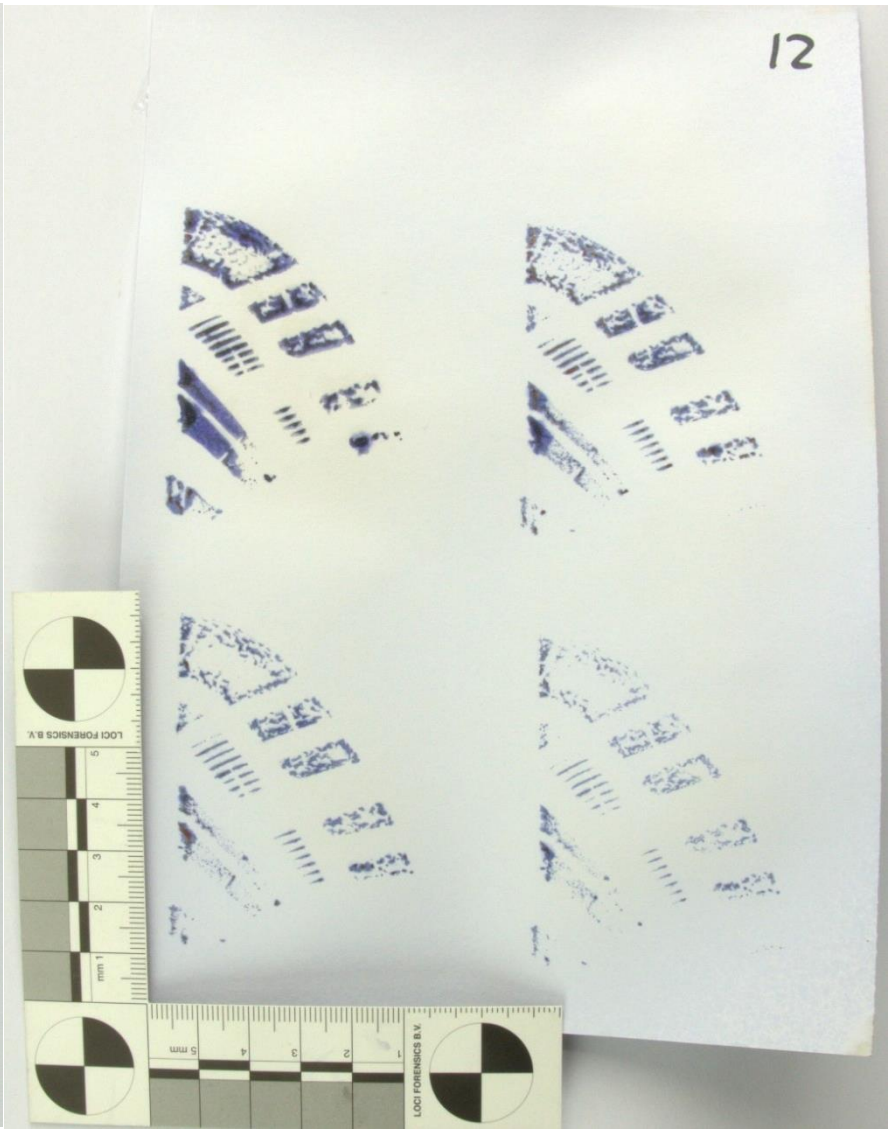


Print	Score
LB	
RB	
LO	
RO	

(A)LCV 2



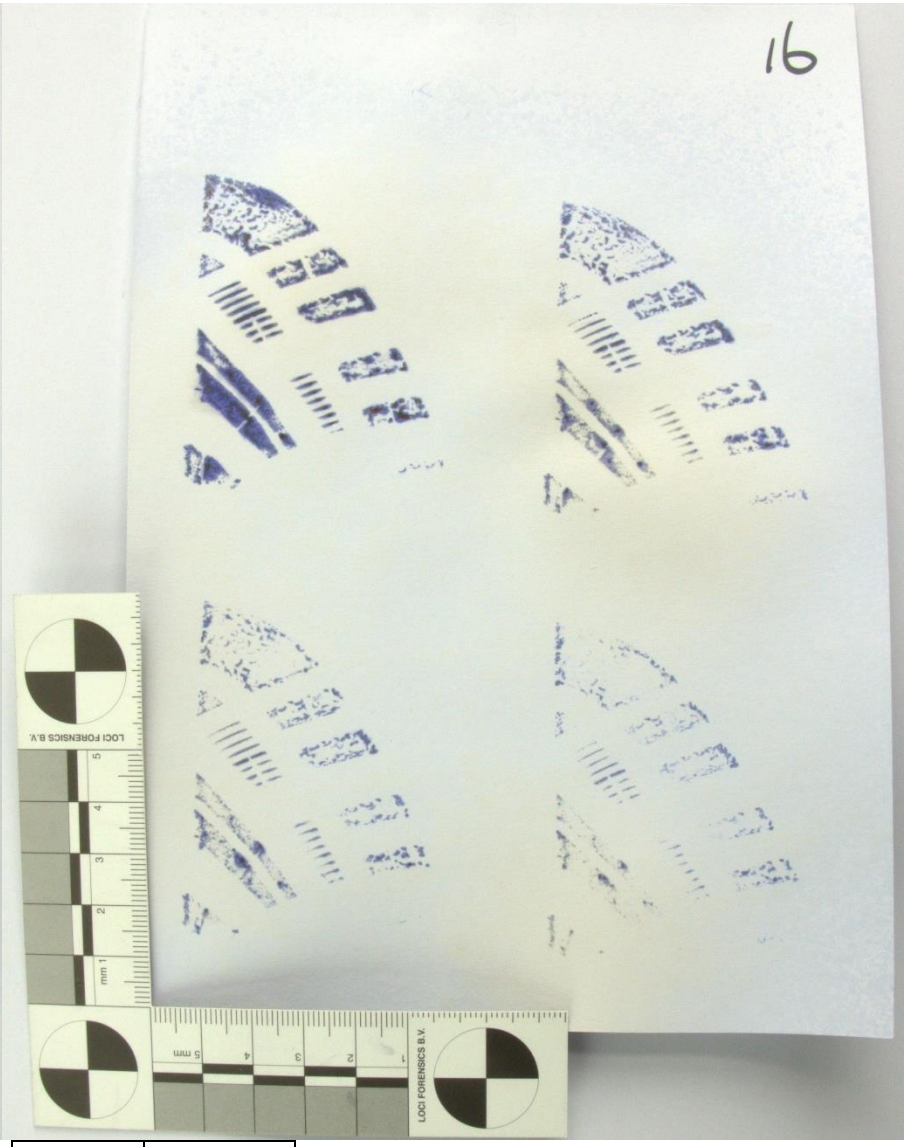
Print	Score
LB	
RB	
LO	
RO	



Print	Score
LB	
RB	
LO	
RO	

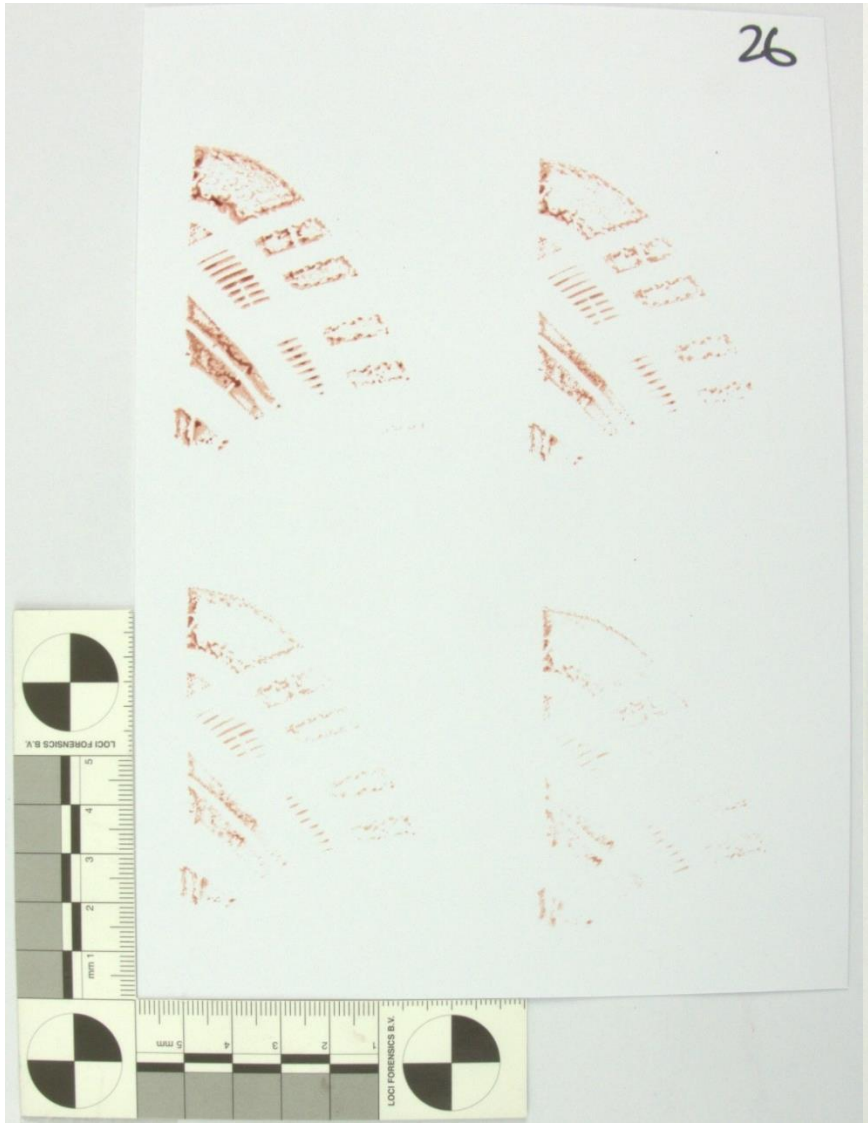
(A)LCV 3

Print	Score
LB	
RB	
LO	
RO	

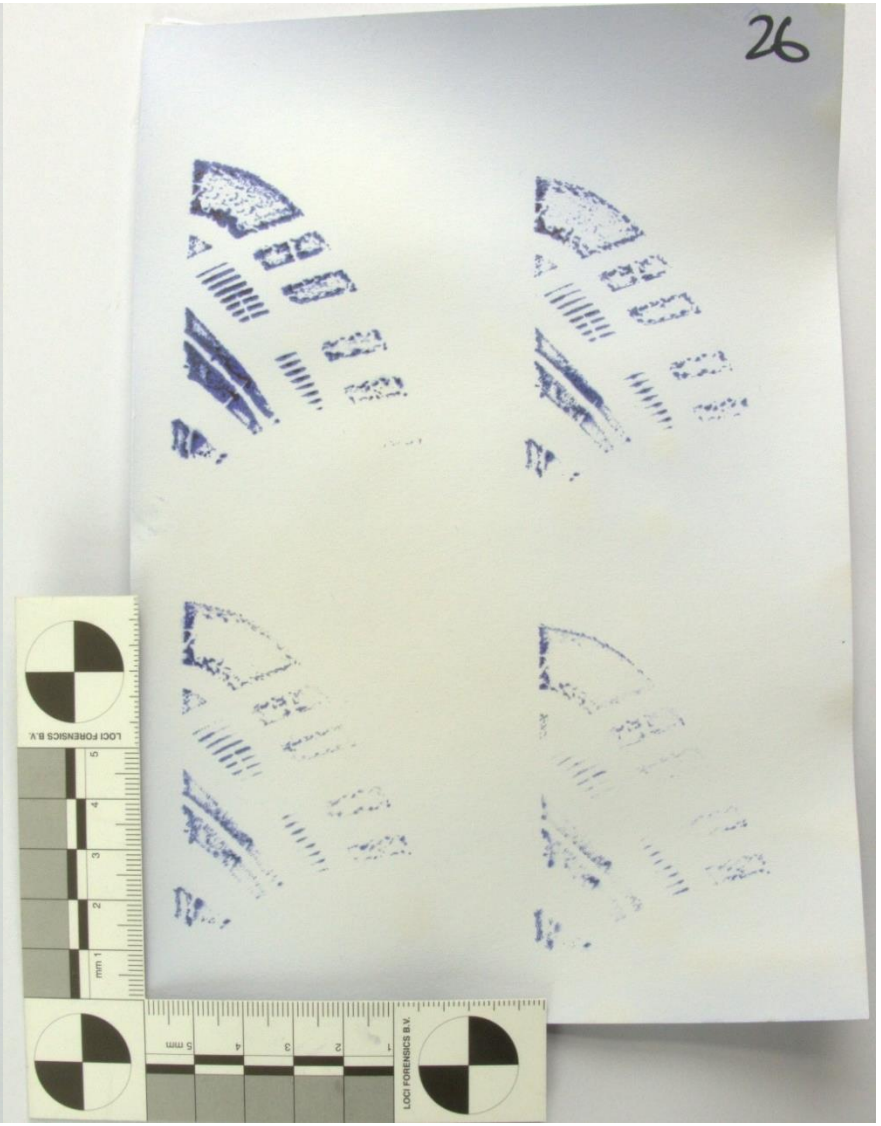


Print	Score
LB	
RB	
LO	
RO	

(A)LCV 4



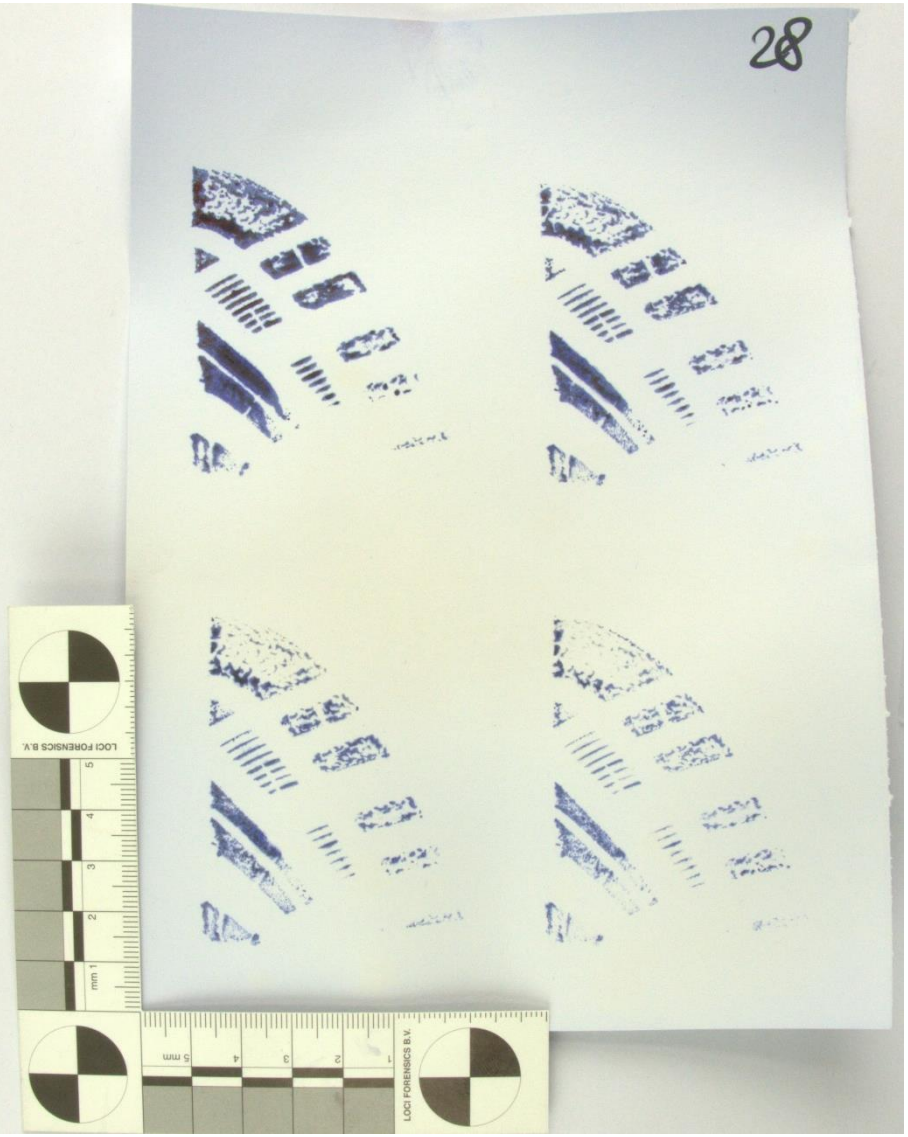
Print	Score
LB	
RB	
LO	
RO	



Print	Score
LB	
RB	
LO	
RO	

(A)LCV 5

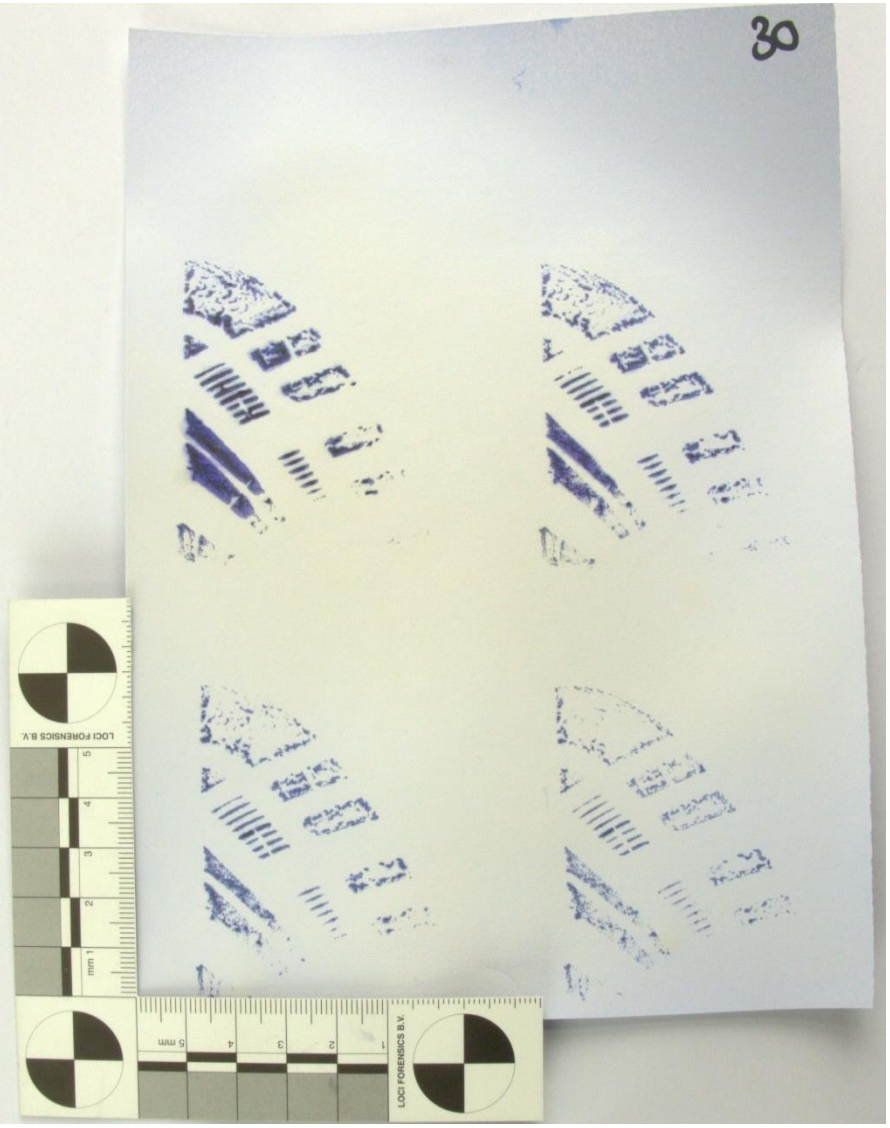
Print	Score
LB	
RB	
LO	
RO	



Print	Score
LB	
RB	
LO	
RO	

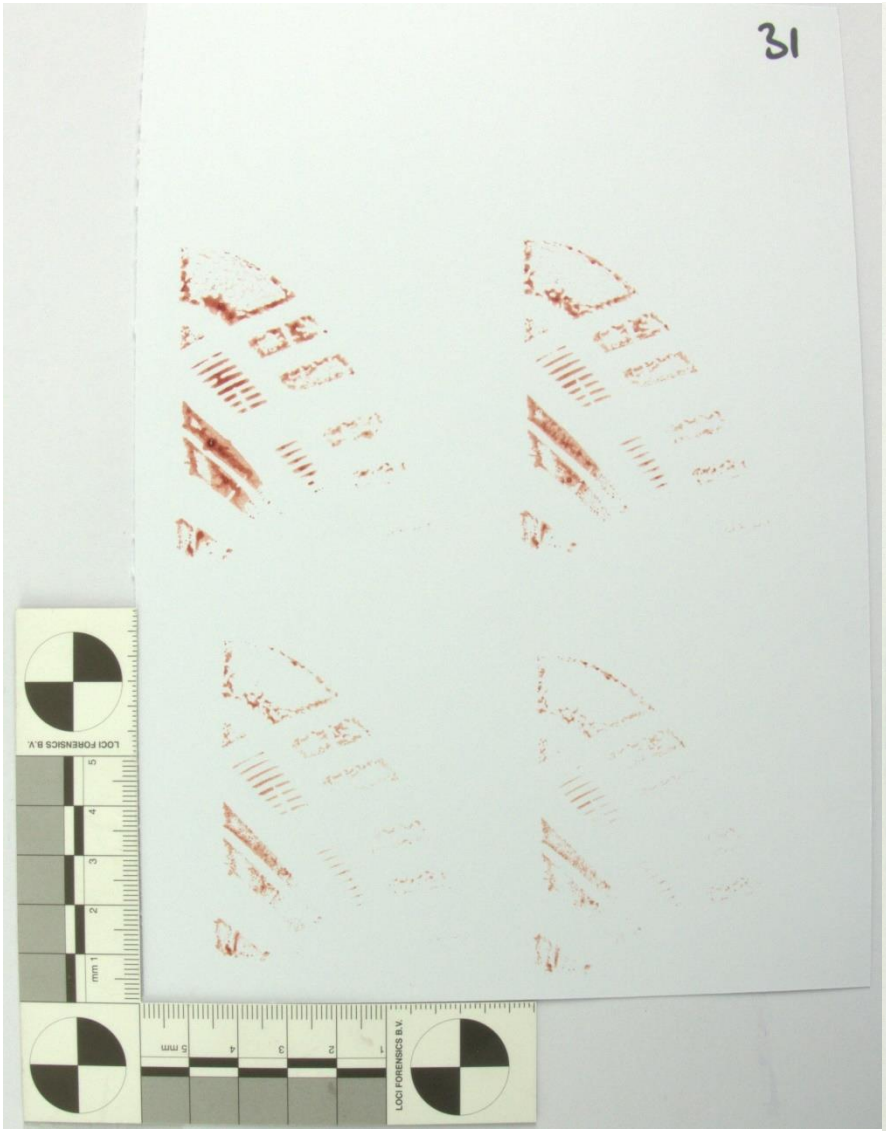
(A)LCV 6

Print	Score
LB	
RB	
LO	
RO	

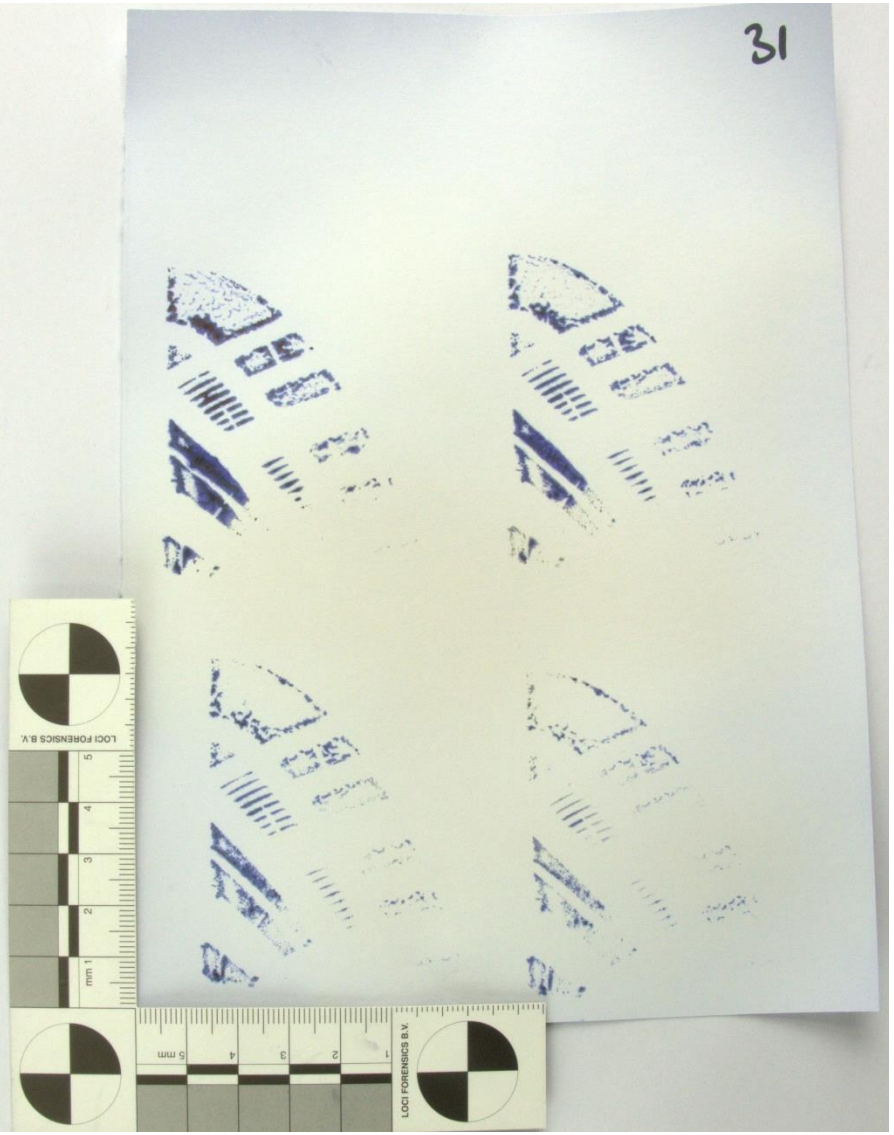


Print	Score
LB	
RB	
LO	
RO	

(A)LCV 7



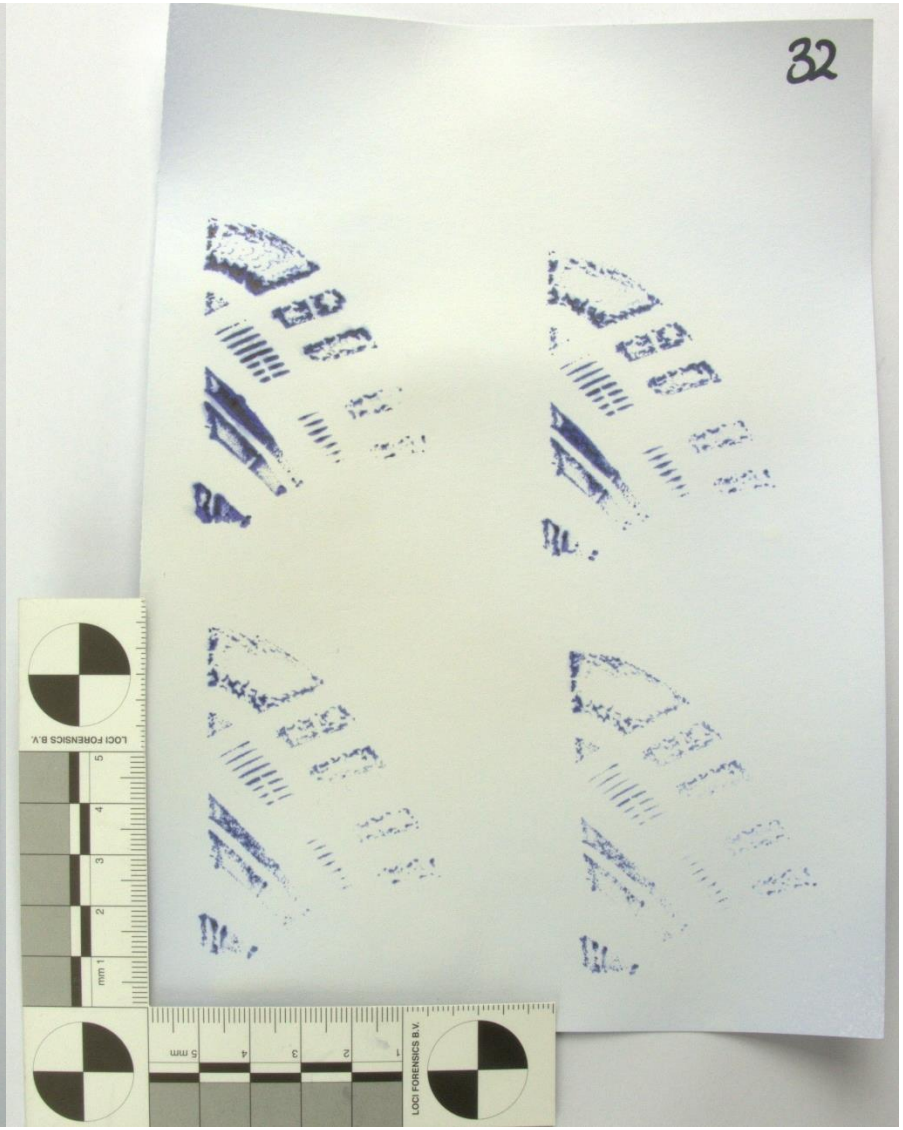
Print	Score
LB	
RB	
LO	
RO	



Print	Score
LB	
RB	
LO	
RO	

(A)LCV 8

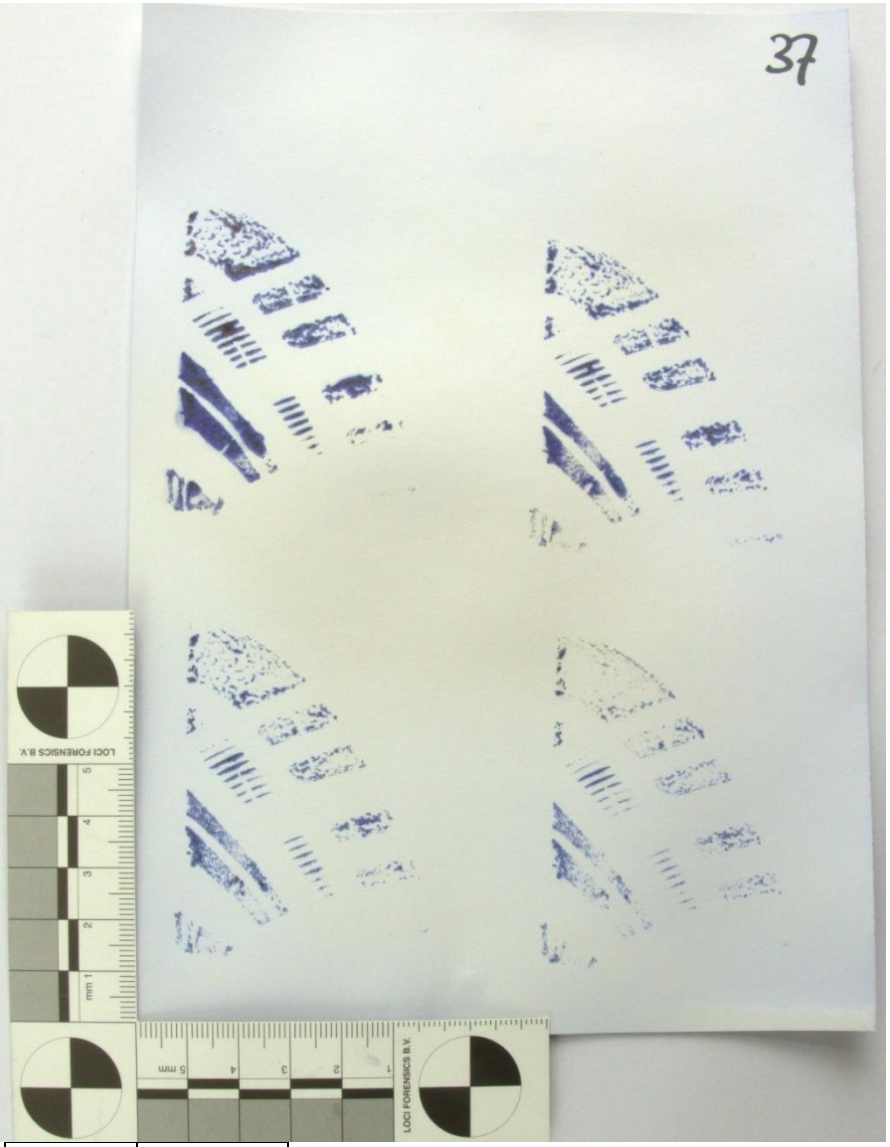
Print	Score
LB	
RB	
LO	
RO	



Print	Score
LB	
RB	
LO	
RO	

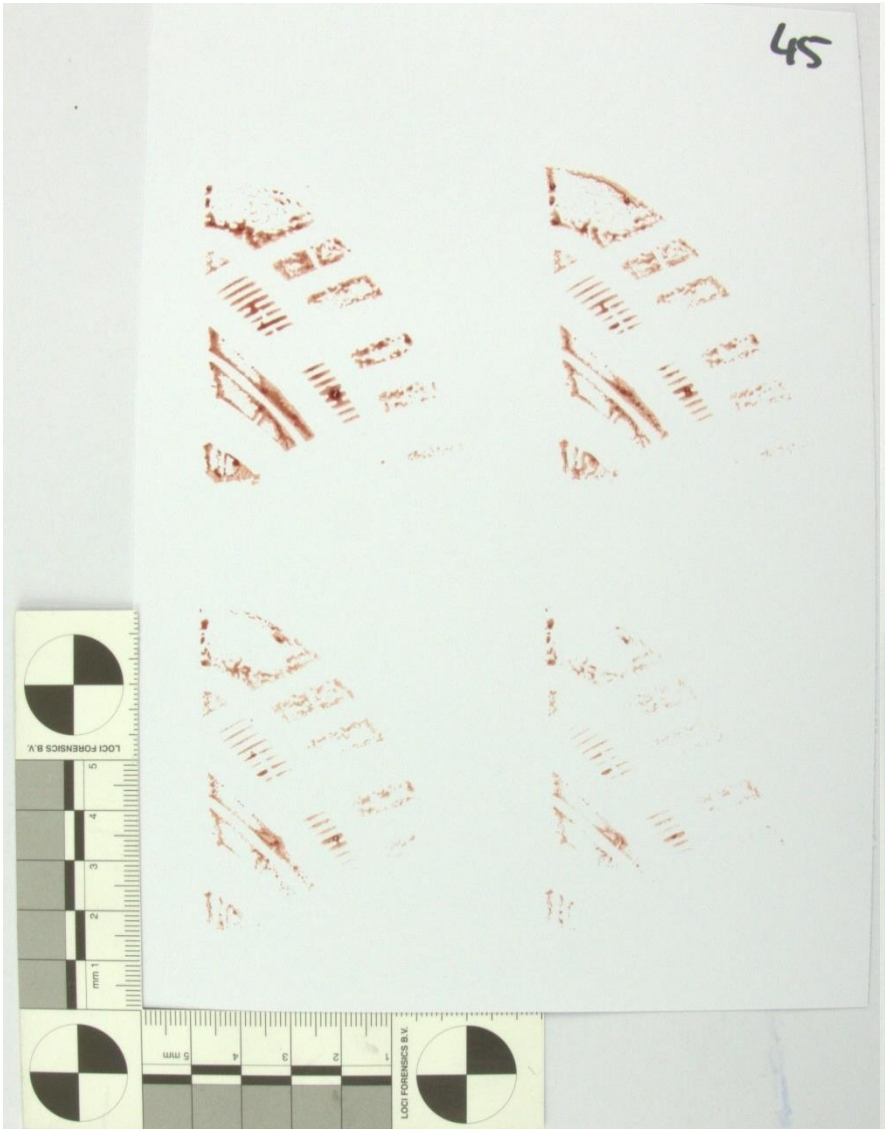
(A)LCV 9

Print	Score
LB	
RB	
LO	
RO	

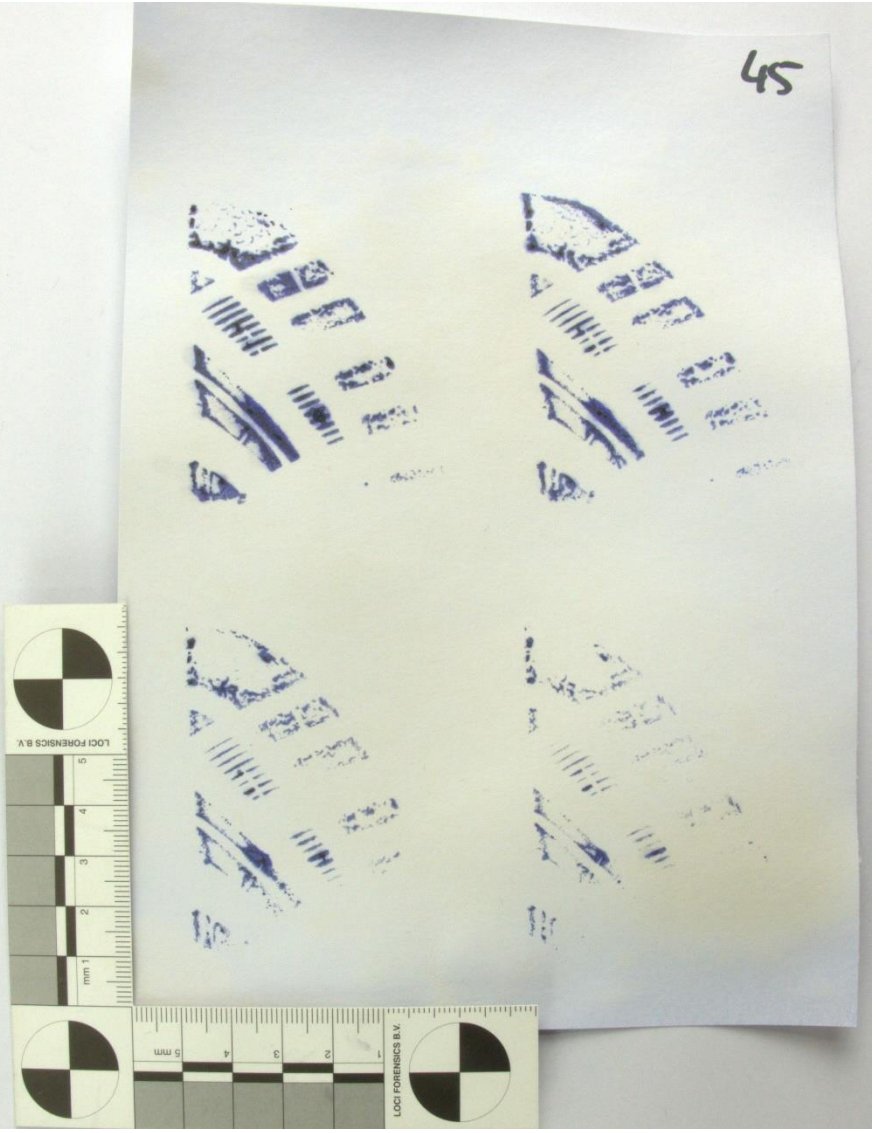


Print	Score
LB	
RB	
LO	
RO	

(A)LCV 10

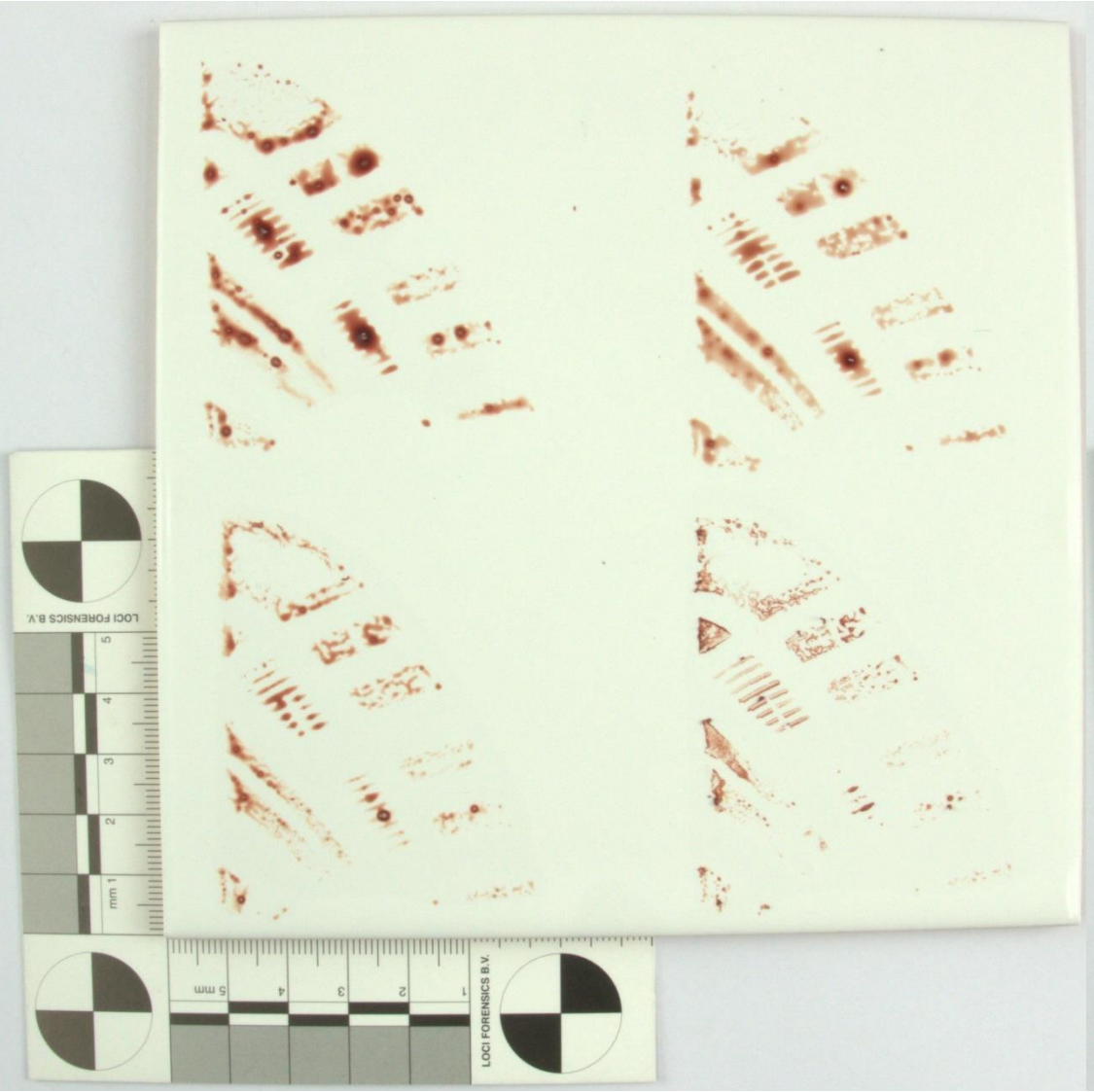


Print	Score
LB	
RB	
LO	
RO	

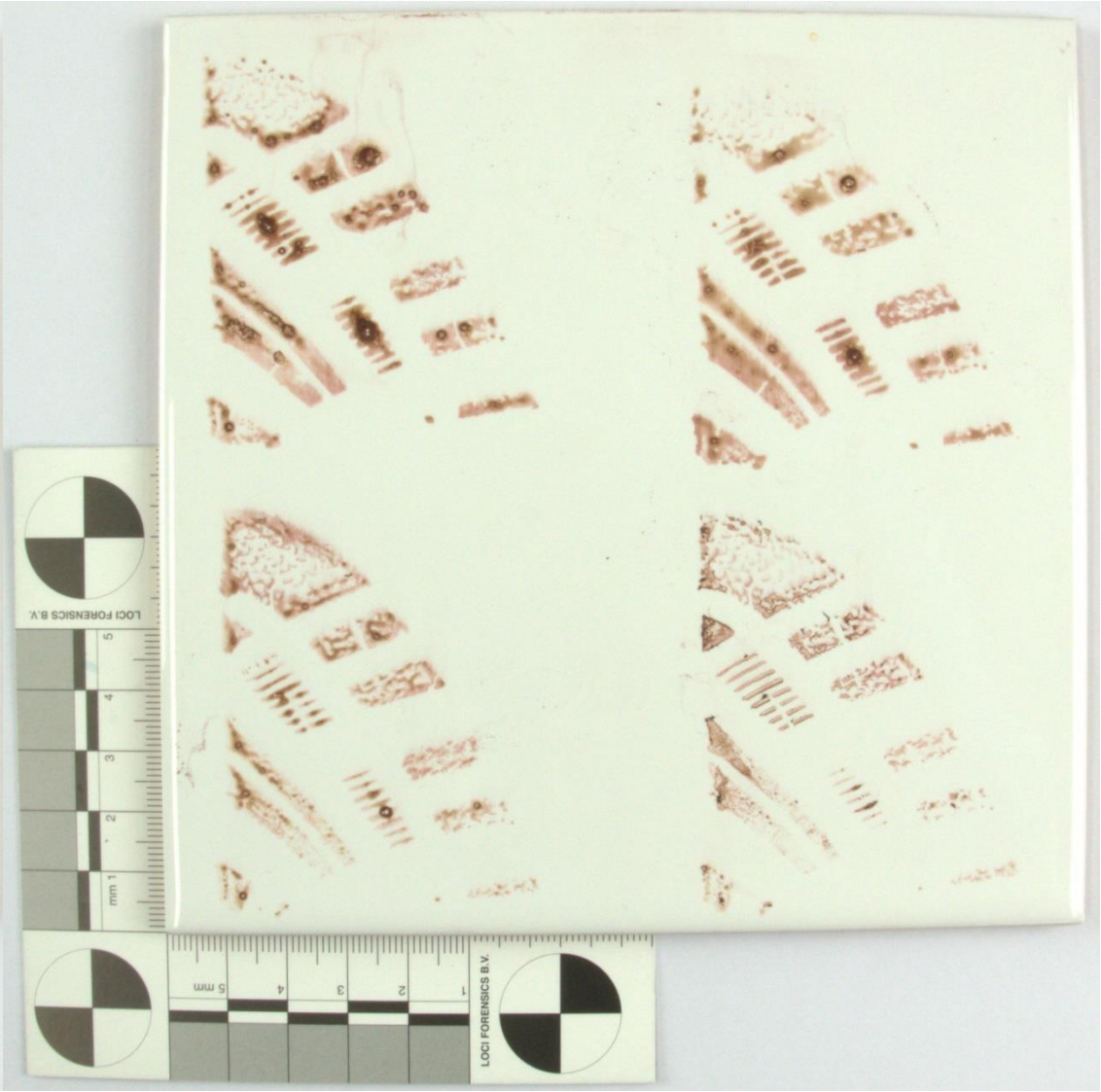


Print	Score
LB	
RB	
LO	
RO	

Iron oxide 1

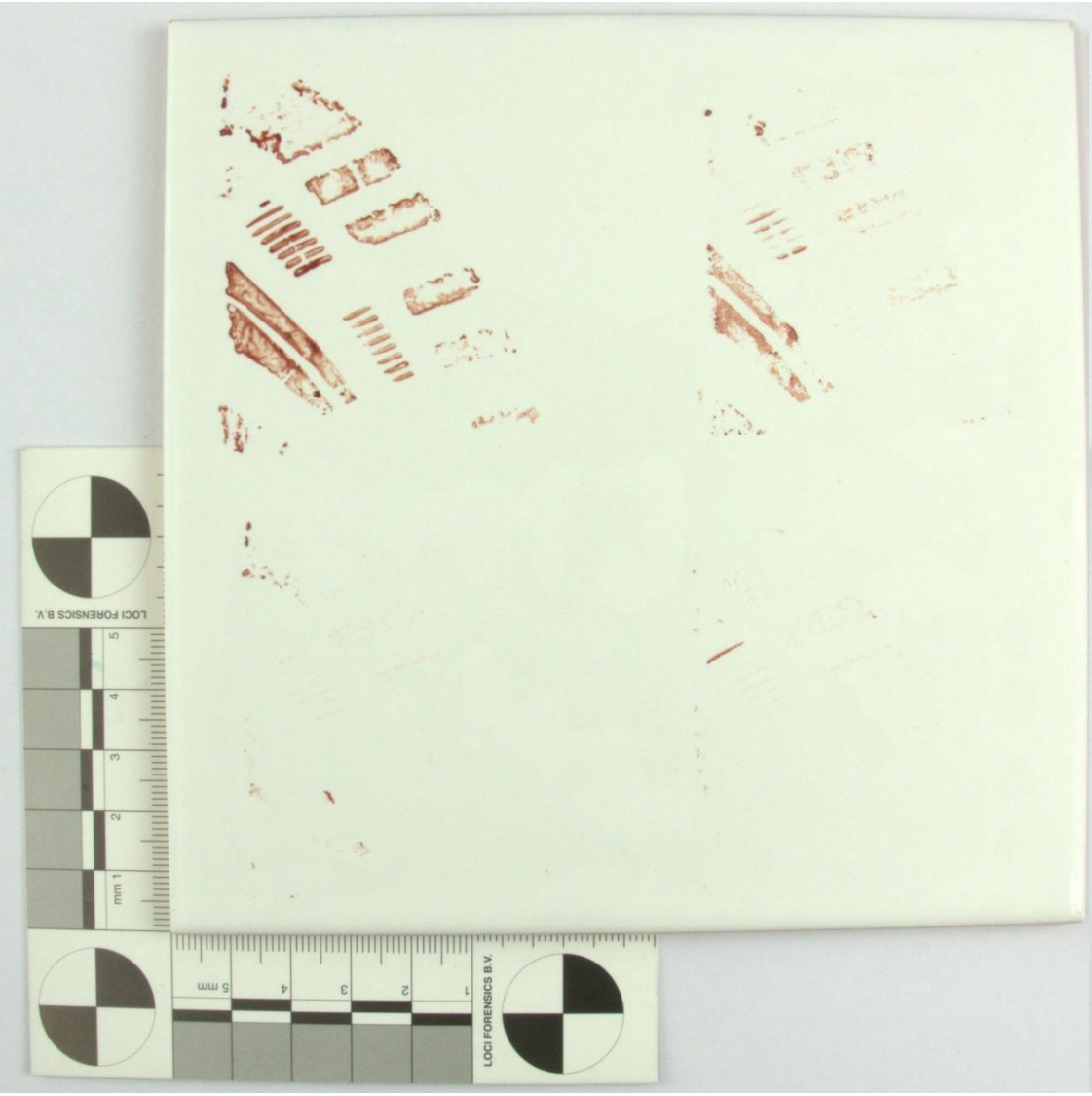


Print	Score
LB	
RB	
LO	
RO	



Print	Score
LB	
RB	
LO	
RO	

Iron oxide 2

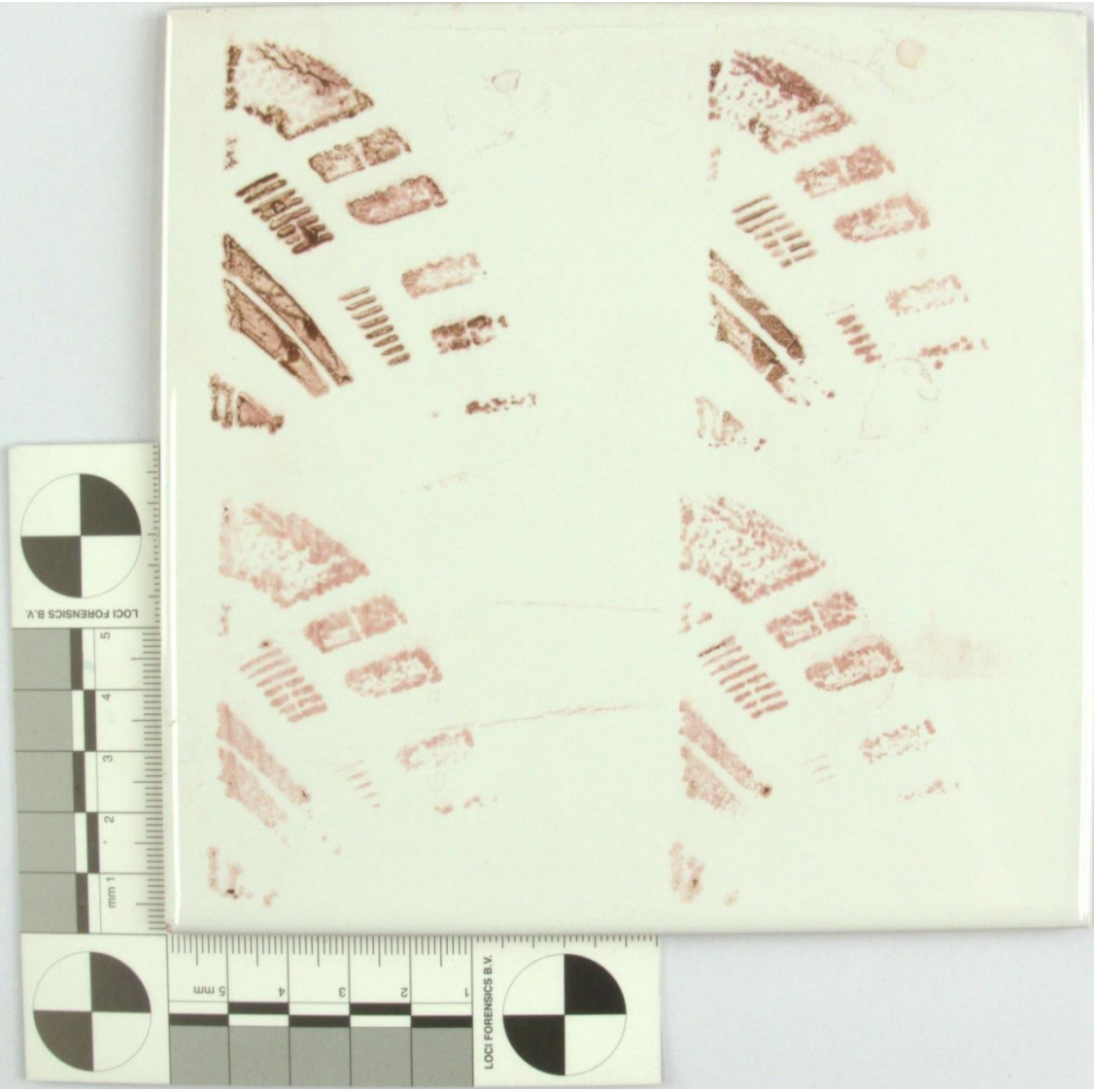


Print	Score
LB	
RB	
LO	
RO	

Print	Score
LB	
RB	
LO	
RO	

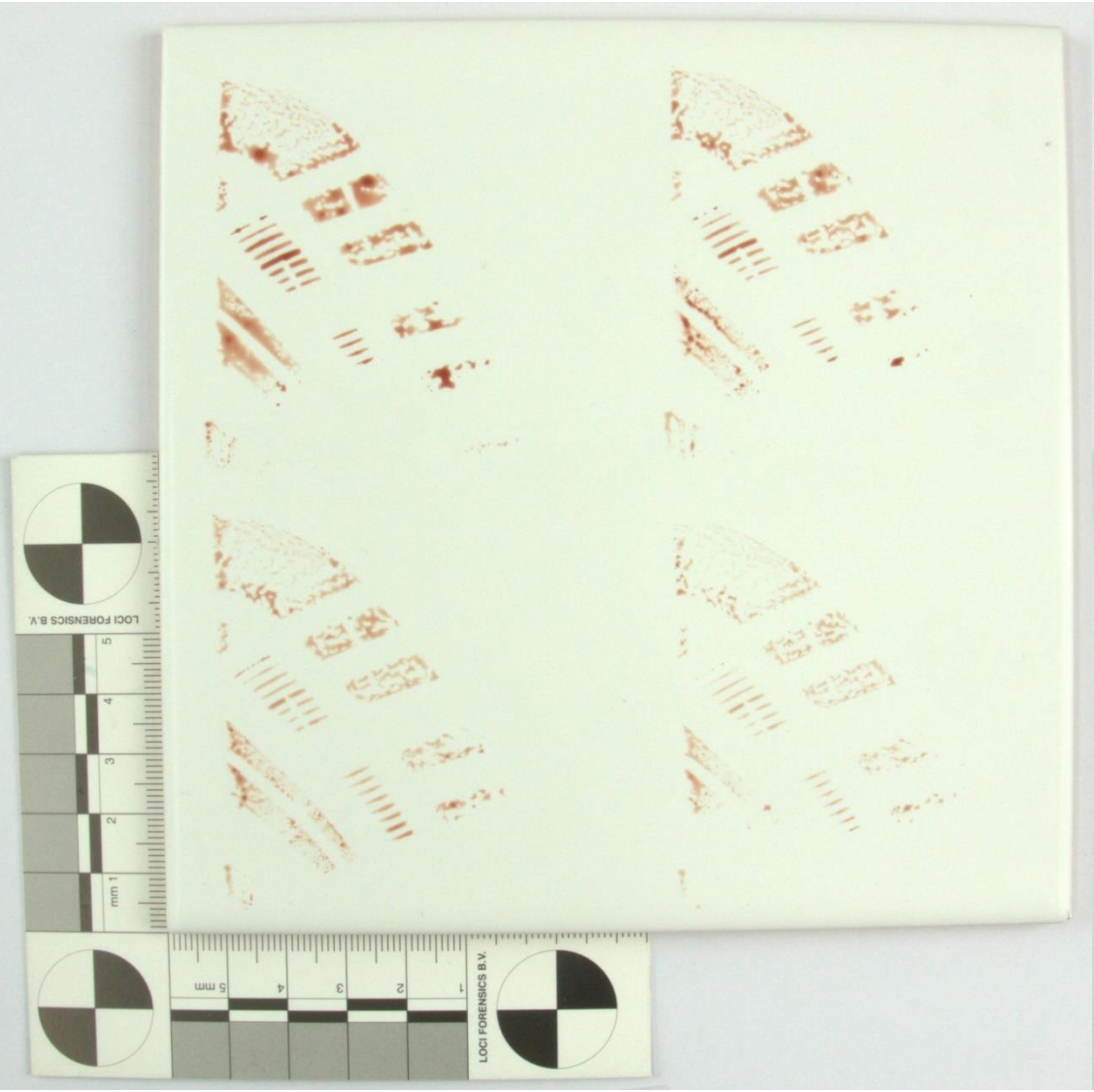
Iron oxide 3

Print	Score
LB	
RB	
LO	
RO	

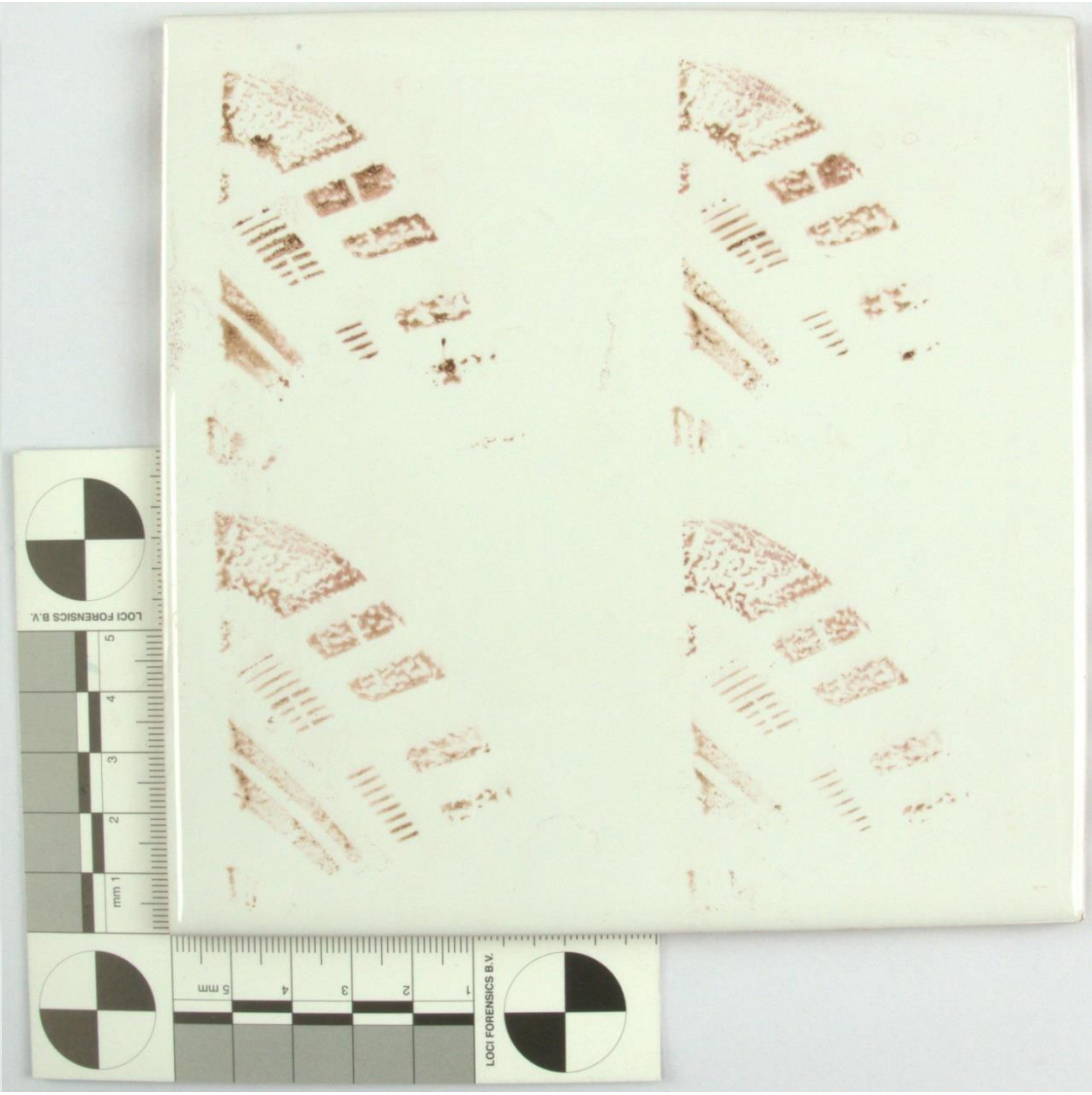


Print	Score
LB	
RB	
LO	
RO	

Iron oxide 4

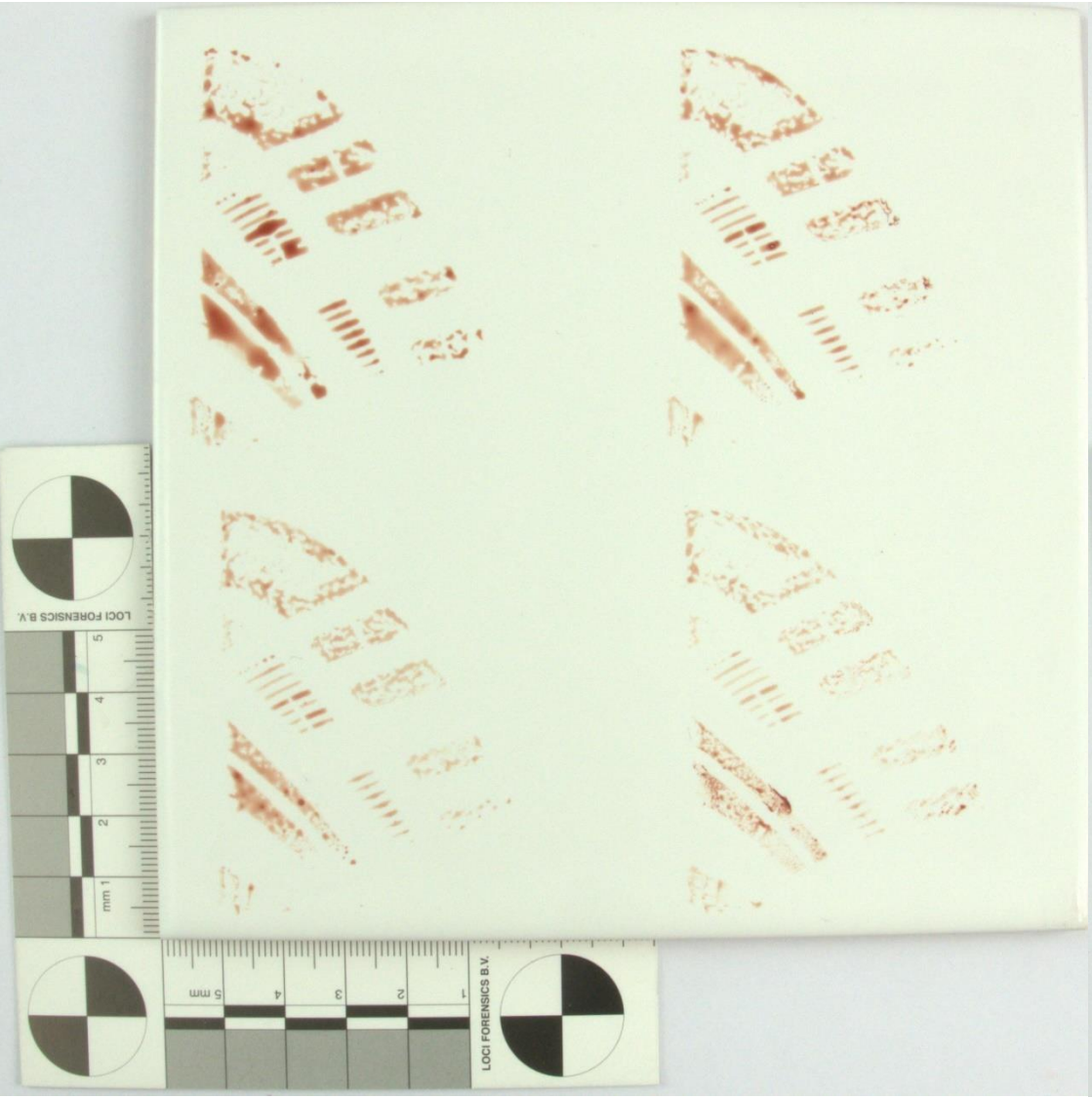


Print	Score
LB	
RB	
LO	
RO	

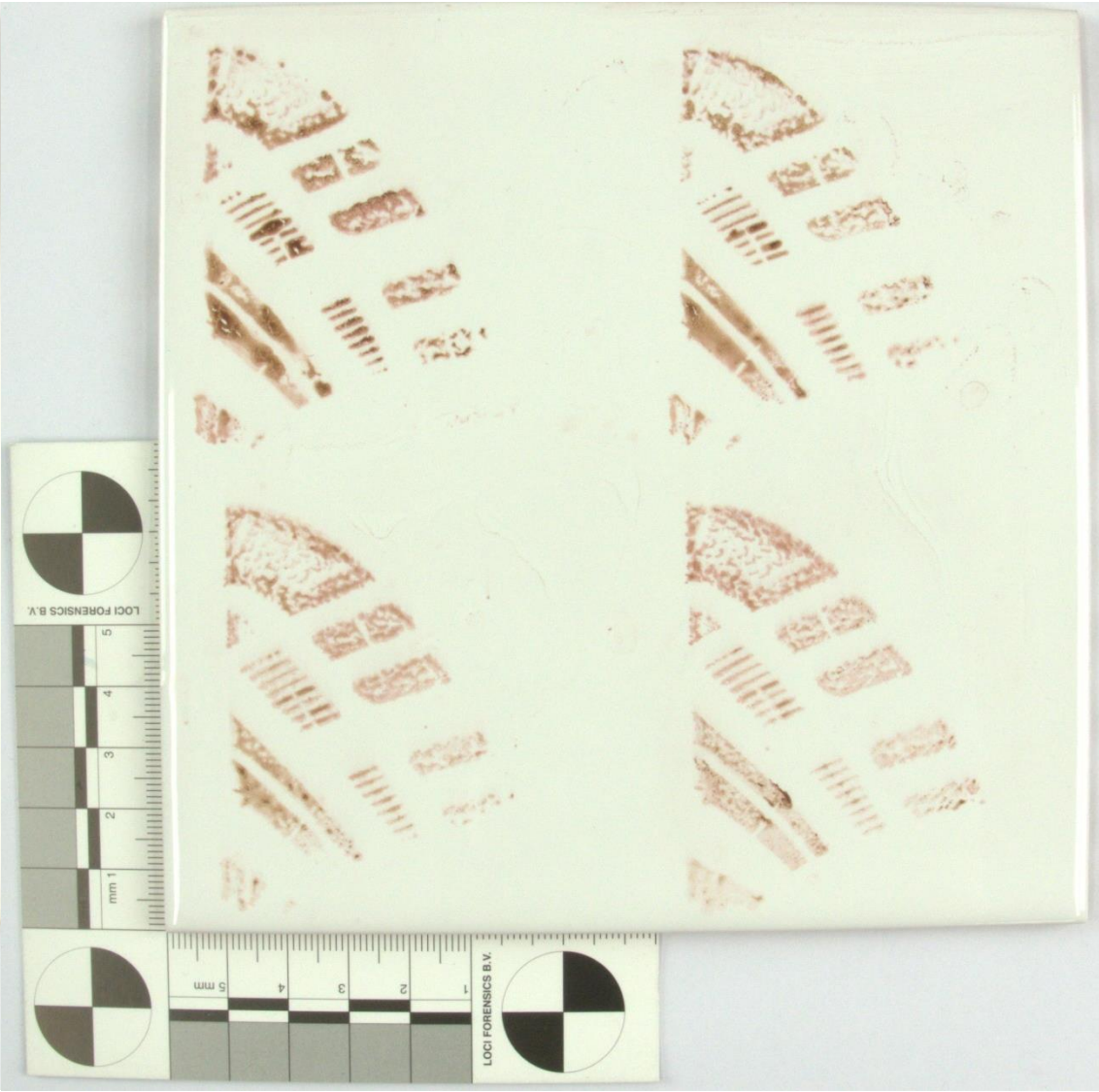


Print	Score
LB	
RB	
LO	
RO	

Iron oxide 5

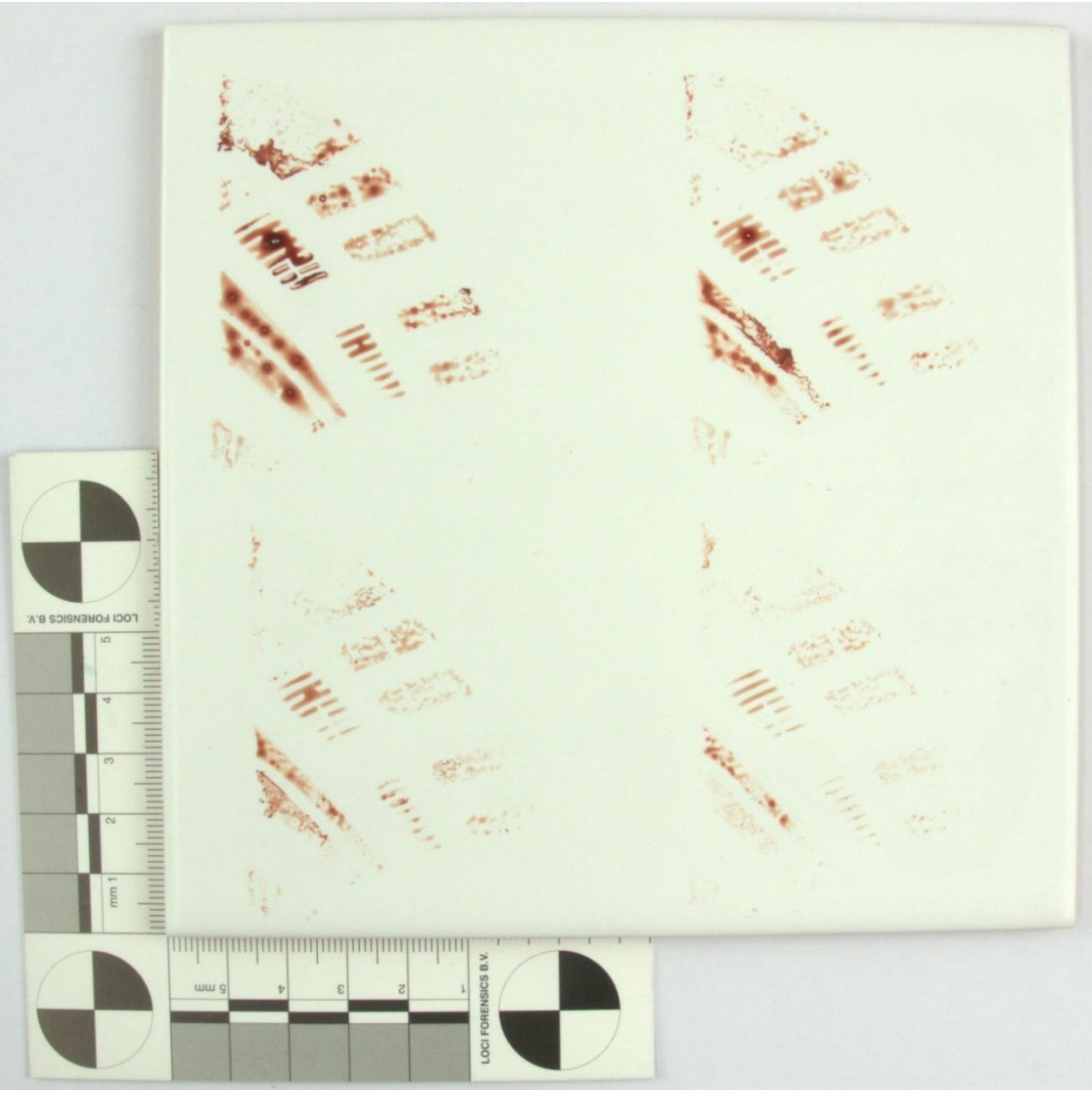


Print	Score
LB	
RB	
LO	
RO	

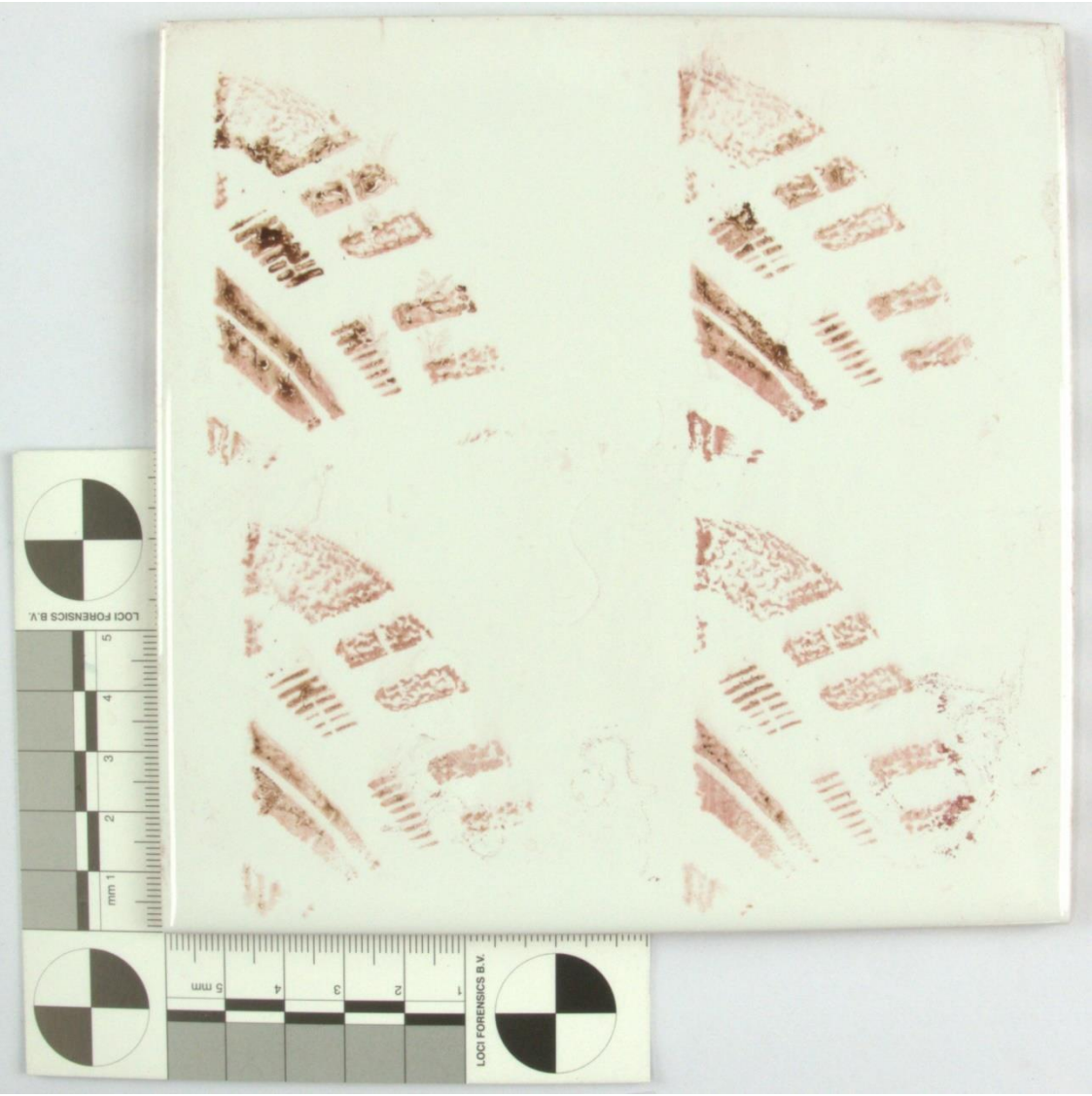


Print	Score
LB	
RB	
LO	
RO	

Iron oxide 6

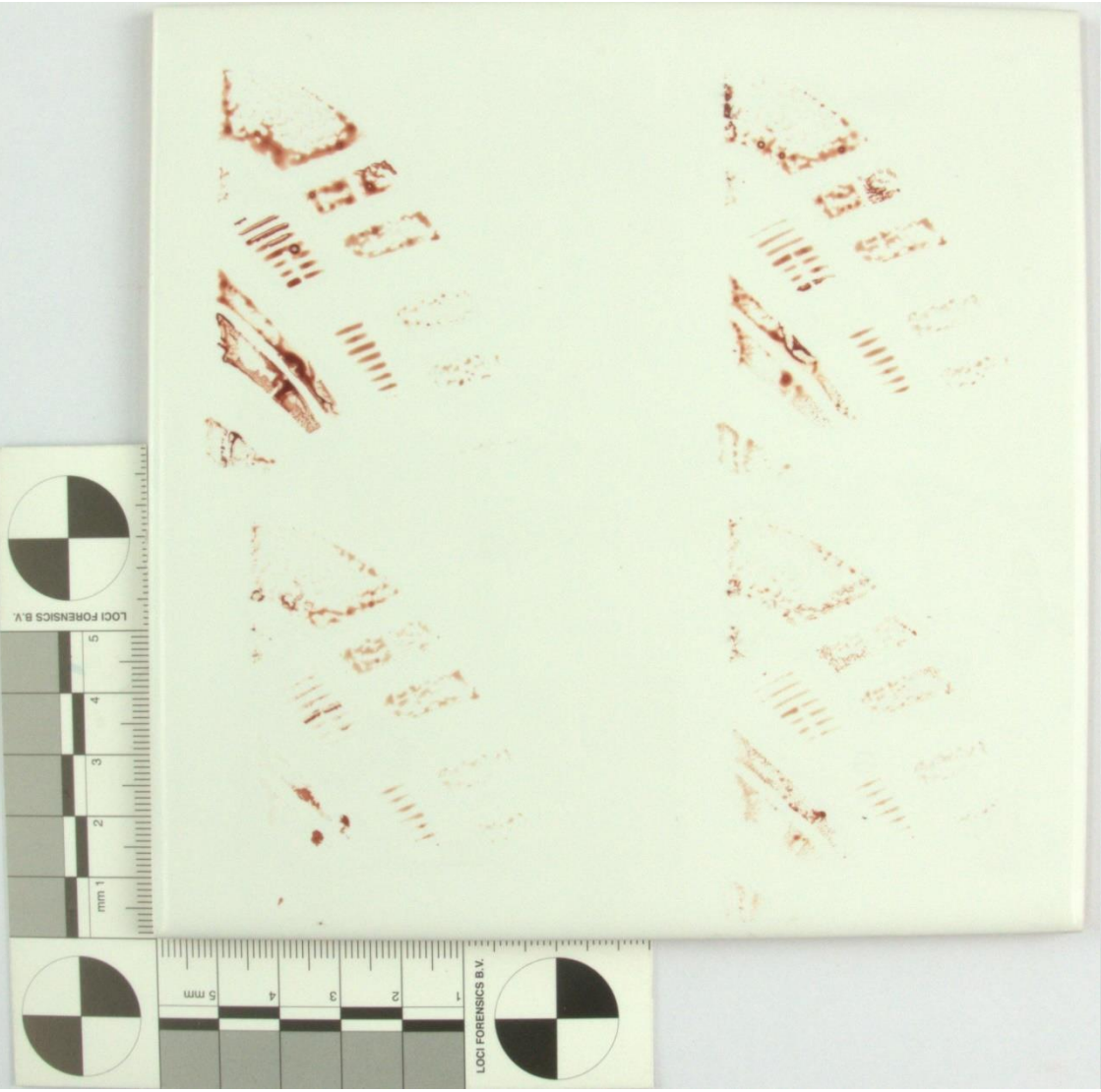


Print	Score
LB	
RB	
LO	
RO	



Print	Score
LB	
RB	
LO	
RO	

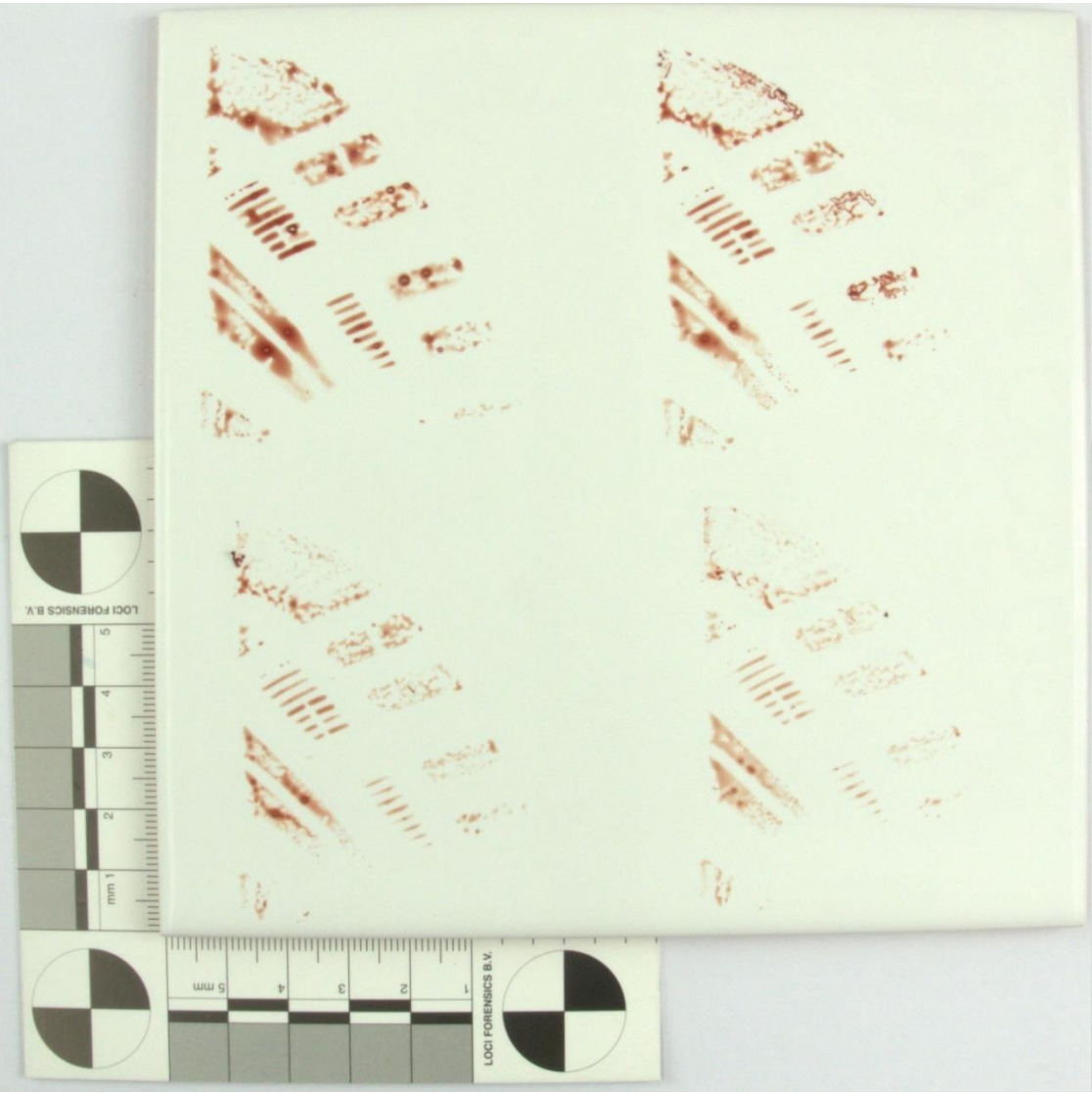
Iron oxide 7



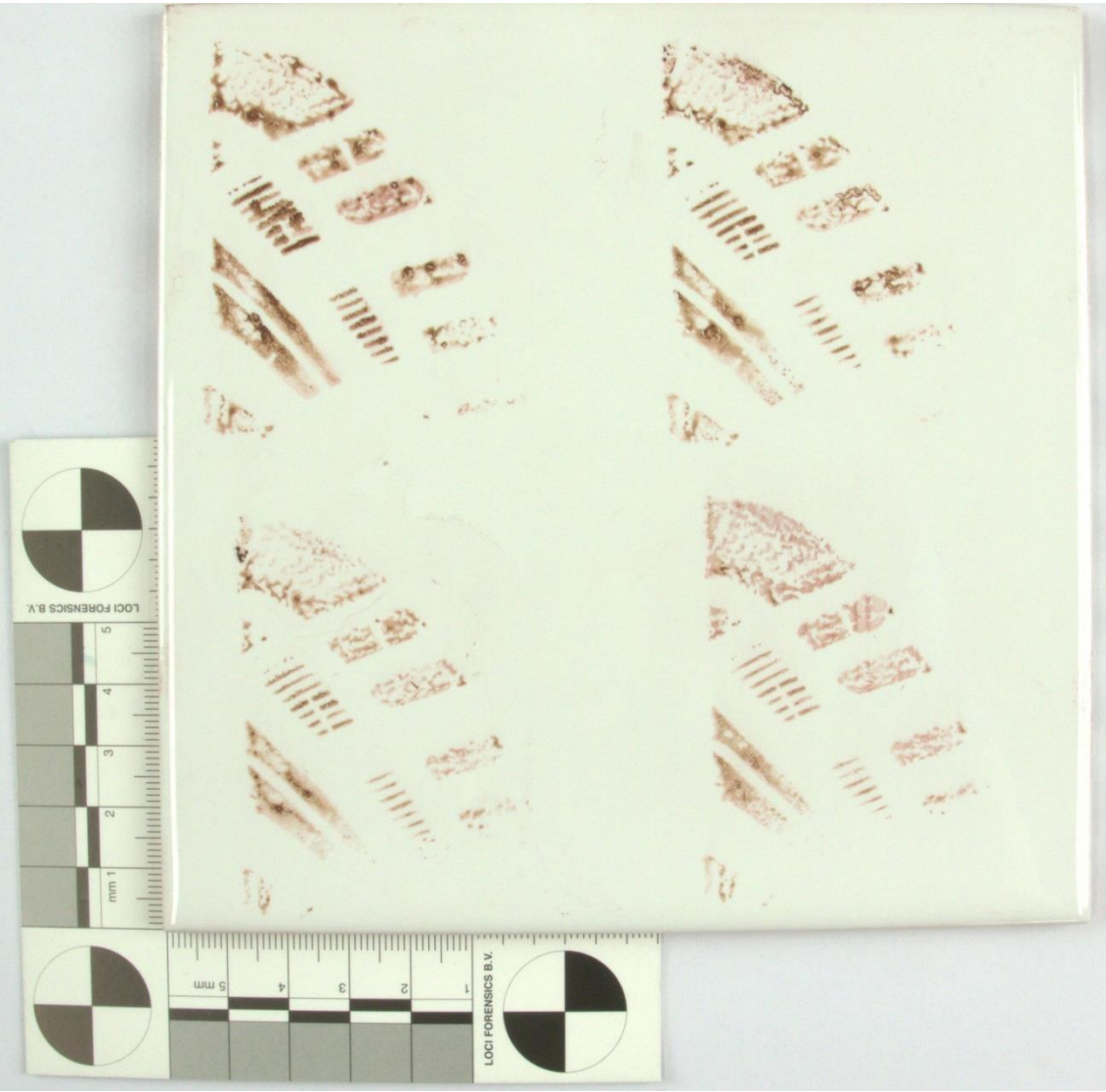
Print	Score
LB	
RB	
LO	
RO	

Print	Score
LB	
RB	
LO	
RO	

Iron oxide 8



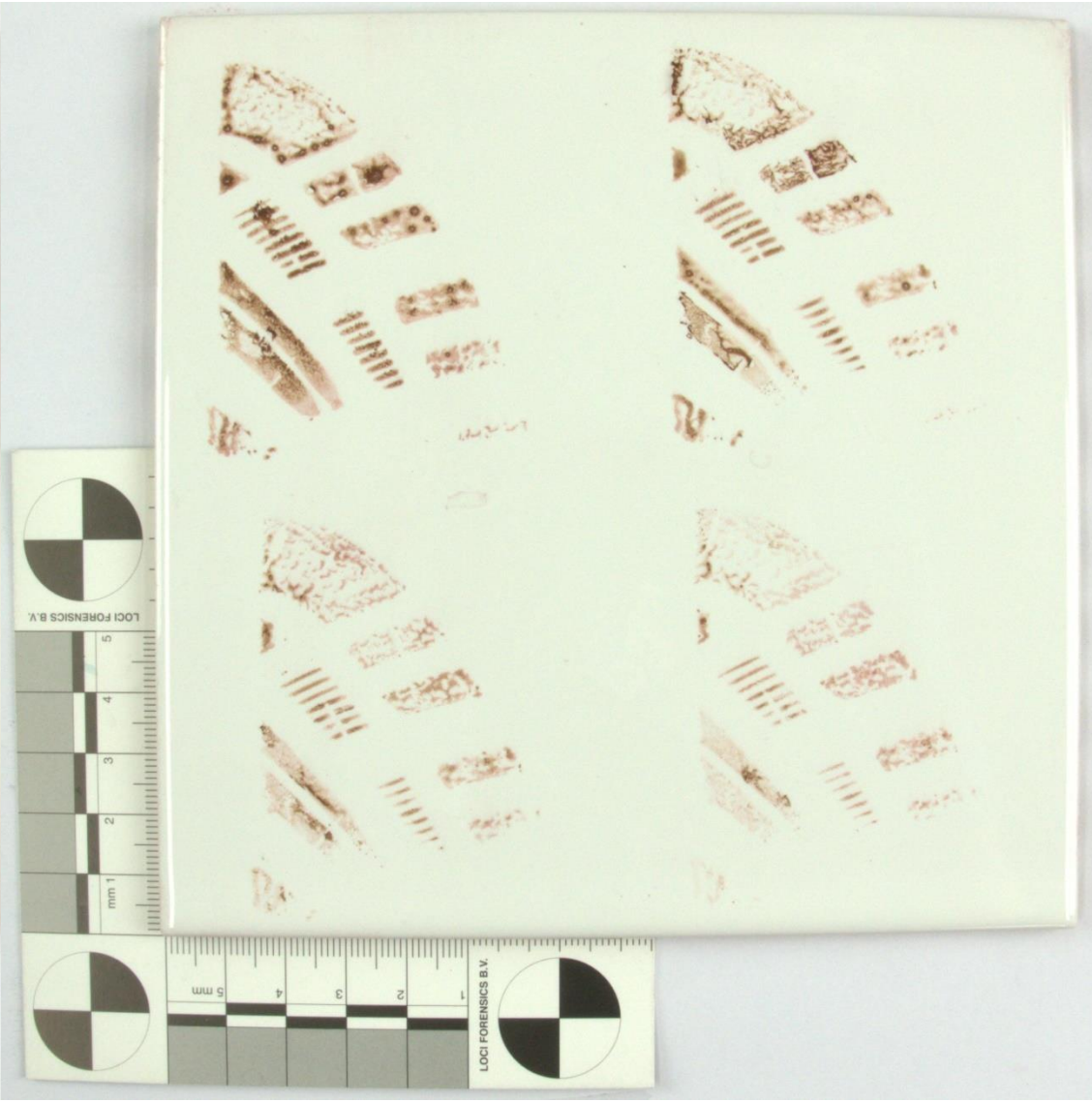
Print	Score
LB	
RB	
LO	
RO	



Print	Score
LB	
RB	
LO	
RO	

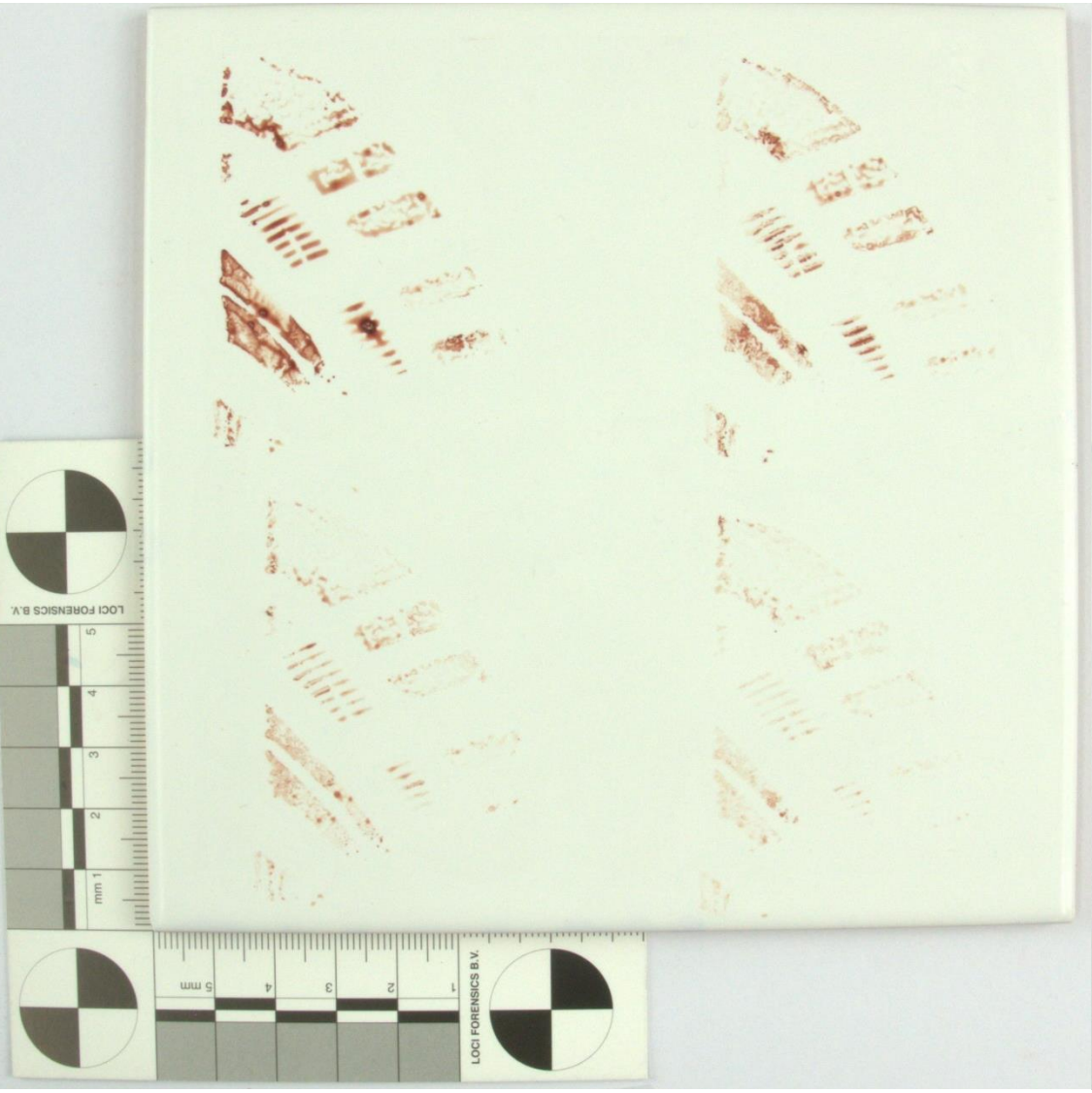
Iron oxide 9

Print	Score
LB	
RB	
LO	
RO	

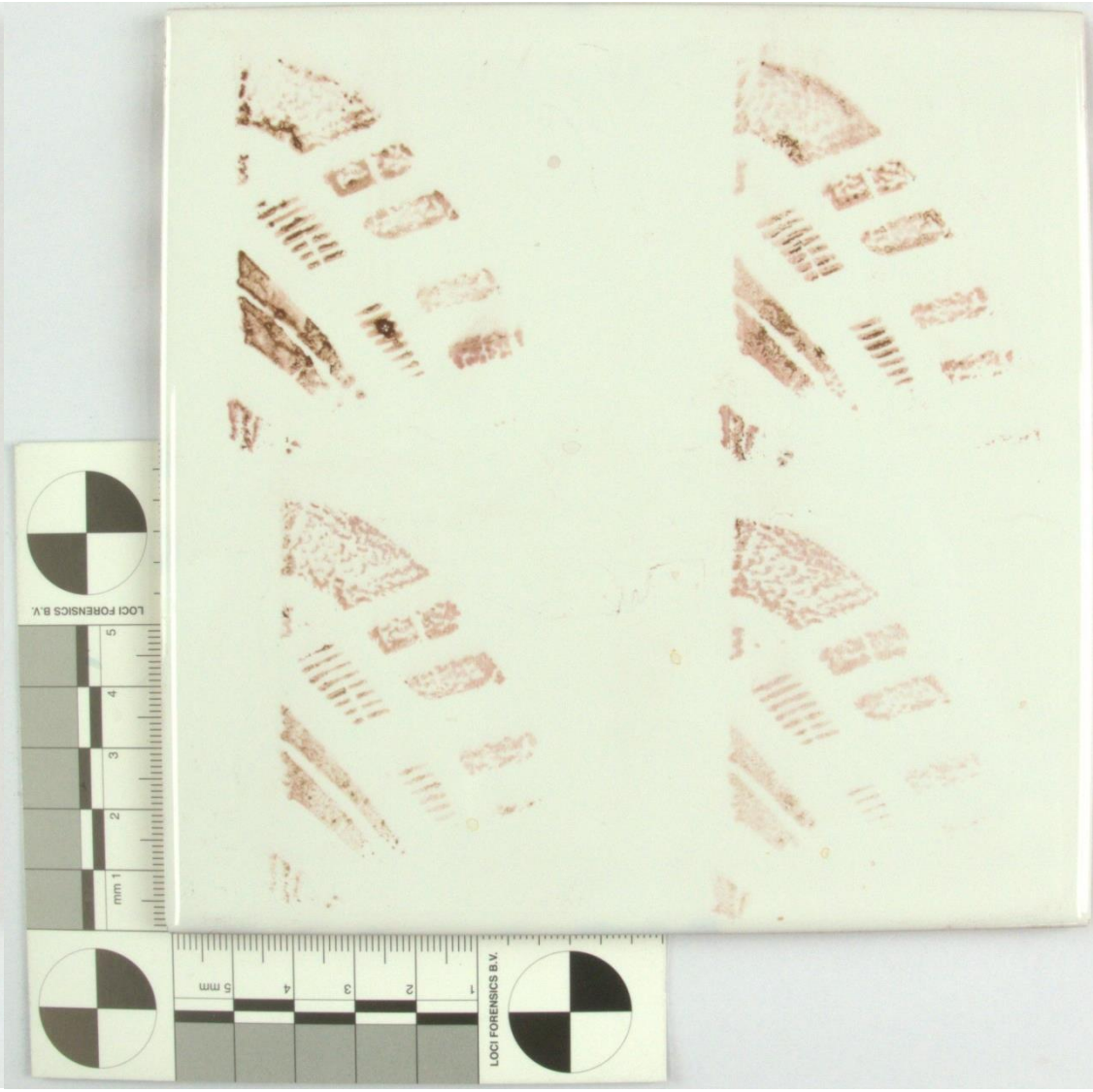


Print	Score
LB	
RB	
LO	
RO	

Iron oxide 10



Print	Score
LB	
RB	
LO	
RO	



Print	Score
LB	
RB	
LO	
RO	

Appendix 7: Calculating sample size

At this appendix, the sample size has been calculated with a post-hoc power analysis to check if a good number of samples were investigated to investigate what the chance is that false negatives (β) are rejected. The post-hoc power analysis was performed with the computer program G*Power 3.1.9.7.

The Tail has been set on One, because it has been investigated whether the treated tiles gave a more visible result than the untreated tiles. See figure 27 and 28.

The 'Effect size d' and the 'α err prob' were already set automatically.

The Sample size has been set for each group (before and after treatment) on 50 samples.

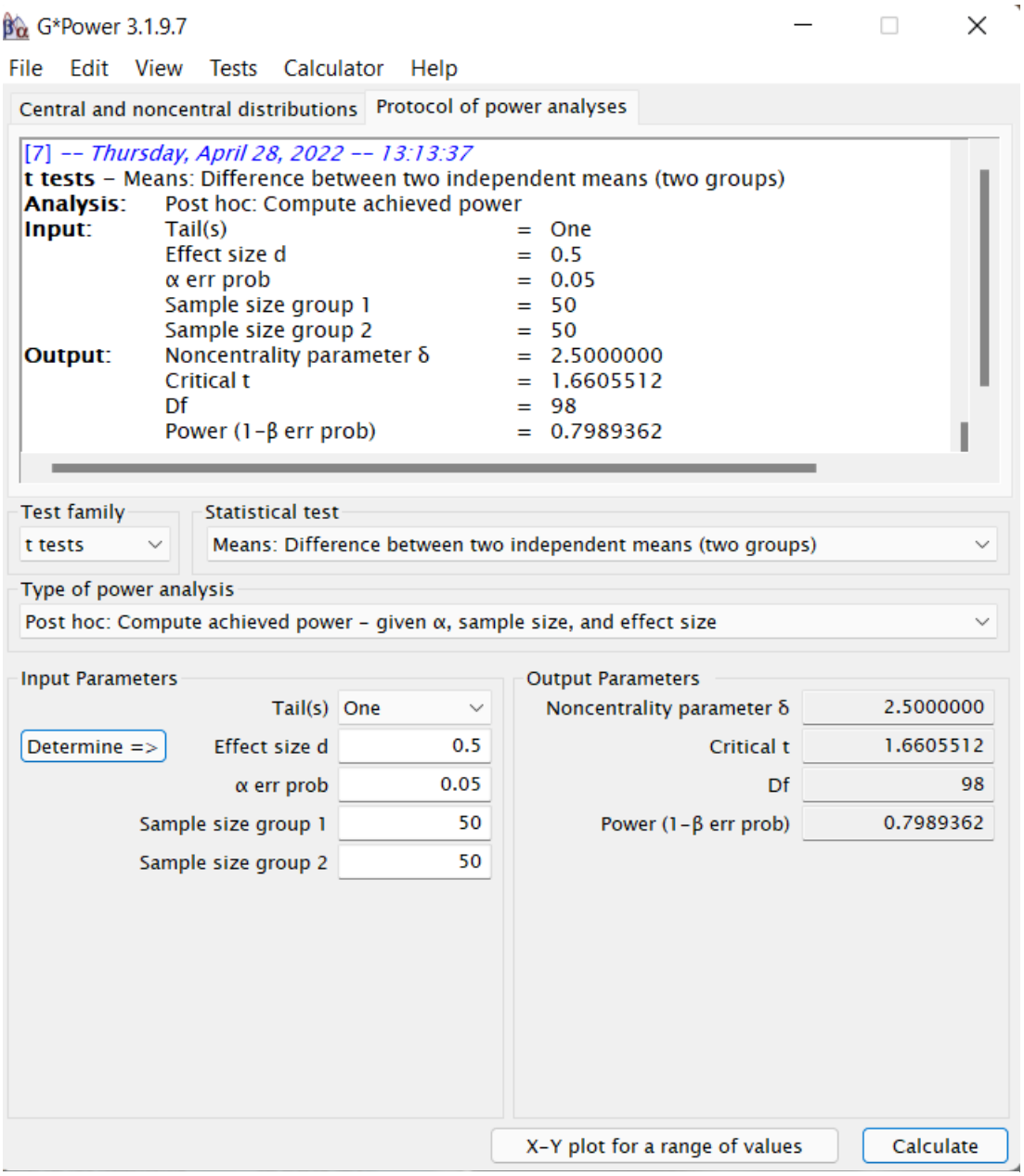


Figure 28: Results sample size calculation with a post-how power analysis

The Power (1- β) has been calculated at 0.7989362 = 79,89%

The desired Power (1- β) is 95%, which means that the calculated sample size for both groups is 88 samples.

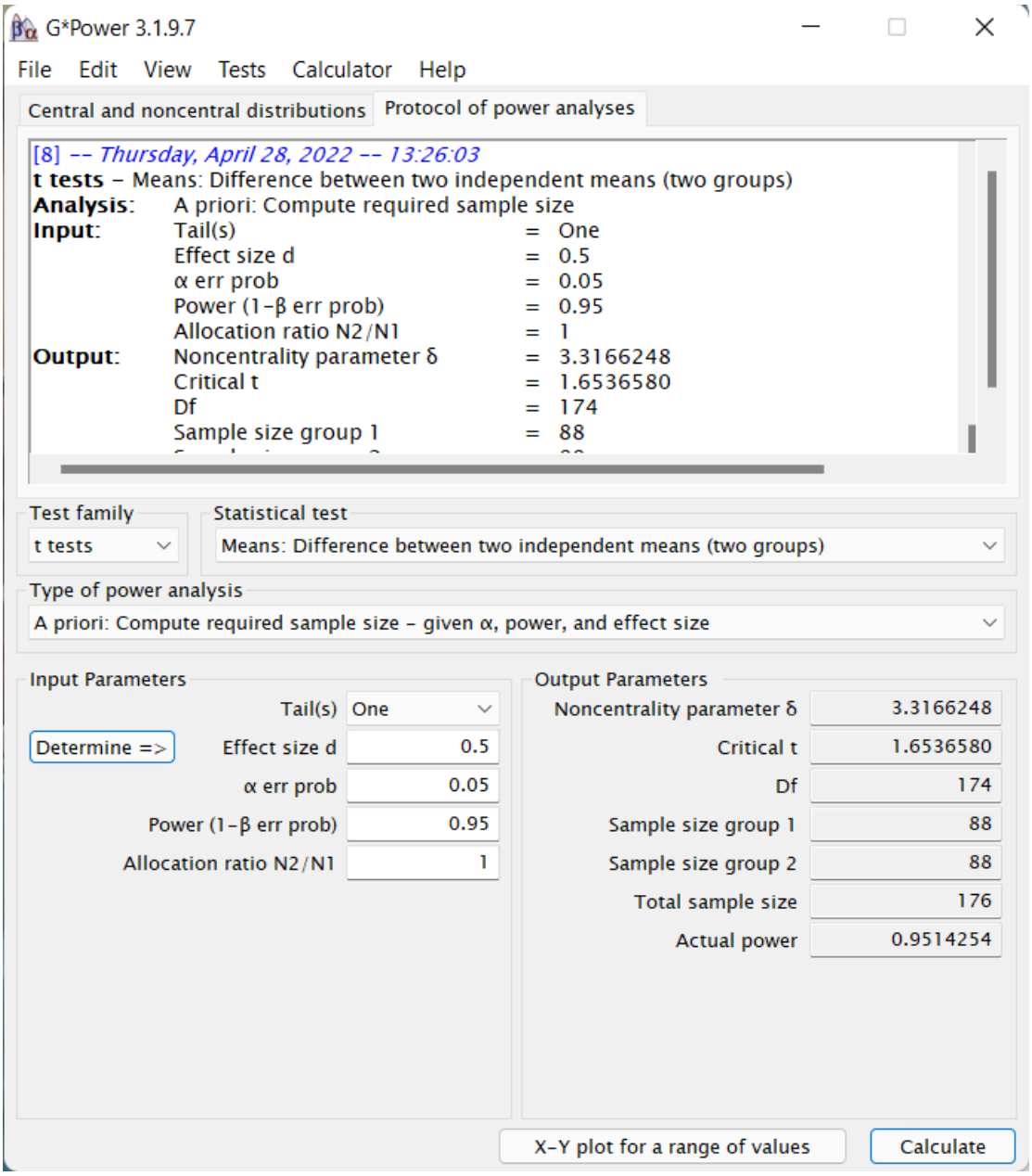
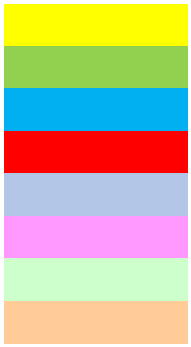


Figure 29: Desired sample size for a power (1- β) of 95%

Appendix 8: Raw data results

Legenda



Results Upper left (LB)
Results Upper right (RB)
Results Bottom left (LO)
Results Bottom right (RO)
Amido Black (AB)
Hungarian Red (HR)
(A)LCV
Iron oxide (IO)

Upper left

Table 10: Raw data shoeprint grading with the granted scores per rater of the upper left prints, before treatment, with the four Visual Blood Enhancement Techniques

Person:	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P
	Before	Before	Before	Before	Before	Before	Before	Before	Before	Before	Before	Before	Before	Before	Before	Before
	LB	LB	LB	LB	LB	LB	LB	LB	LB	LB	LB	LB	LB	LB	LB	LB
AB 1	2	3	4	3	2	3	2	3	2	2	2	3	2	3	3	4
2	2	4	1	2	3	3	3	3	2	2	2	2	2	3	3	4
3	1	2	1	1	2	2	3	2	2	2	1	2	1	4	3	3
4	1	2	1	1	1	3	2	2	1	2	1	1	1	2	3	3
5	2	2	1	2	3	2	2	2	1	1	1	2	2	2	4	4
6	1	2	1	1	2	2	2	2	1	1	1	1	1	2	3	3
7	1	2	1	2	2	3	2	2	1	1	1	3	2	2	3	3
8	1	2	2	2	2	3	2	2	2	2	1	2	1	1	3	3
9	1	3	2	1	2	3	2	2	2	2	1	1	2	2	3	2
10	2	2	1	2	2	2	1	2	1	1	1	2	1	2	3	3
HR 11	4	3	1	3	2	2	4	3	2	2	1	2	2	3	4	4
12	4	4	2	3	3	2	4	3	2	0	1	2	2	4	4	4
13	4	4	3	3	3	3	4	3	3	2	1	3	3	4	4	4
14	2	2	1	2	2	2	3	2	2	2	1	2	2	2	3	3
15	2	2	1	1	2	2	1	2	2	1	1	1	1	1	3	3
16	2	2	1	1	1	2	1	2	2	1	1	2	1	2	3	3
17	2	2	2	2	2	2	1	2	2	2	1	3	2	2	3	3
18	3	3	1	1	2	2	1	2	2	1	2	2	3	2	3	4
19	4	3	1	3	2	2	3	2	2	2	1	2	3	3	3	3
20	4	3	3	3	3	3	2	3	3	1	1	2	3	3	3	4
(A)LCV 21	3	3	2	2	3	3	3	2	3	1	2	2	2	3	4	4
22	3	3	1	2	3	3	2	2	2	1	2	2	2	3	4	4
23	3	3	2	2	3	3	2	2	3	2	2	3	3	3	4	4
24	3	3	2	2	3	2	2	2	3	1	2	3	3	3	4	4
25	2	3	2	2	3	2	2	2	2	1	2	2	2	2	4	4
26	2	3	1	2	3	2	3	2	2	1	2	2	2	2	4	4
27	2	3	2	2	2	2	2	2	2	1	2	2	2	2	3	4
28	3	3	2	2	3	2	2	2	2	1	3	3	2	2	3	4

29	3	3	2	2	3	2	2	2	3	1	2	2	2	3	4	4
30	2	3	1	3	3	2	2	2	3	1	3	2	3	3	4	3
IO 31	1	1	0	1	2	1	2	1	1	0	1	1	1	2	2	2
32	3	3	2	2	2	2	2	2	2	2	2	3	3	3	3	4
33	2	3	1	3	2	2	3	3	2	2	1	2	3	3	4	4
34	2	2	1	1	2	2	3	2	1	2	1	2	1	2	3	3
35	1	2	1	2	2	2	3	2	1	1	1	2	2	2	3	3
36	1	3	1	2	2	2	3	2	1	1	1	1	2	2	3	3
37	1	3	2	2	2	2	3	2	2	1	2	2	2	3	3	2
38	2	3	1	2	2	2	3	2	2	1	1	1	2	3	3	3
39	2	3	1	2	2	2	3	2	2	1	1	1	2	2	3	2
40	2	3	2	2	2	2	3	2	1	1	1	2	2	2	3	3

Table 11: Raw data shoeprint grading with the granted scores per rater of the upper left prints, after treatment, with the four Visual Blood Enhancement Techniques

Person:	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P
	After	After	After	After	After	After	After	After	After	After	After	After	After	After	After	After
	LB	LB	LB	LB	LB	LB	LB	LB	LB	LB	LB	LB	LB	LB	LB	LB
AB 1	2	3	4	3	3	3	4	3	3	2	2	3	2	3	4	4
2	2	3	2	2	3	3	4	3	2	2	2	3	2	4	4	4
3	1	3	1	2	3	2	3	3	2	2	1	2	2	4	4	4
4	1	3	1	2	2	3	2	2	1	2	1	2	2	3	4	4
5	1	2	0	3	3	2	3	3	1	1	2	2	2	2	4	3
6	2	2	1	2	2	3	3	3	2	1	2	3	2	2	4	4
7	2	2	2	3	2	3	3	3	3	2	2	3	2	2	4	4
8	2	2	2	2	2	3	2	2	2	2	2	2	1	2	4	4
9	2	3	2	3	3	3	3	3	3	2	2	2	2	2	4	4
10	2	2	1	2	3	3	2	3	2	1	1	3	2	2	4	4
HR 11	4	3	1	3	3	2	4	3	1	2	1	2	2	3	4	4
12	4	4	2	3	3	3	2	3	2	1	2	2	3	3	4	4
13	4	4	3	3	3	3	4	3	3	3	2	3	3	4	4	4
14	2	2	1	2	2	2	3	3	2	2	2	2	2	2	3	4
15	2	2	1	2	2	2	3	3	2	1	1	2	2	2	4	4
16	3	2	1	2	2	2	2	2	2	1	1	2	2	2	3	3

17	3	3	3	3	3	3	2	2	3	3	1	3	3	3	4	4
18	3	3	1	3	3	2	2	2	2	2	2	2	3	2	4	4
19	4	3	1	2	3	3	3	2	2	1	2	2	2	3	4	4
20	4	3	3	3	3	3	4	3	3	2	2	2	3	3	4	4
(A)LCV 21	3	3	2	3	3	3	3	3	3	2	2	2	2	3	4	4
22	3	3	1	2	3	3	3	2	2	2	2	2	2	3	4	4
23	3	3	2	3	3	3	3	3	3	2	2	3	3	3	4	4
24	3	3	2	2	3	3	3	3	3	2	2	3	3	3	4	4
25	2	3	2	2	3	3	4	2	2	2	2	2	2	3	4	4
26	2	3	1	2	3	2	3	3	2	1	2	2	2	2	4	4
27	3	3	2	2	3	3	3	2	2	2	2	3	2	2	4	4
28	3	3	2	2	3	3	3	2	2	2	3	3	2	2	4	4
29	3	3	2	2	3	2	2	3	3	2	2	2	2	3	4	4
30	4	3	1	3	3	2	3	3	3	1	3	2	2	3	4	3
IO 31	1	3	1	2	2	2	3	2	2	2	1	1	2	3	3	3
32	3	3	2	2	3	2	3	2	2	2	2	4	3	3	4	4
33	3	3	1	3	3	2	4	3	3	2	1	2	3	3	4	4
34	2	2	1	1	2	2	2	3	2	1	1	2	1	2	4	3
35	1	3	1	2	2	2	4	3	1	2	1	2	2	3	4	3
36	2	4	1	2	2	2	4	3	1	2	1	1	3	3	3	3
37	2	3	2	2	2	2	3	3	2	2	2	2	2	3	4	3
38	2	3	1	2	2	2	4	2	2	1	1	1	3	3	4	3
39	2	3	1	2	2	2	4	2	2	1	1	1	2	2	4	3
40	2	4	2	2	2	2	4	3	1	2	1	2	3	3	4	3

Table 12: Frequency table of the data of the upper left prints, before treatment, in combination with the average scores per print

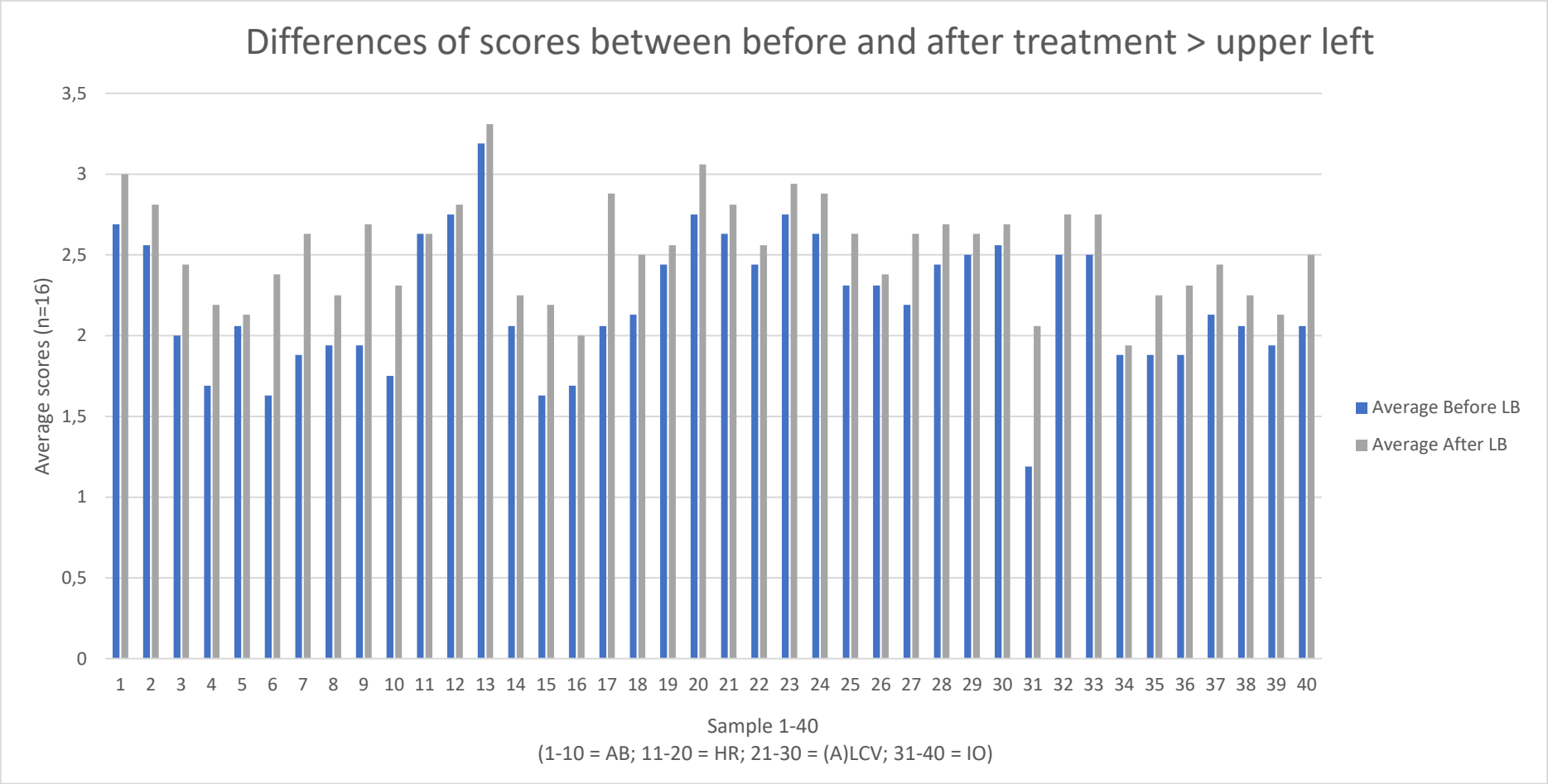
	Data shoeprint LB grading: Before treatment			*Frequency table (number of times the score is granted per shoeprint)*				
VBET	Photo	Score 0	Score 1	Score 2	Score 3	Score 4	Total	Average
AB	1	0	0	7	7	2	16	2,69
AB	2	0	1	7	6	2	16	2,56
AB	3	0	5	7	3	1	16	2
AB	4	0	8	5	3	0	16	1,69
AB	5	0	4	9	1	2	16	2,06
AB	6	0	8	6	2	0	16	1,63
AB	7	0	4	7	4	0	16	1,88
AB	8	0	4	9	3	0	16	1,94
AB	9	0	4	9	3	0	16	1,94
AB	10	0	6	8	2	0	16	1,75
HR	11	0	2	6	4	4	16	2,63
HR	12	1	1	5	3	6	16	2,75
HR	13	0	1	1	8	6	16	3,19
HR	14	0	2	11	3	0	16	2,06
HR	15	0	8	6	2	0	16	1,63
HR	16	0	7	7	2	0	16	1,69
HR	17	0	2	11	3	0	16	2,06
HR	18	0	4	7	4	1	16	2,13
HR	19	0	2	6	7	1	16	2,44
HR	20	0	2	2	10	2	16	2,75
(A)LCV	21	0	1	6	7	2	16	2,63
(A)LCV	22	0	2	7	5	2	16	2,44
(A)LCV	23	0	0	6	8	2	16	2,75
(A)LCV	24	0	1	6	7	2	16	2,63
(A)LCV	25	0	1	11	2	2	16	2,31
(A)LCV	26	0	2	9	3	2	16	2,31
(A)LCV	27	0	1	12	2	1	16	2,19
(A)LCV	28	0	1	8	6	1	16	2,44
(A)LCV	29	0	1	8	5	2	16	2,5
(A)LCV	30	0	2	5	7	2	16	2,56
IO	31	2	9	5	0	0	16	1,19
IO	32	0	0	9	6	1	16	2,5
IO	33	0	2	6	6	2	16	2,5
IO	34	0	5	8	3	0	16	1,88
IO	35	0	5	8	3	0	16	1,88
IO	36	0	6	6	4	0	16	1,88
IO	37	0	2	10	4	0	16	2,13
IO	38	0	4	7	5	0	16	2,06
IO	39	0	4	9	3	0	16	1,94
IO	40	0	3	9	4	0	16	2,06

Table 13: Frequency table of the data of the upper left prints, after treatment, in combination with the average scores per print

	Data shoeprint LB grading: After treatment			*Frequency table (number of times the score is granted per shoeprint)*				
VBET	Photo	Score 0	Score 1	Score 2	Score 3	Score 4	Total	Average
AB	1	0	0	4	8	4	16	3
AB	2	0	0	7	5	4	16	2,81
AB	3	0	3	6	4	3	16	2,44
AB	4	0	4	7	3	2	16	2,19
AB	5	1	3	6	5	1	16	2,13
AB	6	0	2	8	4	2	16	2,38
AB	7	0	0	8	6	2	16	2,63
AB	8	0	1	12	1	2	16	2,25
AB	9	0	0	7	7	2	16	2,69
AB	10	0	3	7	4	2	16	2,31
HR	11	0	3	4	5	4	16	2,63
HR	12	0	1	5	6	4	16	2,81
HR	13	0	0	1	9	6	16	3,31
HR	14	0	1	11	3	1	16	2,25
HR	15	0	3	9	2	2	16	2,19
HR	16	0	3	10	3	0	16	2
HR	17	0	1	2	11	2	16	2,88
HR	18	0	1	8	5	2	16	2,5
HR	19	0	2	6	5	3	16	2,56
HR	20	0	0	3	9	4	16	3,06
(A)LCV	21	0	0	5	9	2	16	2,81
(A)LCV	22	0	1	7	6	2	16	2,56
(A)LCV	23	0	0	3	11	2	16	2,94
(A)LCV	24	0	0	4	10	2	16	2,88
(A)LCV	25	0	0	9	4	3	16	2,63
(A)LCV	26	0	2	8	4	2	16	2,38
(A)LCV	27	0	0	8	6	2	16	2,63
(A)LCV	28	0	0	7	7	2	16	2,69
(A)LCV	29	0	0	8	6	2	16	2,63
(A)LCV	30	0	2	3	9	2	16	2,69
IO	31	0	4	7	5	0	16	2,06
IO	32	0	0	7	6	3	16	2,75
IO	33	0	2	3	8	3	16	2,75
IO	34	0	5	8	2	1	16	1,94
IO	35	0	4	6	4	2	16	2,25
IO	36	0	4	5	5	2	16	2,31
IO	37	0	0	10	5	1	16	2,44
IO	38	0	4	6	4	2	16	2,25
IO	39	0	4	8	2	2	16	2,13
IO	40	0	2	7	4	3	16	2,5

Table 14: Scheme to statistically calculate the significant difference between the average score before and after treatment for the four Visual Blood Enhancement Techniques for the upper left prints

VBET	Photo	Average Before LB	Average After LB	Difference (d) Average Before - Average After	$\bar{d}$ $(n = 10)$	$d - \bar{d}$	$(d - \bar{d})^2$	$\sum (d_i - \bar{d})^2$ $(n = 10)$	Standard deviation $\sqrt{\frac{\sum (d_i - \bar{d})^2}{n-1}}$	Paired t-test $\frac{\lvert\bar{d}\rvert \times \sqrt{n}}{Sd}$	Critical-value (α=5%) (n-1) [58]	Comparison Paired t-test and critical value [58]	Significant difference vs. No significant difference
AB	1	2,69	3	-0,31		0,159	0,02528						
AB	2	2,56	2,81	-0,25		0,219	0,04796						
AB	3	2	2,44	-0,44		0,029	0,00084						
AB	4	1,69	2,19	-0,5		-0,031	0,00096						
AB	5	2,06	2,13	-0,07		0,399	0,1592						
AB	6	1,63	2,38	-0,75	-0,469	-0,281	0,07896	0,50469	$\sqrt{\frac{0,50469}{9}} =$	$\frac{\lvert-0,469\rvert \times \sqrt{10}}{0,23681} =$	2,262	2,262 < 6,2629	Significant difference
AB	7	1,88	2,63	-0,75		-0,281	0,07896		0,23681	6,2629			
AB	8	1,94	2,25	-0,31		0,159	0,02528						
AB	9	1,94	2,69	-0,75		-0,281	0,07896						
AB	10	1,75	2,31	-0,56		-0,091	0,00828						
HR	11	2,63	2,63	0		0,261	0,06812						
HR	12	2,75	2,81	-0,06		0,201	0,0404						
HR	13	3,19	3,31	-0,12		0,141	0,01988						
HR	14	2,06	2,25	-0,19		0,071	0,00504						
HR	15	1,63	2,19	-0,56	-0,261	-0,299	0,0894	0,57989	$\sqrt{\frac{0,57989}{9}} =$	$\frac{\lvert-0,261\rvert \times \sqrt{10}}{0,25384} =$	2,262	2,262 < 3,2515	Significant difference
HR	16	1,69	2	-0,31		-0,049	0,0024		0,25384	3,2515			
HR	17	2,06	2,88	-0,82		-0,559	0,31248						
HR	18	2,13	2,5	-0,12		-0,141	0,01988						
HR	19	2,44	2,56	-0,12		-0,141	0,01988						
HR	20	2,75	3,06	-0,31		-0,049	0,0024						
(A)LCV	21	2,63	2,81	-0,18		0,028	0,00078						
(A)LCV	22	2,44	2,56	-0,12		0,088	0,00774						
(A)LCV	23	2,75	2,94	-0,19		0,018	0,00032						
(A)LCV	24	2,63	2,88	-0,25		-0,042	0,0023						
(A)LCV	25	2,31	2,63	-0,32		-0,112	0,01254						
(A)LCV	26	2,31	2,38	-0,07	-0,208	0,138	0,01904	0,1105	$\sqrt{\frac{0,1105}{9}} =$	$\frac{\lvert-0,208\rvert \times \sqrt{10}}{0,11081} =$	2,262	2,262 < 5,9359	Significant difference
(A)LCV	27	2,19	2,63	-0,44		-0,232	0,05382		0,11081	5,9359			
(A)LCV	28	2,44	2,69	-0,25		-0,042	0,00176						
(A)LCV	29	2,5	2,63	-0,13		0,078	0,00608						
(A)LCV	30	2,56	2,69	-0,13		0,078	0,00608						
IO	31	1,19	2,06	-0,87		-0,559	0,31248						
IO	32	2,5	2,75	-0,25		0,061	0,00372						
IO	33	2,5	2,75	-0,25		0,061	0,00372						
IO	34	1,88	1,94	-0,06		0,251	0,063						
IO	35	1,88	2,25	-0,37	-0,311	-0,059	0,00348	0,44449	$\sqrt{\frac{0,44449}{9}} =$	$\frac{\lvert-0,311\rvert \times \sqrt{10}}{0,22223} =$	2,262	2,262 < 4,4255	Significant difference
IO	36	1,88	2,31	-0,43		-0,119	0,01416		0,22223	4,4255			
IO	37	2,13	2,44	-0,31		0,001	1E-06						
IO	38	2,06	2,25	-0,19		0,121	0,01464						
IO	39	1,94	2,13	-0,19		0,121	0,01464						
IO	40	2,06	2,5	-0,19		0,121	0,01464						



Graph 1: Differences of scores between before and after treatment with the four Visual Blood Enhancement Techniques (Amido Black, Hungarian Red, (A)LCV and Iron oxide) for the upper left prints

Upper right

Table 15: Raw data shoeprint grading with the granted scores per rater of the upper right prints, before treatment, with the four Visual Blood Enhancement Techniques

Person:	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P
	Before	Before	Before	Before	Before	Before	Before	Before	Before	Before	Before	Before	Before	Before	Before	Before
	RB	RB	RB	RB	RB	RB	RB	RB	RB	RB	RB	RB	RB	RB	RB	RB
AB 1	0	1	1	1	1	0	1	1	1	0	1	1	1	1	2	1
2	0	1	0	1	1	1	1	1	1	0	1	1	0	1	1	1
3	0	1	0	0	1	0	0	1	1	0	0	0	0	1	1	1
4	0	1	0	0	0	1	0	1	1	0	0	0	0	1	1	1
5	1	2	0	1	2	1	1	1	1	1	1	1	1	1	3	2
6	1	2	1	1	1	1	1	1	1	1	1	1	1	1	3	2
7	0	2	1	1	1	1	1	1	1	1	1	1	1	1	3	2
8	0	1	0	1	1	1	1	1	1	1	1	1	1	1	2	2
9	0	1	0	0	1	1	0	1	1	0	0	0	0	1	1	1
10	0	1	0	0	1	1	0	0	1	0	0	0	0	1	1	1
HR 11	1	2	1	1	1	2	2	2	1	1	1	1	1	2	3	3
12	2	2	1	2	2	1	2	2	1	1	1	2	1	2	3	3
13	0	1	0	1	1	1	1	1	1	0	0	0	1	1	1	1
14	1	1	0	1	1	1	1	1	1	1	1	1	1	1	2	2
15	0	1	0	1	1	1	0	1	1	0	0	0	1	1	2	1
16	0	1	0	0	0	0	0	0	1	0	0	0	0	1	1	1
17	0	1	0	1	1	1	0	1	1	0	0	1	1	1	2	2
18	0	1	0	1	1	1	0	1	1	0	1	0	1	1	1	2
19	0	1	0	0	1	1	0	0	1	0	0	0	1	1	1	1
20	0	1	0	0	1	1	1	1	1	0	0	0	1	1	1	1
(A)LCV 21	3	2	1	1	2	3	3	2	2	1	1	2	1	2	3	3
22	2	3	1	1	2	2	2	2	2	1	1	1	2	2	3	3
23	3	2	1	1	2	2	2	2	2	1	1	2	2	3	3	3
24	1	2	2	1	2	2	1	1	2	1	1	2	2	2	3	3
25	2	2	2	2	3	2	2	2	2	1	1	2	2	2	4	4
26	1	3	1	1	2	2	3	2	2	1	1	2	2	2	4	3
27	2	2	2	2	2	2	2	2	2	1	1	2	2	2	3	3
28	3	2	2	1	2	2	1	1	2	1	1	2	2	2	3	3

29	4	2	2	2	2	2	2	2	2	1	1	2	2	2	3	3
30	2	2	1	3	2	2	2	2	3	1	2	2	3	3	3	3
IO 31	1	2	1	1	2	2	2	2	1	1	1	1	1	2	2	1
32	0	2	0	1	1	1	1	1	1	1	1	1	1	1	2	2
33	1	2	1	1	1	1	1	2	1	1	1	1	1	1	2	2
34	2	2	1	1	2	2	3	2	1	1	1	2	1	2	3	3
35	1	2	2	2	2	2	3	2	2	1	2	2	2	2	3	3
36	1	2	1	1	1	2	2	1	1	1	1	1	1	2	3	2
37	1	2	1	1	1	2	2	1	1	1	1	1	2	2	3	2
38	2	2	1	2	2	2	3	2	1	1	1	1	1	2	3	2
39	2	2	1	2	2	2	3	2	1	1	1	2	2	1	3	2
40	1	2	1	1	1	2	3	2	1	1	1	1	1	1	2	2

Table 16: Raw data shoeprint grading with the granted scores per rater of the upper right prints, after treatment, with the four Visual Blood Enhancement Techniques

Person:	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P
	After	After	After	After	After	After	After	After	After	After	After	After	After	After	After	After
	RB	RB	RB	RB	RB	RB	RB	RB	RB	RB	RB	RB	RB	RB	RB	RB
AB 1	1	3	3	2	2	3	3	2	2	2	1	2	1	2	3	3
2	1	2	1	1	2	3	2	2	2	1	2	2	1	2	3	2
3	1	3	2	1	2	2	4	2	2	1	1	1	1	3	3	2
4	1	2	1	1	1	2	1	1	1	2	1	2	1	2	3	3
5	2	2	1	2	3	3	3	3	3	2	2	2	2	1	4	3
6	2	2	1	1	2	3	3	3	2	1	1	2	1	2	4	4
7	1	2	1	1	2	2	2	2	2	1	1	2	1	1	3	3
8	2	2	2	2	2	2	2	2	2	2	1	2	2	1	4	3
9	2	2	1	2	2	2	3	3	3	1	1	2	1	1	4	3
10	2	2	1	2	2	2	2	2	2	1	1	1	1	1	4	3
HR 11	4	3	1	3	2	2	4	2	2	1	1	1	2	3	4	3
12	4	3	2	3	2	2	2	3	2	1	1	2	2	3	4	4
13	1	2	1	2	2	2	2	2	1	1	1	1	1	2	2	3
14	2	2	1	1	2	2	3	2	2	1	2	2	1	2	3	3
15	2	2	1	1	1	2	2	2	2	1	1	1	1	1	3	3
16	0	2	0	1	1	1	1	1	1	1	0	1	1	1	1	2

17	2	1	1	1	2	2	1	1	1	1	1	2	1	1	2	3
18	2	2	1	1	2	2	1	2	1	1	1	1	1	1	3	2
19	0	2	1	1	2	1	1	1	1	1	1	1	1	1	2	2
20	1	2	0	1	2	1	1	2	1	1	1	1	1	1	2	2
(A)LCV 21	3	2	1	1	2	3	2	2	2	1	1	1	1	2	3	4
22	2	3	1	2	2	2	3	2	2	1	2	2	2	3	4	4
23	3	3	2	2	2	3	3	2	2	1	1	2	2	2	4	3
24	2	3	2	2	2	2	2	2	3	1	1	2	2	2	3	3
25	2	2	2	2	3	3	4	2	2	1	2	2	2	2	4	4
26	2	3	1	2	2	3	3	2	2	1	1	2	2	2	4	4
27	3	2	2	2	2	2	3	2	2	1	2	3	2	2	4	3
28	3	3	2	2	2	2	3	2	2	2	2	3	2	2	4	4
29	3	2	2	2	2	2	2	2	3	1	2	2	2	2	4	4
30	4	3	1	3	3	3	3	2	3	1	2	2	2	3	4	3
IO 31	1	3	2	3	2	3	3	2	2	3	2	1	2	3	3	3
32	3	3	1	2	2	2	2	1	2	2	2	2	2	1	3	3
33	3	2	1	3	3	2	3	2	2	2	2	2	2	3	3	3
34	2	2	1	2	2	2	3	2	2	1	1	2	1	2	4	3
35	2	3	2	2	2	2	4	3	1	2	3	2	2	2	4	3
36	1	2	1	1	2	2	4	2	2	2	1	1	2	2	3	3
37	2	2	2	2	2	2	3	2	1	1	1	2	2	2	3	3
38	2	3	2	2	2	2	4	2	2	1	2	1	2	2	4	3
39	2	3	1	2	2	2	4	3	1	1	2	1	2	1	4	3
40	2	3	1	2	2	2	4	3	2	2	1	1	2	2	4	4

Table 17: Frequency table of the data of the upper right prints, before treatment, in combination with the average scores per print

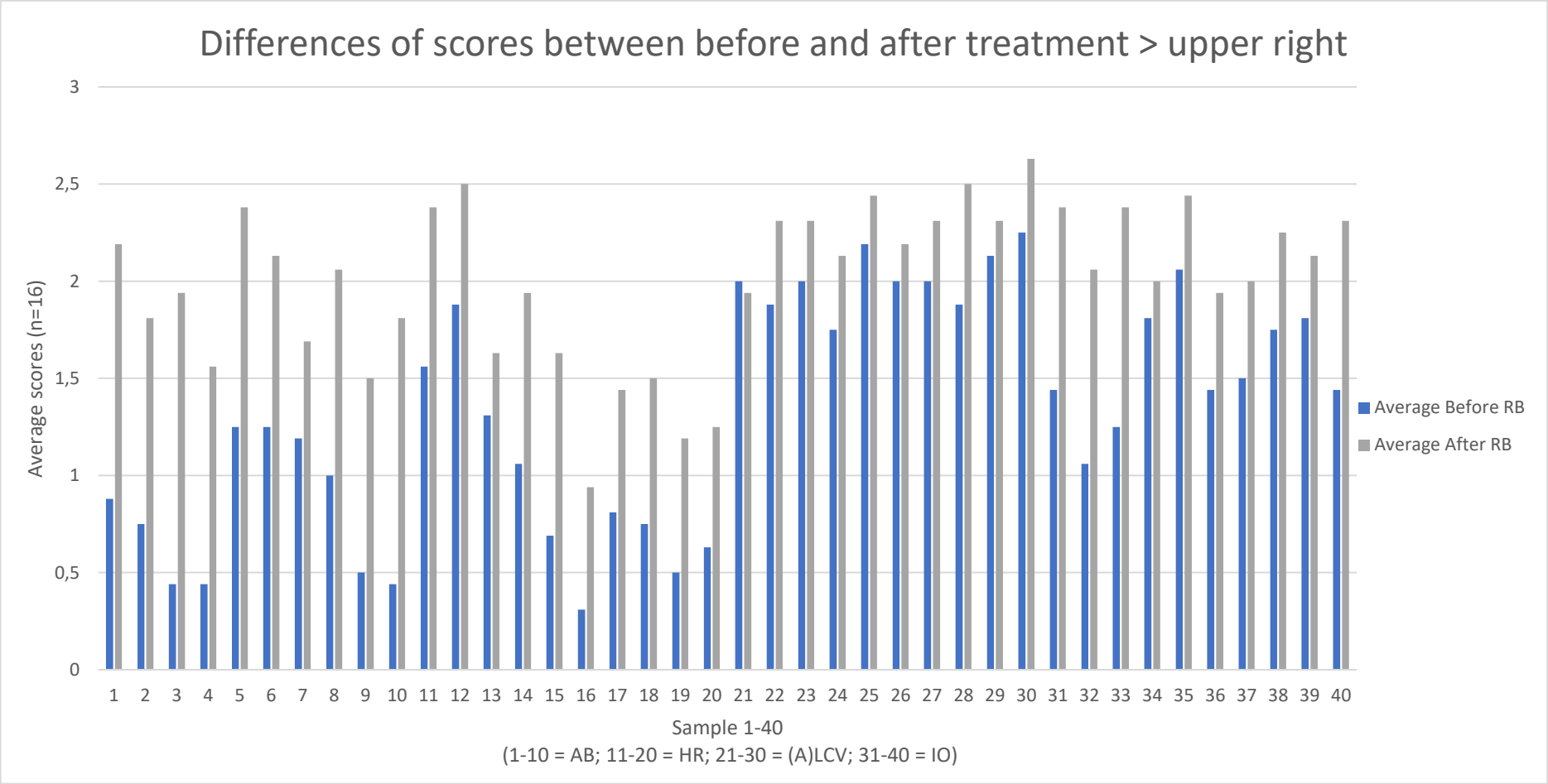
	Data shoeprint RB grading: Before treatment			*Frequency table (number of times the score is granted per shoeprint)*				
VBET	Photo	Score 0	Score 1	Score 2	Score 3	Score 4	Total	Average
AB	1	3	12	1	0	0	16	0,88
AB	2	4	12	0	0	0	16	0,75
AB	3	9	7	0	0	0	16	0,44
AB	4	9	7	0	0	0	16	0,44
AB	5	1	11	3	1	0	16	1,25
AB	6	0	13	2	1	0	16	1,25
AB	7	1	12	2	1	0	16	1,19
AB	8	2	12	2	0	0	16	1
AB	9	8	8	0	0	0	16	0,5
AB	10	9	7	0	0	0	16	0,44
HR	11	0	9	5	2	0	16	1,56
HR	12	0	5	8	3	0	16	1,88
HR	13	0	11	5	0	0	16	1,31
HR	14	1	13	2	0	0	16	1,06
HR	15	6	9	1	0	0	16	0,69
HR	16	11	5	0	0	0	16	0,31
HR	17	5	9	2	0	0	16	0,81
HR	18	5	10	1	0	0	16	0,75
HR	19	8	8	0	0	0	16	0,5
HR	20	6	10	0	0	0	16	0,63
(A)LCV	21	0	5	6	5	0	16	2
(A)LCV	22	0	5	8	3	0	16	1,88
(A)LCV	23	0	4	8	4	0	16	2
(A)LCV	24	0	6	8	2	0	16	1,75
(A)LCV	25	0	2	11	1	2	16	2,19
(A)LCV	26	0	5	7	3	1	16	2
(A)LCV	27	0	2	12	2	0	16	2
(A)LCV	28	0	5	8	3	0	16	1,88
(A)LCV	29	0	2	11	2	1	16	2,13
(A)LCV	30	0	2	8	6	0	16	2,25
IO	31	0	9	7	0	0	16	1,44
IO	32	2	11	3	0	0	16	1,06
IO	33	0	12	4	0	0	16	1,25
IO	34	0	6	7	3	0	16	1,81
IO	35	0	2	11	3	0	16	2,06
IO	36	0	10	5	1	0	16	1,44
IO	37	0	9	6	1	0	16	1,5
IO	38	0	6	8	2	0	16	1,75
IO	39	0	5	9	2	0	16	1,81
IO	40	0	10	5	1	0	16	1,44

Table 18: Frequency table of the data of the upper right prints, after treatment, in combination with the average scores per print

	Data shoeprint RB grading: After treatment			*Frequency table (number of times the score is granted per shoeprint)*				
VBET	Photo	Score 0	Score 1	Score 2	Score 3	Score 4	Total	Average
AB	1	0	3	7	6	0	16	2,19
AB	2	0	5	9	2	0	16	1,81
AB	3	0	6	6	3	1	16	1,94
AB	4	0	9	5	2	0	16	1,56
AB	5	0	2	7	6	1	16	2,38
AB	6	0	5	6	3	2	16	2,13
AB	7	0	7	7	2	0	16	1,69
AB	8	0	2	12	1	1	16	2,06
AB	9	0	5	6	4	1	16	1,5
AB	10	0	6	8	1	1	16	1,81
HR	11	0	4	5	4	3	16	2,38
HR	12	0	2	7	4	3	16	2,5
HR	13	0	7	8	1	0	16	1,63
HR	14	0	4	9	3	0	16	1,94
HR	15	0	8	6	2	0	16	1,63
HR	16	3	11	2	0	0	16	0,94
HR	17	0	10	5	1	0	16	1,44
HR	18	0	9	6	1	0	16	1,5
HR	19	1	11	4	0	0	16	1,19
HR	20	1	10	5	0	0	16	1,25
(A)LCV	21	0	6	6	3	1	16	1,94
(A)LCV	22	0	2	9	3	2	16	2,31
(A)LCV	23	0	2	8	5	1	16	2,31
(A)LCV	24	0	2	10	4	0	16	2,13
(A)LCV	25	0	1	10	2	3	16	2,44
(A)LCV	26	0	3	8	3	2	16	2,19
(A)LCV	27	0	1	10	4	1	16	2,31
(A)LCV	28	0	0	10	4	2	16	2,5
(A)LCV	29	0	1	11	2	2	16	2,31
(A)LCV	30	0	2	4	8	2	16	2,63
IO	31	0	2	6	8	0	16	2,38
IO	32	0	3	9	4	0	16	2,06
IO	33	0	1	8	7	0	16	2,38
IO	34	0	4	9	2	1	16	2
IO	35	0	1	9	4	2	16	2,44
IO	36	0	5	8	2	1	16	1,94
IO	37	0	3	10	3	0	16	2
IO	38	0	2	10	2	2	16	2,25
IO	39	0	5	6	3	2	16	2,13
IO	40	0	3	8	2	3	16	2,31

Table 19: Scheme to statistically calculate the significant difference between the average score before and after treatment for the four Visual Blood Enhancement Techniques for the upper right prints

VBET	Photo	Average Before RB	Average After RB	Difference (d) Average Before - Average After	$\bar{d}$ $(n = 10)$	$d - \bar{d}$	$(d - \bar{d})^2$	$\sum(d_i - \bar{d})^2$ $(n = 10)$	Standard deviation $\sqrt{\frac{\sum(d_i - \bar{d})^2}{n-1}}$	Paired t-test $\frac{\lvert\bar{d}\rvert \times \sqrt{n}}{Sd}$	Critical value (α=5%) (n-1) [58]	Comparison Paired t-test and t-value [58]	Significant difference vs. No significant difference
AB	1	0,88	2,19	-1,31		-0,217	0,04709						
AB	2	0,75	1,81	-1,06		0,033	0,00109						
AB	3	0,44	1,94	-1,5		-0,407	0,16565		$\sqrt{\frac{0,69941}{9}} =$	$\frac{\lvert-1,093\rvert \times \sqrt{10}}{0,27877} =$			
AB	4	0,44	1,56	-1,12		-0,027	0,00073						
AB	5	1,25	2,38	-1,13	-1,093	-0,037	0,00137	0,69941	0,27877	12,3986	2,262	2,262 < 12,3986	Significant difference
AB	6	1,25	2,13	-0,88		0,213	0,04537						
AB	7	1,19	1,69	-0,5		0,593	0,35165						
AB	8	1	2,06	-1,06		0,033	0,00109						
AB	9	0,5	1,5	-1		0,093	0,00865						
AB	10	0,44	1,81	-1,37		-0,277	0,07673						
HR	11	1,56	2,38	-0,82		-0,13	0,0169						
HR	12	1,88	2,5	-0,62		0,07	0,0049						
HR	13	1,31	1,63	-0,32		0,37	0,1369		$\sqrt{\frac{0,273}{9}} =$	$\frac{\lvert-0,69\rvert \times \sqrt{10}}{0,17416} =$			
HR	14	1,06	1,94	-0,88		-0,19	0,0361						
HR	15	0,69	1,63	-0,94		-0,25	0,0625						
HR	16	0,31	0,94	-0,63	-0,69	0,06	0,0036	0,273	0,17416	12,5285	2,262	2,262 < 12,5285	Significant difference
HR	17	0,81	1,44	-0,63		0,06	0,0036						
HR	18	0,75	1,5	-0,75		-0,06	0,0036						
HR	19	0,5	1,19	-0,69		0	0						
HR	20	0,63	1,25	-0,62		0,07	0,0049						
(A)LCV	21	2	1,94	0,06		0,359	0,12888						
(A)LCV	22	1,88	2,31	-0,43		-0,131	0,01716						
(A)LCV	23	2	2,31	-0,31		-0,011	0,00012		$\sqrt{\frac{0,29089}{9}} =$	$\frac{\lvert-0,299\rvert \times \sqrt{10}}{0,17978} =$			
(A)LCV	24	1,75	2,13	-0,38		-0,081	0,00656						
(A)LCV	25	2,19	2,44	-0,25	-0,299	0,049	0,0024	0,29089	0,17978	5,2593	2,262	2,262 < 5,2593	Significant difference
(A)LCV	26	2	2,19	-0,19		0,109	0,01188						
(A)LCV	27	2	2,31	-0,31		-0,011	0,00012						
(A)LCV	28	1,88	2,5	-0,62		-0,321	0,10304						
(A)LCV	29	2,13	2,31	-0,18		0,119	0,01416						
(A)LCV	30	2,25	2,63	-0,38		-0,081	0,00656						
IO	31	1,44	2,38	-0,94		-0,325	0,10563						
IO	32	1,06	2,06	-1		-0,385	0,14823						
IO	33	1,25	2,38	-1,13		-0,515	0,26523		$\sqrt{\frac{0,88725}{9}} =$	$\frac{\lvert-0,615\rvert \times \sqrt{10}}{0,31398} =$			
IO	34	1,81	2	-0,19		0,425	0,18063						
IO	35	2,06	2,44	-0,38		0,235	0,05523						
IO	36	1,44	1,94	-0,5	-0,615	0,115	0,01323	0,88725	0,31398	6,194	2,262	2,262 < 6,194	Significant difference
IO	37	1,5	2	-0,5		0,115	0,01323						
IO	38	1,75	2,25	-0,5		0,115	0,01323						
IO	39	1,81	2,13	-0,32		0,295	0,08703						
IO	40	1,44	2,31	-0,69		-0,075	0,00563						



Graph 2: Differences of scores between before and after treatment with the four Visual Blood Enhancement Techniques (Amido Black, Hungarian Red, (A)LCV and Iron oxide) for the upper right prints

Bottom left

Table 20: Raw data shoeprint grading with the granted scores per rater of the bottom left prints, before treatment, with the four Visual Blood Enhancement Techniques

Person:	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P
	Before	Before	Before	Before	Before	Before	Before	Before	Before	Before	Before	Before	Before	Before	Before	Before
	LO	LO	LO	LO	LO	LO	LO	LO	LO	LO	LO	LO	LO	LO	LO	LO
AB 1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0
2	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
5	0	1	0	1	1	0	1	0	1	0	0	0	0	1	1	1
6	0	1	0	0	0	0	0	0	1	0	0	0	0	1	1	1
7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0	1	0	0	0	0	1	1	1
9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	1	0	0	0	0	1	1	0
HR 11	0	1	0	0	0	1	0	1	1	0	0	0	1	1	1	1
12	0	1	0	1	1	1	0	1	1	0	0	0	1	1	1	2
13	0	0	0	0	0	0	0	0	1	0	0	0	0	1	1	1
14	0	1	0	1	1	1	0	1	1	0	0	1	1	1	1	1
15	0	1	0	0	0	0	0	0	1	0	0	0	0	0	1	0
16	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
17	0	1	0	1	1	1	0	1	1	0	0	0	1	1	1	1
18	0	1	0	1	1	1	0	1	1	0	1	1	1	1	1	2
19	0	1	0	0	0	0	0	0	1	0	0	0	0	1	1	1
20	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0
(A)LCV 21	1	2	1	1	2	2	1	1	1	1	1	1	1	1	3	3
22	1	2	1	1	2	1	1	1	1	1	1	1	1	1	3	3
23	1	2	1	1	2	1	1	1	1	1	1	1	1	1	2	2
24	0	2	1	1	1	1	1	1	1	1	1	1	1	1	1	2
25	1	2	1	1	2	1	2	1	1	1	1	1	2	1	2	3
26	1	2	1	1	2	1	2	2	1	1	1	1	1	1	3	2
27	1	2	2	1	1	1	0	1	1	1	1	1	1	1	3	2
28	2	2	1	1	1	1	1	1	2	1	1	1	1	1	2	2

29	1	2	1	1	1	1	1	1	1	1	1	1	1	1	2	2
30	1	2	1	1	1	1	1	1	2	1	1	1	2	1	2	2
IO 31	1	2	1	1	2	1	1	1	1	1	1	1	1	1	2	2
32	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
33	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
34	2	2	1	1	1	1	2	2	2	1	1	1	1	2	3	3
35	1	3	2	2	2	1	2	2	1	1	1	2	2	1	2	3
36	1	2	1	1	1	1	2	1	1	1	1	1	1	1	2	2
37	0	1	0	1	1	1	1	1	1	1	1	1	1	1	2	1
38	2	2	1	1	1	1	2	1	1	1	1	1	1	1	2	2
39	1	1	1	1	1	1	2	2	1	1	1	1	1	1	2	1
40	1	2	1	1	1	1	2	1	1	1	1	1	1	1	1	2

Table 21: Raw data shoeprint grading with the granted scores per rater of the bottom left prints, after treatment, with the four Visual Blood Enhancement Techniques

Person:	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P
	After	After	After	After	After	After	After	After	After	After	After	After	After	After	After	After
	LO	LO	LO	LO	LO	LO	LO	LO	LO	LO	LO	LO	LO	LO	LO	LO
AB 1	1	2	1	2	2	2	1	1	2	2	1	1	1	1	3	3
2	0	2	1	1	1	1	1	1	1	1	1	1	1	1	2	1
3	1	2	1	1	1	1	1	1	1	0	1	1	1	1	2	2
4	0	1	0	1	1	1	0	0	1	0	0	1	1	1	2	2
5	2	2	1	1	2	2	2	2	3	2	1	1	2	1	4	2
6	1	2	1	1	1	2	2	2	1	1	1	1	1	1	3	2
7	1	2	1	1	1	1	1	1	1	1	1	1	1	1	3	2
8	1	2	1	1	1	2	1	2	1	1	1	1	1	1	3	2
9	2	2	1	1	2	2	2	2	3	1	1	1	1	1	4	2
10	2	2	1	1	2	2	2	2	2	1	1	1	1	1	4	2
HR 11	1	2	1	1	1	2	1	2	1	1	1	1	1	1	2	2
12	1	3	1	2	1	1	1	2	1	1	1	1	1	1	2	2
13	0	2	1	1	2	1	1	2	1	1	1	1	1	1	2	2
14	2	2	1	1	2	1	1	2	1	1	1	1	1	1	2	2
15	1	2	1	1	1	1	1	1	1	1	1	1	1	1	3	2
16	0	1	0	1	1	1	1	1	1	0	0	0	1	1	1	1

17	1	1	0	1	2	1	0	1	1	0	1	1	1	1	2	2
18	2	2	1	1	2	2	1	2	1	1	1	1	1	2	3	3
19	0	2	0	1	2	1	1	1	1	0	1	1	1	1	2	2
20	0	2	0	1	1	1	1	1	1	0	1	1	1	1	2	2
(A)LCV 21	2	2	1	1	2	3	1	2	1	1	1	1	1	1	3	4
22	1	3	1	1	2	2	2	2	2	1	1	2	1	2	3	3
23	2	2	2	1	2	2	2	2	2	1	1	1	2	1	3	3
24	2	2	1	1	1	2	2	1	2	1	1	1	2	1	2	2
25	2	2	1	2	2	2	2	2	2	1	1	2	2	1	4	3
26	2	2	1	1	1	1	2	2	2	1	1	1	1	1	4	3
27	2	2	2	2	2	2	2	2	2	1	1	2	1	1	3	3
28	3	2	1	1	1	2	2	2	2	1	1	2	1	1	3	3
29	3	2	1	1	2	2	1	2	2	1	1	1	2	1	3	3
30	3	2	1	2	2	1	2	2	2	1	1	1	2	1	3	2
IO 31	2	3	1	2	2	2	2	2	2	2	1	1	1	1	3	3
32	1	2	1	1	2	1	1	1	2	1	1	1	2	1	3	3
33	2	2	1	2	2	2	2	2	2	2	1	1	1	2	2	2
34	2	2	1	1	2	2	3	2	2	2	1	2	1	2	4	3
35	2	3	2	2	2	2	4	2	2	1	1	2	2	1	4	3
36	2	3	1	2	2	2	4	2	2	2	1	2	3	2	3	3
37	1	2	1	1	2	1	2	2	1	1	1	1	1	1	3	3
38	2	3	2	2	2	2	3	2	1	1	1	1	2	1	3	3
39	2	3	1	2	2	2	3	2	2	1	1	1	1	1	3	2
40	2	2	1	2	2	3	3	2	2	2	1	2	2	2	3	3

Table 22: Frequency table of the data of the bottom left prints, before treatment, in combination with the average scores per print

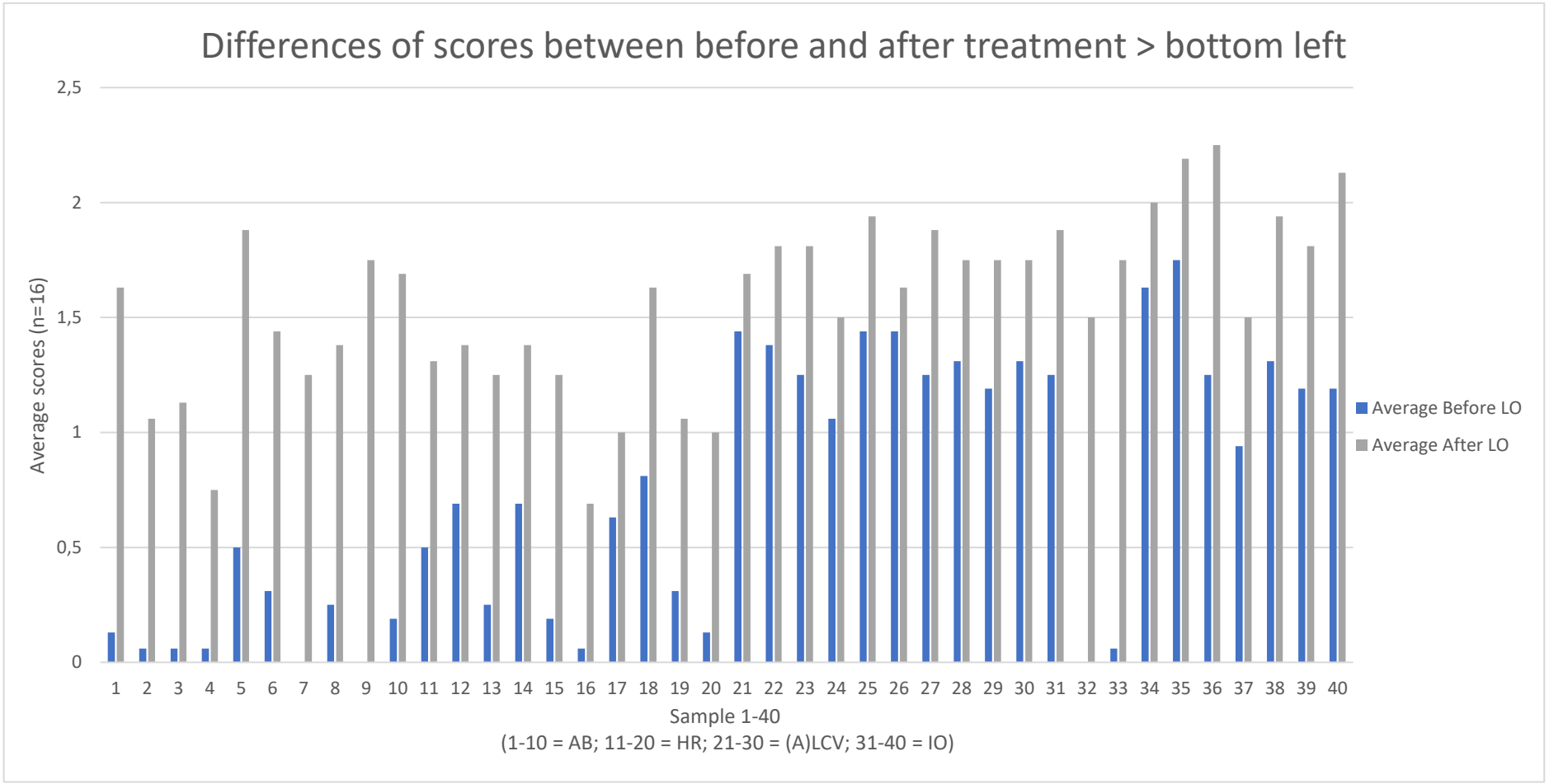
	Data shoeprint LO grading: Before treatment			*Frequency table (number of times the score is granted per shoeprint)*				
VBET	Photo	Score 0	Score 1	Score 2	Score 3	Score 4	Total	Average
AB	1	14	2	0	0	0	16	0,13
AB	2	15	1	0	0	0	16	0,06
AB	3	15	1	0	0	0	16	0,06
AB	4	15	1	0	0	0	16	0,06
AB	5	8	8	0	0	0	16	0,5
AB	6	11	5	0	0	0	16	0,31
AB	7	16	0	0	0	0	16	0
AB	8	12	4	0	0	0	16	0,25
AB	9	16	0	0	0	0	16	0
AB	10	13	3	0	0	0	16	0,19
HR	11	8	8	0	0	0	16	0,5
HR	12	6	9	1	0	0	16	0,69
HR	13	12	4	0	0	0	16	0,25
HR	14	5	11	0	0	0	16	0,69
HR	15	13	3	0	0	0	16	0,19
HR	16	15	1	0	0	0	16	0,06
HR	17	6	10	0	0	0	16	0,63
HR	18	4	11	1	0	0	16	0,81
HR	19	11	5	0	0	0	16	0,31
HR	20	14	2	0	0	0	16	0,13
(A)LCV	21	0	11	3	2	0	16	1,44
(A)LCV	22	0	12	2	2	0	16	1,38
(A)LCV	23	0	12	4	0	0	16	1,25
(A)LCV	24	1	13	2	0	0	16	1,06
(A)LCV	25	0	10	5	1	0	16	1,44
(A)LCV	26	0	10	5	1	0	16	1,44
(A)LCV	27	1	11	3	1	0	16	1,25
(A)LCV	28	0	11	5	0	0	16	1,31
(A)LCV	29	0	13	3	0	0	16	1,19
(A)LCV	30	0	11	5	0	0	16	1,31
IO	31	0	12	4	0	0	16	1,25
IO	32	16	0	0	0	0	16	0
IO	33	15	1	0	0	0	16	0,06
IO	34	0	8	6	2	0	16	1,63
IO	35	0	6	8	2	0	16	1,75
IO	36	0	12	4	0	0	16	1,25
IO	37	2	13	1	0	0	16	0,94
IO	38	0	11	5	0	0	16	1,31
IO	39	0	13	3	0	0	16	1,19
IO	40	0	13	3	0	0	16	1,19

Table 23: Frequency table of the data of the bottom left prints, after treatment, in combination with the average scores per print

	Data shoeprint LO grading: After treatment			*Frequency table (number of times the score is granted per shoeprint)*				
VBET	Photo	Score 0	Score 1	Score 2	Score 3	Score 4	Total	Average
AB	1	0	8	6	2	0	16	1,63
AB	2	1	13	2	0	0	16	1,06
AB	3	1	12	3	0	0	16	1,13
AB	4	6	8	2	0	0	16	0,75
AB	5	0	5	9	1	1	16	1,88
AB	6	0	10	5	1	0	16	1,44
AB	7	0	13	2	1	0	16	1,25
AB	8	0	11	4	1	0	16	1,38
AB	9	0	7	7	1	1	16	1,75
AB	10	0	7	8	0	1	16	1,69
HR	11	0	11	5	0	0	16	1,31
HR	12	0	11	4	1	0	16	1,38
HR	13	1	10	5	0	0	16	1,25
HR	14	0	10	6	0	0	16	1,38
HR	15	0	13	2	1	0	16	1,25
HR	16	5	11	0	0	0	16	0,69
HR	17	3	10	3	0	0	16	1
HR	18	0	8	6	2	0	16	1,63
HR	19	3	9	4	0	0	16	1,06
HR	20	3	10	3	0	0	16	1
(A)LCV	21	0	9	4	2	1	16	1,69
(A)LCV	22	0	6	7	3	0	16	1,81
(A)LCV	23	0	5	9	2	0	16	1,81
(A)LCV	24	0	8	8	0	0	16	1,5
(A)LCV	25	0	4	10	1	1	16	1,94
(A)LCV	26	0	9	5	1	1	16	1,63
(A)LCV	27	0	4	10	2	0	16	1,88
(A)LCV	28	0	7	6	3	0	16	1,75
(A)LCV	29	0	7	6	3	0	16	1,75
(A)LCV	30	0	6	8	2	0	16	1,75
IO	31	0	5	8	3	0	16	1,88
IO	32	0	10	4	2	0	16	1,5
IO	33	0	4	12	0	0	16	1,75
IO	34	0	4	9	2	1	16	2
IO	35	0	3	9	2	2	16	2,19
IO	36	0	2	9	4	1	16	2,25
IO	37	0	10	4	2	0	16	1,5
IO	38	0	5	7	4	0	16	1,94
IO	39	0	6	7	3	0	16	1,81
IO	40	0	2	10	4	0	16	2,13

Table 24: Scheme to statistically calculate the significant difference between the average score before and after treatment for the four Visual Blood Enhancement Techniques for the bottom left prints

VBET	Photo	Average Before LO	Average After LO	Difference (d) Average Before - Average After	$\bar{d}$ $(n = 10)$	$d - \bar{d}$	$(d - \bar{d})^2$	$\sum(d_i - \bar{d})^2$ $(n = 10)$	Standard deviation $\sqrt{\frac{\sum(d_i - \bar{d})^2}{n-1}}$	Paired t-test $\frac{\lvert\bar{d}\rvert \times \sqrt{n}}{Sd}$	Critical value (α=5%) (n-1) [58]	Comparison Paired t-test and t-value [58]	Significant difference vs. No significant difference
AB	1	0,13	1,63	-1,5		-0,26	0,0676						
AB	2	0,06	1,06	-1		0,24	0,0576						
AB	3	0,06	1,13	-1,07		0,17	0,0289		$\sqrt{\frac{0,8282}{9}} =$	$\frac{\lvert-1,24\rvert \times \sqrt{10}}{0,30335} =$			
AB	4	0,06	0,75	-0,69		0,55	0,3025						
AB	5	0,5	1,88	-1,38	-1,24	-0,14	0,0196	0,8282	0,30335	12,9264	2,262	2,262 < 12,9264	Significant difference
AB	6	0,31	1,44	-1,13		0,11	0,0121						
AB	7	0	1,25	-1,25		-0,01	0,0001						
AB	8	0,25	1,38	-1,13		0,11	0,0121						
AB	9	0	1,75	-1,75		-0,51	0,2601						
AB	10	0,19	1,69	-1,5		-0,26	0,0676						
HR	11	0,5	1,31	-0,81		-0,041	0,00168						
HR	12	0,69	1,38	-0,69		0,079	0,00624						
HR	13	0,25	1,25	-1		-0,231	0,05336		$\sqrt{\frac{0,34378}{9}} =$	$\frac{\lvert-0,769\rvert \times \sqrt{10}}{0,19544} =$			
HR	14	0,69	1,38	-0,69		0,079	0,00624						
HR	15	0,19	1,25	-1,06		-0,291	0,08468						
HR	16	0,06	0,69	-0,63	-0,769	0,139	0,01921	0,34378	0,19544	12,4427	2,262	2,262 < 12,4427	Significant difference
HR	17	0,63	1	-0,37		0,399	0,1592						
HR	18	0,81	1,63	-0,82		-0,051	0,0026						
HR	19	0,31	1,06	-0,75		0,019	0,00036						
HR	20	0,13	1	-0,87		-0,101	0,0102						
(A)LCV	21	1,44	1,69	-0,25		0,194	0,03764						
(A)LCV	22	1,38	1,81	-0,43		0,014	0,0002						
(A)LCV	23	1,25	1,81	-0,56		-0,116	0,01346		$\sqrt{\frac{0,16704}{9}} =$	$\frac{\lvert-0,444\rvert \times \sqrt{10}}{0,13624} =$			
(A)LCV	24	1,06	1,5	-0,44		0,004	1,6E-05						
(A)LCV	25	1,44	1,94	-0,5		-0,056	0,00314						
(A)LCV	26	1,44	1,63	-0,19	-0,444	0,254	0,06452	0,16704	0,13624	10,3057	2,262	2,262 < 10,3057	Significant difference
(A)LCV	27	1,25	1,88	-0,63		-0,186	0,0346						
(A)LCV	28	1,31	1,75	-0,44		0,004	1,6E-05						
(A)LCV	29	1,19	1,75	-0,56		-0,116	0,01346						
(A)LCV	30	1,31	1,75	-0,44		0,004	1,6E-05						
IO	31	1,25	1,88	-0,63		0,208	0,04326						
IO	32	0	1,5	-1,5		-0,662	0,43824						
IO	33	0,06	1,75	-1,69		-0,852	0,7259		$\sqrt{\frac{1,78956}{9}} =$	$\frac{\lvert-0,838\rvert \times \sqrt{10}}{0,44591} =$			
IO	34	1,63	2	-0,37		0,468	0,21902						
IO	35	1,75	2,19	-0,44		0,398	0,1584						
IO	36	1,25	2,25	-1	-0,838	-0,162	0,02624	1,78956	0,44591	5,9429	2,262	2,262 < 5,9429	Significant difference
IO	37	0,94	1,5	-0,56		0,278	0,07728						
IO	38	1,31	1,94	-0,63		0,208	0,04326						
IO	39	1,19	1,81	-0,62		0,218	0,04752						
IO	40	1,19	2,13	-0,94		-0,102	0,0104						



Graph 3: Differences of scores between before and after treatment with the four Visual Blood Enhancement Techniques (Amido Black, Hungarian Red, (A)LCV and Iron oxide) for the bottom left prints

Bottom right

Table 25: Raw data shoeprint grading with the granted scores per rater of the bottom right prints, before treatment, with the four Visual Blood Enhancement Techniques

Person:	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P
	Before	Before	Before	Before	Before	Before	Before	Before	Before	Before	Before	Before	Before	Before	Before	Before
	RO	RO	RO	RO	RO	RO	RO	RO	RO	RO	RO	RO	RO	RO	RO	RO
AB 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
5	0	1	0	0	1	0	0	0	1	0	0	0	0	1	1	1
6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
HR 11	0	1	0	0	0	0	0	1	1	0	0	0	0	1	1	1
12	0	1	0	1	0	1	0	1	1	0	0	0	1	1	1	1
13	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
14	0	1	0	1	1	0	0	0	1	0	0	0	0	1	1	1
15	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
16	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
17	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0
18	0	2	0	1	1	1	0	1	1	0	1	1	1	1	1	1
19	0	1	0	0	0	0	0	0	1	0	0	0	0	1	1	0
20	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
(A)LCV 21	1	2	1	1	1	1	1	1	1	1	1	1	1	1	2	2
22	1	2	0	1	1	1	1	1	1	0	1	1	1	1	2	2
23	0	1	0	1	1	1	1	1	1	0	1	0	1	1	1	2
24	0	1	0	1	1	1	0	0	1	0	1	1	1	1	1	1
25	1	1	1	1	2	1	1	1	1	0	1	1	1	1	2	2
26	1	1	1	1	1	1	2	1	1	1	1	1	1	1	2	2
27	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	1
28	1	1	0	1	1	1	0	1	1	1	1	1	1	1	1	1

29	0	1	0	1	1	1	0	1	1	0	1	1	1	1	1	1
30	0	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1
IO 31	2	2	1	1	2	2	1	1	1	1	1	2	1	1	2	3
32	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
33	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
34	0	2	1	1	1	1	1	1	1	1	1	1	1	1	2	2
35	0	2	1	2	2	1	1	1	1	1	1	1	1	1	2	2
36	0	2	1	1	1	1	1	1	1	1	1	1	1	1	2	1
37	0	1	1	1	1	1	2	1	1	1	1	1	1	1	2	1
38	1	1	1	1	1	1	2	1	1	1	1	1	1	1	2	1
39	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
40	0	1	0	1	1	1	1	1	1	0	1	0	1	1	1	1

Table 26: Raw data shoeprint grading with the granted scores per rater of the bottom right prints, after treatment, with the four Visual Blood Enhancement Techniques

Person:	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P
	After	After	After	After	After	After	After	After	After	After	After	After	After	After	After	After
	RO	RO	RO	RO	RO	RO	RO	RO	RO	RO	RO	RO	RO	RO	RO	RO
AB 1	1	2	1	1	2	2	1	1	2	1	1	1	1	1	3	2
2	0	2	1	1	1	1	1	1	1	1	1	1	1	1	2	1
3	0	2	1	1	1	1	1	1	1	0	1	1	1	1	2	2
4	0	1	0	1	1	1	0	0	1	0	0	0	0	1	1	1
5	1	2	0	1	2	2	2	2	3	1	1	1	1	1	4	2
6	1	2	1	1	1	2	2	2	1	1	1	1	1	1	3	2
7	0	1	1	1	1	1	1	1	1	1	1	1	1	1	3	2
8	1	2	1	1	1	2	1	1	1	1	1	1	1	1	3	2
9	2	2	1	1	1	2	1	2	2	1	1	1	1	1	3	2
10	1	1	0	1	1	1	2	1	1	1	1	1	1	1	3	2
HR 11	0	1	0	1	1	1	1	1	1	1	1	0	1	1	2	2
12	1	2	1	1	1	1	1	1	1	1	1	1	1	1	2	2
13	0	1	1	1	1	1	1	1	1	1	1	1	1	1	2	2
14	2	2	1	1	1	1	1	2	1	1	1	1	1	1	2	2
15	0	1	1	1	1	1	1	1	1	1	1	0	1	1	2	2
16	0	1	0	1	1	1	0	1	1	0	0	0	1	1	1	1

17	0	1	0	1	1	1	0	1	1	0	0	0	1	1	1	1
18	2	2	1	1	2	2	2	2	1	1	1	1	1	2	3	2
19	0	1	0	1	1	1	1	1	1	0	1	1	1	1	2	1
20	0	1	0	1	1	1	1	1	1	0	1	0	1	1	2	1
(A)LCV 21	1	2	1	1	1	2	1	1	1	1	1	1	1	1	2	3
22	1	2	1	1	2	1	2	1	1	1	1	1	1	1	3	3
23	1	2	0	1	1	1	2	1	1	1	1	0	1	1	3	2
24	1	2	1	1	1	1	1	1	2	1	1	1	1	1	2	2
25	2	2	1	2	2	1	2	1	2	1	1	1	2	1	4	2
26	2	2	1	1	1	1	2	2	1	1	1	1	1	1	4	2
27	2	1	1	1	1	1	2	1	1	1	1	1	1	1	3	1
28	2	1	1	1	1	1	2	1	1	1	1	1	1	1	3	2
29	2	2	1	1	1	1	1	1	1	1	1	1	1	1	2	2
30	2	2	1	1	1	1	1	1	1	1	1	1	1	1	2	1
IO 31	2	2	1	1	3	2	1	2	1	2	1	2	1	1	3	3
32	1	2	1	1	2	1	1	1	1	1	1	1	1	1	2	2
33	2	2	1	2	2	1	2	2	2	2	1	1	1	1	2	2
34	1	2	1	1	1	1	2	2	2	1	1	2	1	1	3	3
35	2	3	2	2	2	2	4	2	2	1	1	2	2	1	4	4
36	2	2	1	2	2	2	4	2	2	2	1	2	2	2	3	3
37	1	3	1	1	2	1	2	1	1	1	1	1	1	1	3	3
38	2	3	1	1	2	2	3	1	1	2	1	1	1	1	3	3
39	2	3	1	2	2	1	3	2	2	1	1	1	1	1	3	2
40	1	2	1	1	2	2	2	2	2	2	1	1	1	1	3	2

Table 27: Frequency table of the data of the bottom right prints, before treatment, in combination with the average scores per print

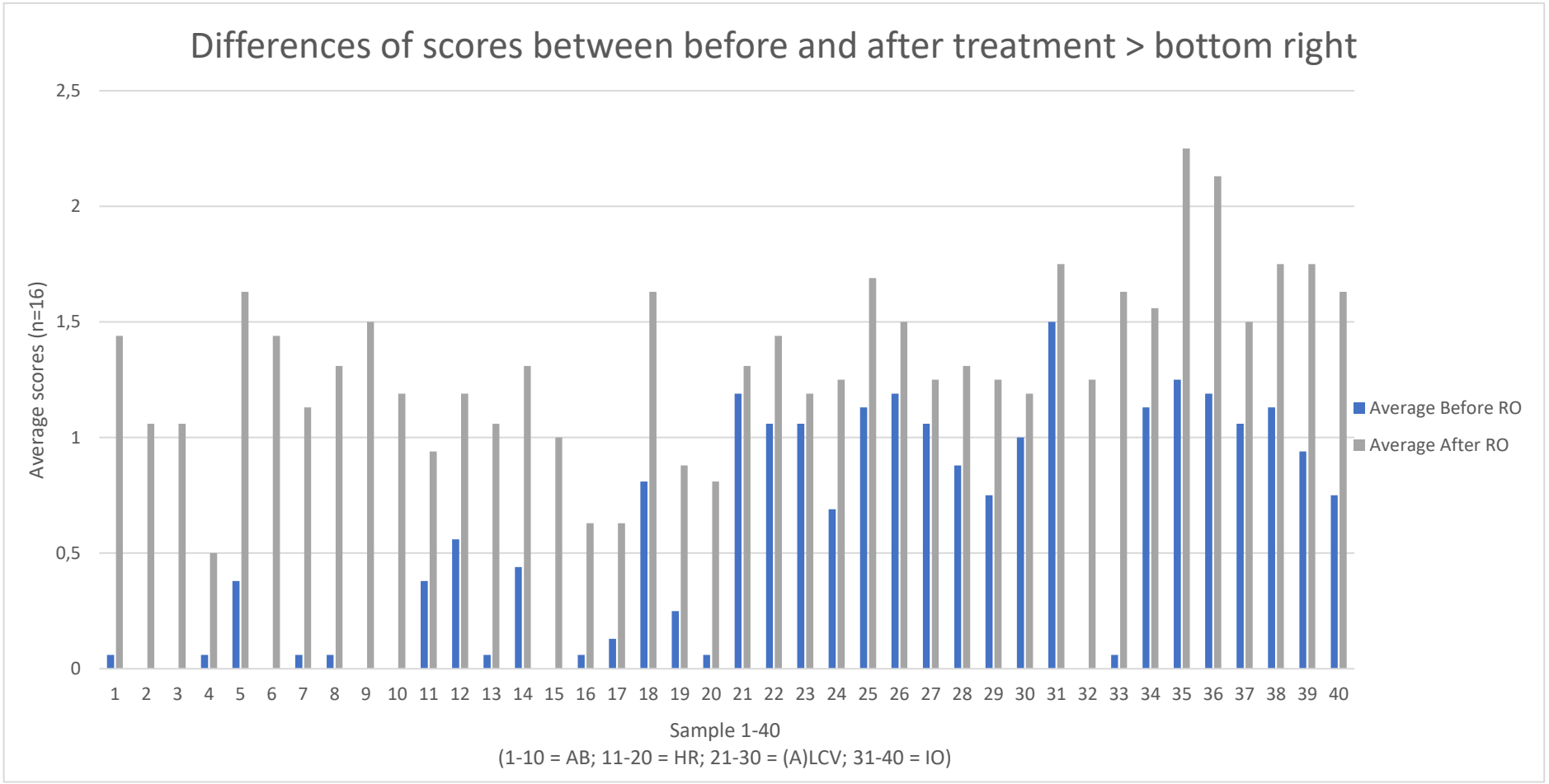
	Data shoeprint RO grading: Before treatment			*Frequency table (number of times the score is granted per shoeprint)*				
VBET	Photo	Score 0	Score 1	Score 2	Score 3	Score 4	Total	Average
AB	1	15	1	0	0	0	16	0,06
AB	2	16	0	0	0	0	16	0
AB	3	15	1	0	0	0	16	0,06
AB	4	15	1	0	0	0	16	0,06
AB	5	10	6	0	0	0	16	0,38
AB	6	16	0	0	0	0	16	0
AB	7	15	1	0	0	0	16	0,06
AB	8	15	1	0	0	0	16	0,06
AB	9	16	0	0	0	0	16	0
AB	10	16	0	0	0	0	16	0
HR	11	10	6	0	0	0	16	0,38
HR	12	7	9	0	0	0	16	0,56
HR	13	15	1	0	0	0	16	0,06
HR	14	9	7	0	0	0	16	0,44
HR	15	16	0	0	0	0	16	0
HR	16	15	1	0	0	0	16	0,06
HR	17	14	2	0	0	0	16	0,13
HR	18	4	11	1	0	0	16	0,81
HR	19	12	4	0	0	0	16	0,25
HR	20	15	1	0	0	0	16	0,06
(A)LCV	21	0	13	3	0	0	16	1,19
(A)LCV	22	2	11	3	0	0	16	1,06
(A)LCV	23	2	11	3	0	0	16	1,06
(A)LCV	24	5	11	0	0	0	16	0,69
(A)LCV	25	1	12	3	0	0	16	1,13
(A)LCV	26	0	13	3	0	0	16	1,19
(A)LCV	27	0	15	1	0	0	16	1,06
(A)LCV	28	2	14	0	0	0	16	0,88
(A)LCV	29	4	12	0	0	0	16	0,75
(A)LCV	30	1	14	1	0	0	16	1
IO	31	0	9	6	1	0	16	1,5
IO	32	16	0	0	0	0	16	0
IO	33	15	1	0	0	0	16	0,06
IO	34	1	12	3	0	0	16	1,13
IO	35	1	10	5	0	0	16	1,25
IO	36	1	13	2	0	0	16	1,19
IO	37	1	13	2	0	0	16	1,06
IO	38	0	14	2	0	0	16	1,13
IO	39	1	15	0	0	0	16	0,94
IO	40	4	12	0	0	0	16	0,75

Table 28: Frequency table of the data of the bottom right prints, after treatment, in combination with the average scores per print

	Data shoeprint RO grading: After treatment			*Frequency table (number of times the score is granted per shoeprint)*				
VBET	Photo	Score 0	Score 1	Score 2	Score 3	Score 4	Total	Average
AB	1	0	10	5	1	0	16	1,44
AB	2	1	13	2	0	0	16	1,06
AB	3	2	11	3	0	0	16	1,06
AB	4	8	8	0	0	0	16	0,5
AB	5	1	7	6	1	1	16	1,63
AB	6	0	10	5	1	0	16	1,44
AB	7	1	13	1	1	0	16	1,13
AB	8	0	12	3	1	0	16	1,31
AB	9	0	9	6	1	0	16	1,5
AB	10	1	12	2	1	0	16	1,19
HR	11	3	11	2	0	0	16	0,94
HR	12	0	13	3	0	0	16	1,19
HR	13	1	13	2	0	0	16	1,06
HR	14	0	11	5	0	0	16	1,31
HR	15	2	12	2	0	0	16	1
HR	16	6	10	0	0	0	16	0,63
HR	17	6	10	0	0	0	16	0,63
HR	18	0	7	8	1	0	16	1,63
HR	19	3	12	1	0	0	16	0,88
HR	20	4	11	1	0	0	16	0,81
(A)LCV	21	0	12	3	1	0	16	1,31
(A)LCV	22	0	11	3	2	0	16	1,44
(A)LCV	23	2	10	3	1	0	16	1,19
(A)LCV	24	0	12	4	0	0	16	1,25
(A)LCV	25	0	7	8	0	1	16	1,69
(A)LCV	26	0	10	5	0	1	16	1,5
(A)LCV	27	0	13	2	1	0	16	1,25
(A)LCV	28	0	12	3	1	0	16	1,31
(A)LCV	29	0	12	4	0	0	16	1,25
(A)LCV	30	0	13	3	0	0	16	1,19
IO	31	0	7	6	3	0	16	1,75
IO	32	0	12	4	0	0	16	1,25
IO	33	0	6	10	0	0	16	1,63
IO	34	0	9	5	2	0	16	1,56
IO	35	0	3	9	1	3	16	2,25
IO	36	0	2	11	2	1	16	2,13
IO	37	0	11	2	3	0	16	1,5
IO	38	0	8	4	4	0	16	1,75
IO	39	0	7	6	3	0	16	1,75
IO	40	0	7	8	1	0	16	1,63

Table 29: Scheme to statistically calculate the significant difference between the average score before and after treatment for the four Visual Blood Enhancement Techniques for the bottom right prints

VBET	Photo	Average Before RO	Average After RO	Difference (d) Average Before - Average After	$\bar{d}$ $(n = 10)$	$d - \bar{d}$	$(d - \bar{d})^2$	$\sum(d_i - \bar{d})^2$ $(n = 10)$	Standard deviation $\sqrt{\frac{\sum(d_i - \bar{d})^2}{n-1}}$	Paired t-test $\frac{\lvert\bar{d}\rvert \times \sqrt{n}}{Sd}$	Critical value (α=5%) (n-1) [58]	Comparison Paired t-test and t-value [58]	Significant difference vs. No significant difference
AB	1	0,06	1,44	-1,38		-0,216	0,04666						
AB	2	0	1,06	-1,06		0,104	0,01082						
AB	3	0	1,06	-1,06		0,104	0,01082						
AB	4	0,06	0,5	-0,44		0,724	0,52418						
AB	5	0,38	1,63	-1,25		-0,086	0,0074						
AB	6	0	1,44	-1,44	-1,164	-0,276	0,07618	0,80584	$\sqrt{\frac{0,80584}{9}} =$	$\frac{\lvert-1,164\rvert \times \sqrt{10}}{0,29923} =$	2,262	2,262 < 12,3012	Significant difference
AB	7	0,06	1,13	-1,07		0,094	0,00884		0,29923	12,3012			
AB	8	0,06	1,31	-1,25		-0,086	0,0074						
AB	9	0	1,5	-1,5		-0,336	0,1129						
AB	10	0	1,19	-1,19		-0,026	0,00068						
HR	11	0,38	0,94	-0,56		0,173	0,02993						
HR	12	0,56	1,19	-0,63		0,103	0,01061						
HR	13	0,06	1,06	-1		-0,267	0,07129						
HR	14	0,44	1,31	-0,87		-0,137	0,01877						
HR	15	0	1	-1	-0,733	-0,267	0,07129	0,30121	$\sqrt{\frac{0,30121}{9}} =$	$\frac{\lvert-0,733\rvert \times \sqrt{10}}{0,18294} =$	2,262	2,262 < 12,6705	Significant difference
HR	16	0,06	0,63	-0,57		0,163	0,02657		0,18294	12,6705			
HR	17	0,13	0,63	-0,5		0,233	0,05429						
HR	18	0,81	1,63	-0,82		-0,087	0,00757						
HR	19	0,25	0,88	-0,63		0,103	0,01061						
HR	20	0,06	0,81	-0,75		-0,017	0,00029						
(A)LCV	21	1,19	1,31	-0,12		0,217	0,04709						
(A)LCV	22	1,06	1,44	-0,38		-0,043	0,00185						
(A)LCV	23	1,06	1,19	-0,13		0,207	0,04285						
(A)LCV	24	0,69	1,25	-0,56		-0,233	0,05429						
(A)LCV	25	1,13	1,69	-0,56		-0,233	0,05289						
(A)LCV	26	1,19	1,5	-0,31	-0,337	0,027	0,00073	0,27813	$\sqrt{\frac{0,27813}{9}} =$	$\frac{\lvert-0,337\rvert \times \sqrt{10}}{0,17579} =$	2,262	2,262 < 6,0623	Significant difference
(A)LCV	27	1,06	1,25	-0,19		0,147	0,02161		0,17579	6,0623			
(A)LCV	28	0,88	1,31	-0,43		-0,093	0,00865						
(A)LCV	29	0,75	1,25	-0,5		-0,163	0,02657						
(A)LCV	30	1	1,19	-0,19		0,147	0,02161						
IO	31	1,5	1,75	-0,25		0,569	0,32376						
IO	32	0	1,25	-1,25		-0,431	0,18576						
IO	33	0,06	1,63	-1,57		-0,751	0,564						
IO	34	1,13	1,56	-0,43		0,389	0,15132						
IO	35	1,25	2,25	-1		-0,181	0,03276						
IO	36	1,19	2,13	-0,94	-0,819	-0,121	0,01464	1,45929	$\sqrt{\frac{1,45929}{9}} =$	$\frac{\lvert-0,819\rvert \times \sqrt{10}}{0,40267} =$	2,262	2,262 < 6,4318	Significant difference
IO	37	1,06	1,5	-0,44		0,379	0,14364		0,40267	6,4318			
IO	38	1,13	1,75	-0,62		0,199	0,0396						
IO	39	0,94	1,75	-0,81		0,009	8,1E-05						
IO	40	0,75	1,63	-0,88		-0,061	0,00372						



Graph 4: Differences of scores between before and after treatment with the four Visual Blood Enhancement Techniques (Amido Black, Hungarian Red, (A)LCV and Iron oxide) for the bottom right prints

Appendix 9: Statistical analysis with SPSS

This appendix includes the calculations of the ICC-test with detailed methodology and results.

Intraclass Correlation Coefficient

Calculating the ICC with SPSS:

Step 1: Import the data in the data editor of SPSS

Step 2: Click ‘Analyze’ > ‘Scale’ > ‘Reliability analysis’

Step 3: Move the variables, that need to be analysed, to the ‘Items’ box

Step 4: Click ‘Statistics’ > Select ‘Intraclass Correlation Coefficient’ > Select model ‘Two-way random’ > Select type ‘Absolute agreement’

Step 5: Fill in 95% for the confidence interval

Step 6: Click ‘Continue’ > Click ‘OK’

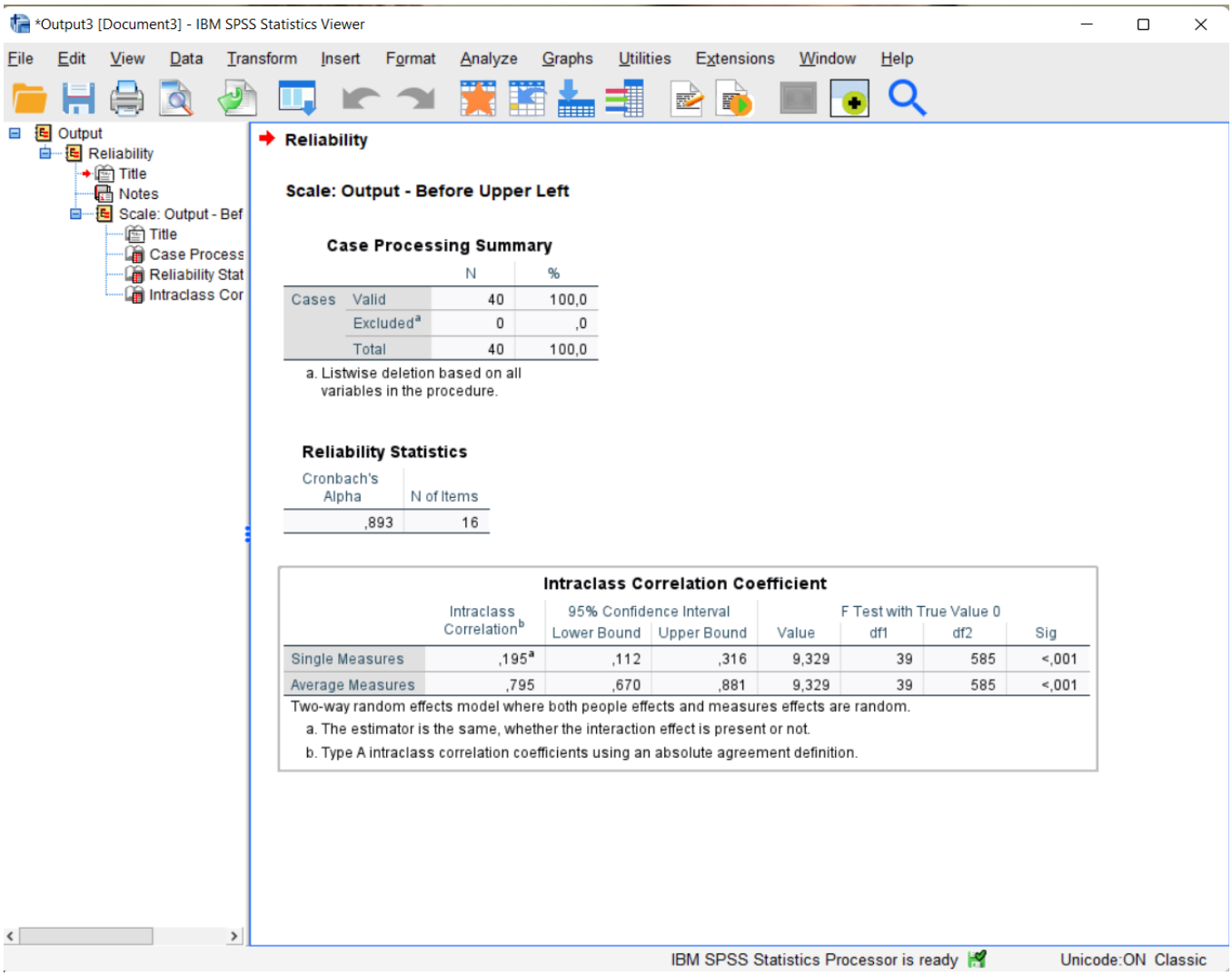


Figure 30: Output of the SPSS reliability analysis based on the shoeprint grading data of the upper left print, before treatment. It contains the corresponding two-way random intraclass correlation coefficient with absolute agreement for multiple evaluators, for single and average measures, with a 95% confidence interval

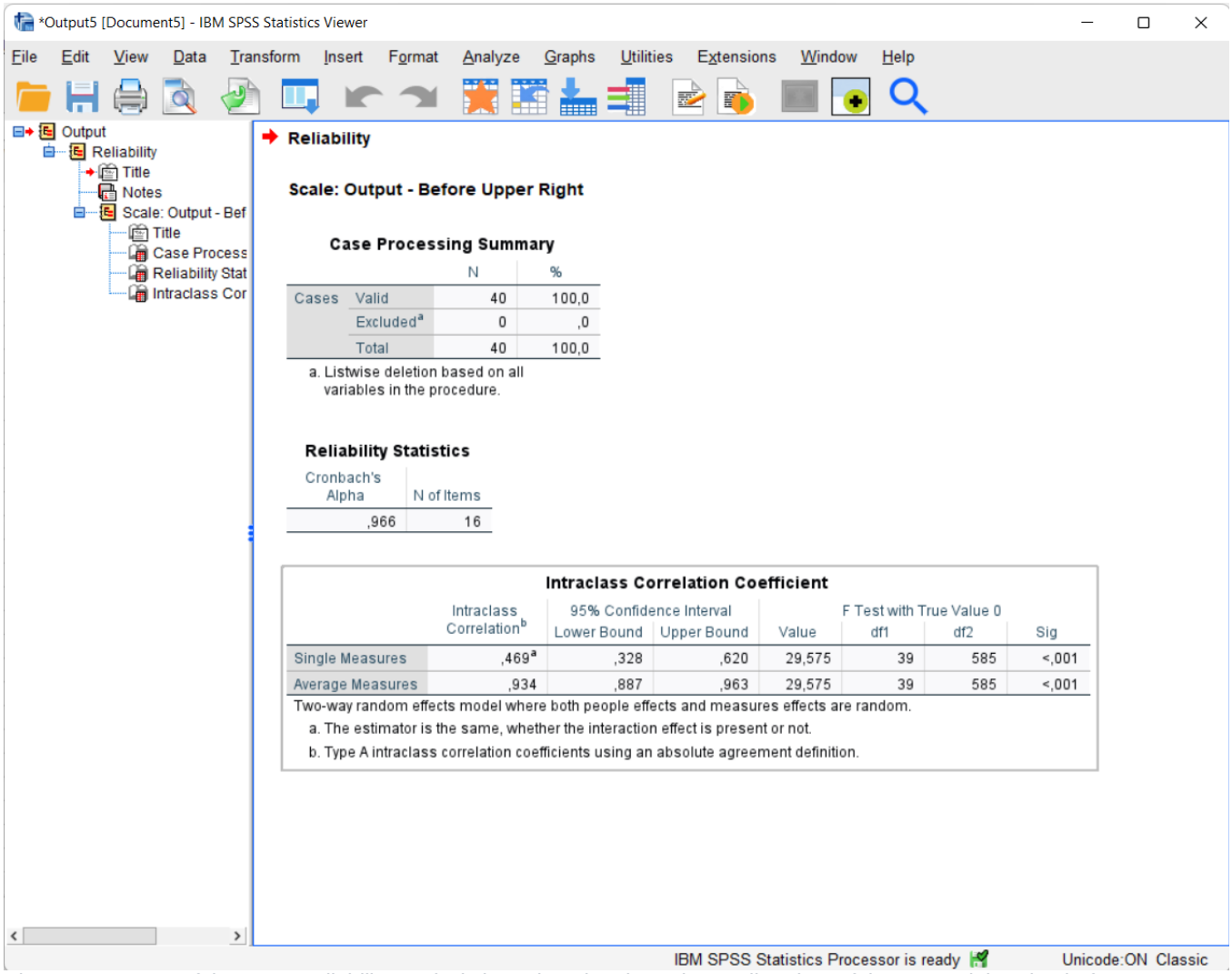


Figure 31: Output of the SPSS reliability analysis based on the shoeprint grading data of the upper right print, before treatment. It contains the corresponding two-way random intraclass correlation coefficient with absolute agreement for multiple evaluators, for single and average measures, with a 95% confidence interval

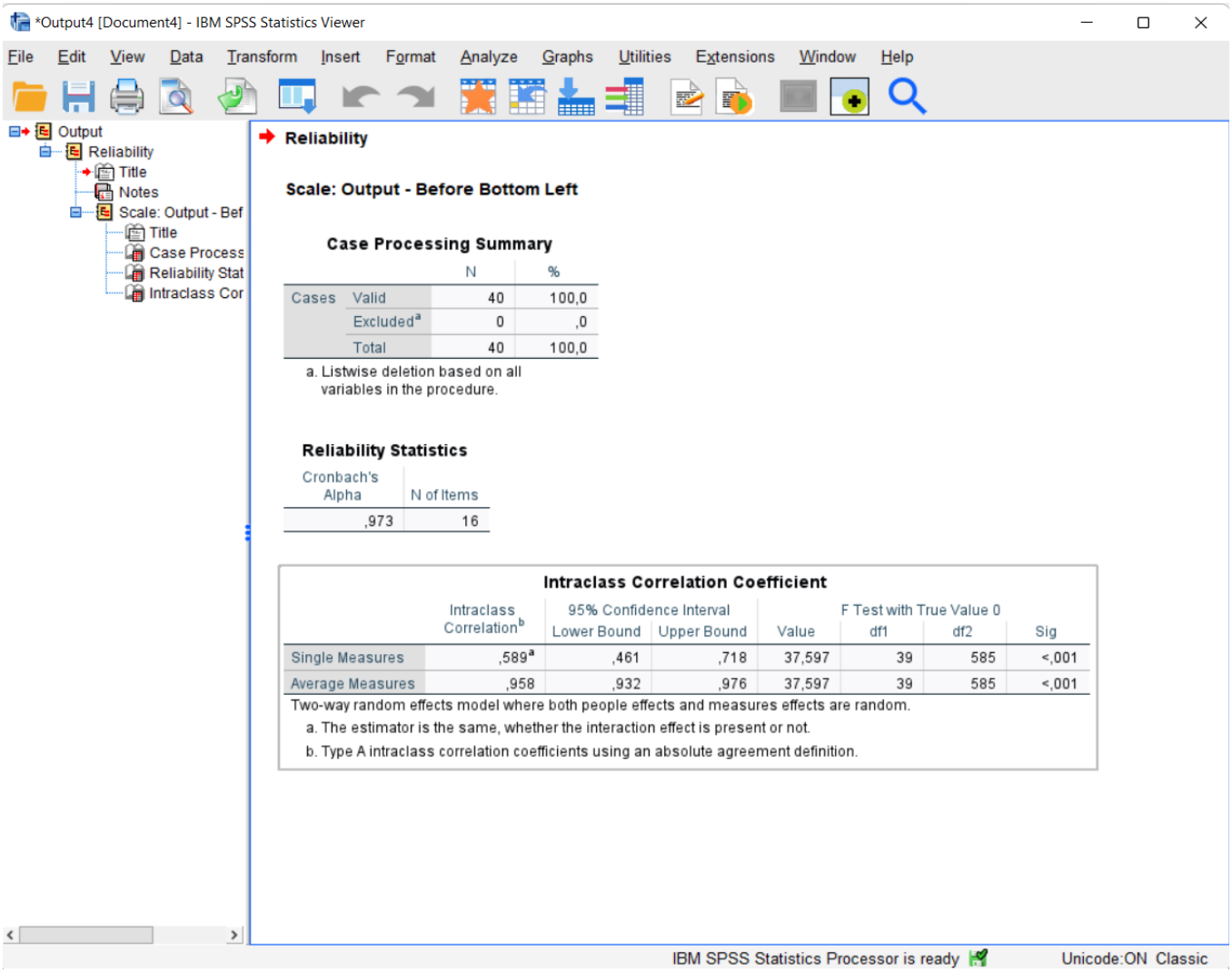


Figure 32: Output of the SPSS reliability analysis based on the shoeprint grading data of the bottom left print, before treatment. It contains the corresponding two-way random intraclass correlation coefficient with absolute agreement for multiple evaluators, for single and average measures, with a 95% confidence interval

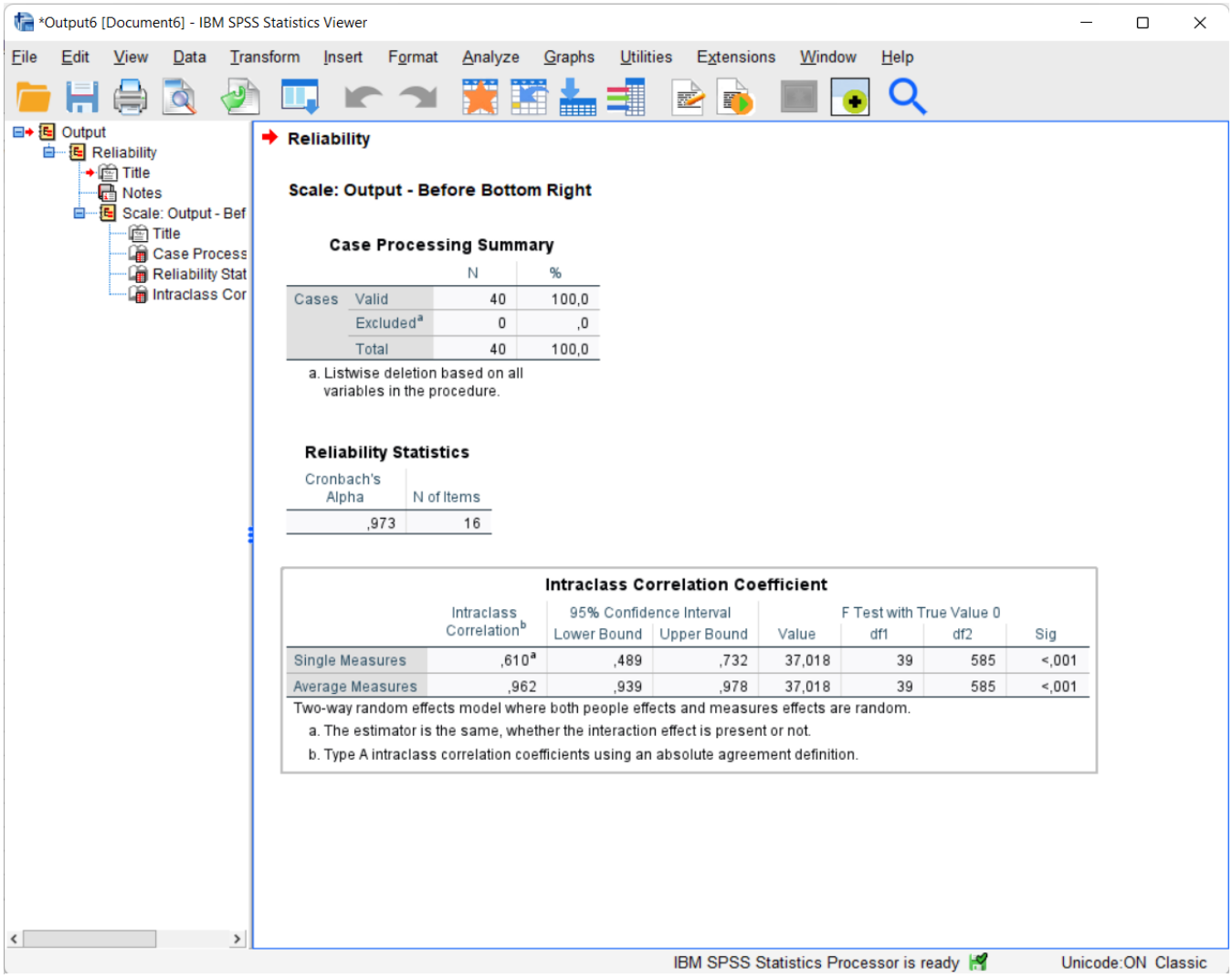


Figure 33: Output of the SPSS reliability analysis based on the shoeprint grading data of the bottom right print, before treatment. It contains the corresponding two-way random intraclass correlation coefficient with absolute agreement for multiple evaluators, for single and average measures, with a 95% confidence interval

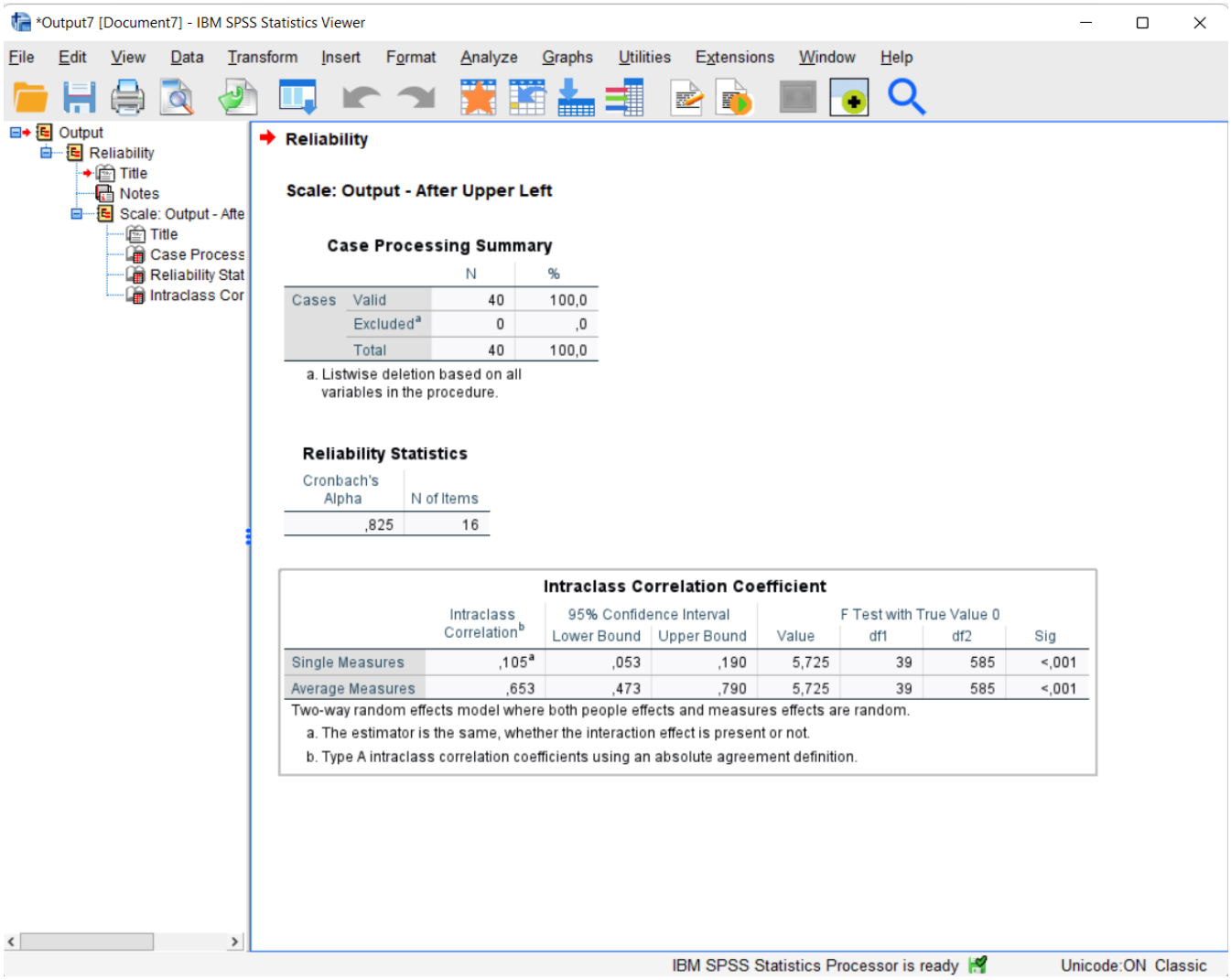


Figure 34: Output of the SPSS reliability analysis based on the shoeprint grading data of the upper left print, after treatment. It contains the corresponding two-way random intraclass correlation coefficient with absolute agreement for multiple evaluators, for single and average measures, with a 95% confidence interval

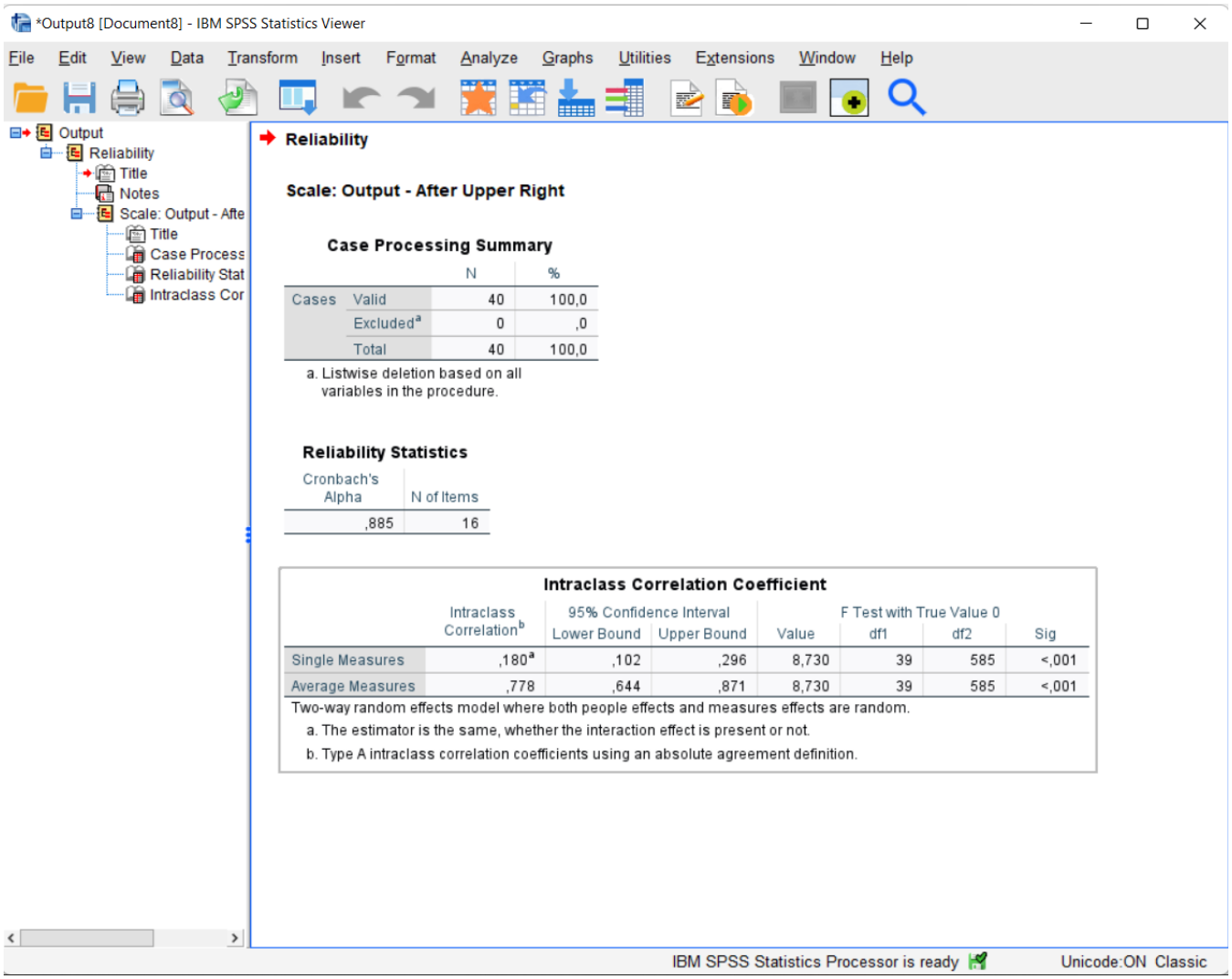


Figure 35: Output of the SPSS reliability analysis based on the shoeprint grading data of the upper right print, after treatment. It contains the corresponding two-way random intraclass correlation coefficient with absolute agreement for multiple evaluators, for single and average measures, with a 95% confidence interval

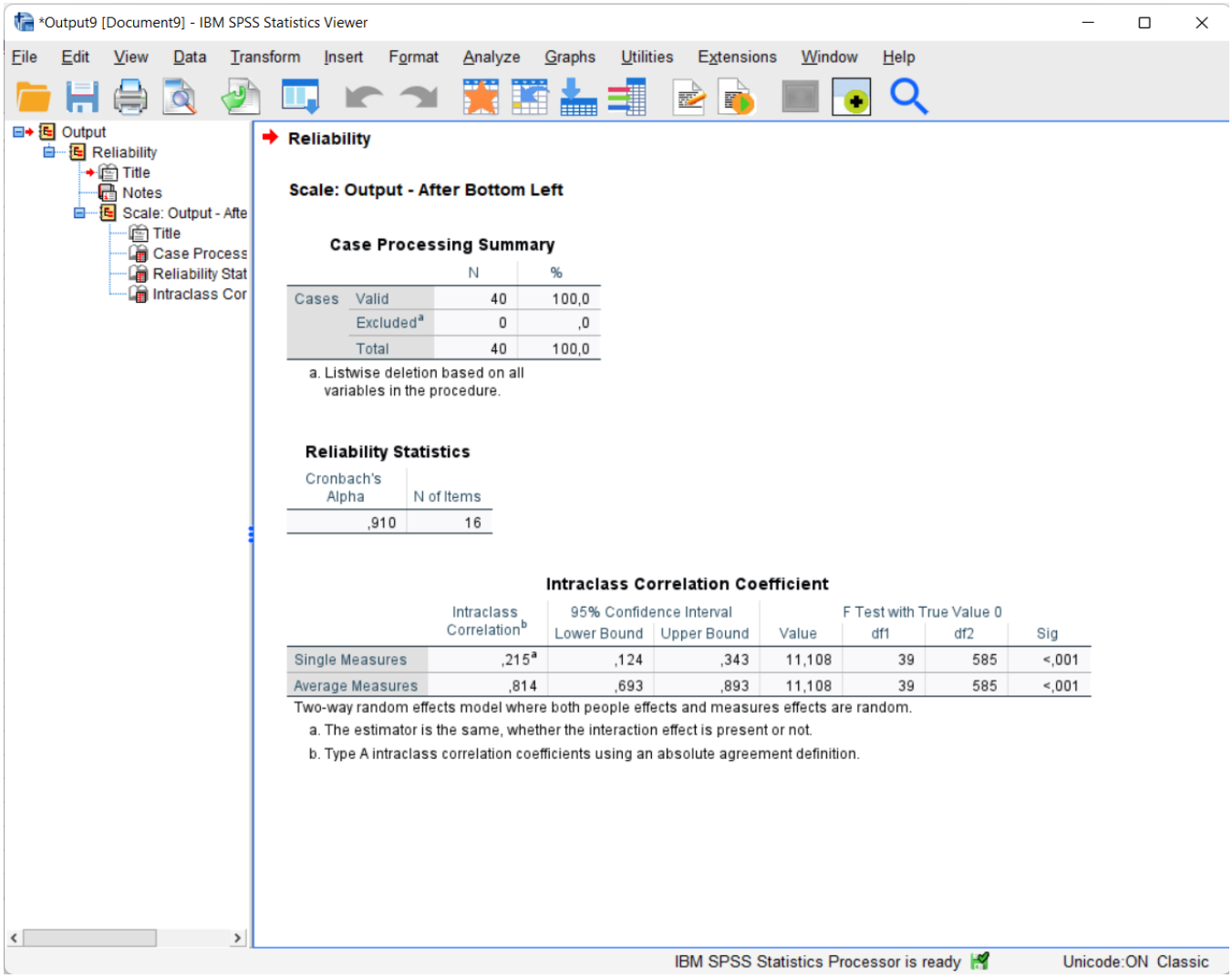


Figure 36: Output of the SPSS reliability analysis based on the shoeprint grading data of the bottom left print, after treatment. It contains the corresponding two-way random intraclass correlation coefficient with absolute agreement for multiple evaluators, for single and average measures, with a 95% confidence interval

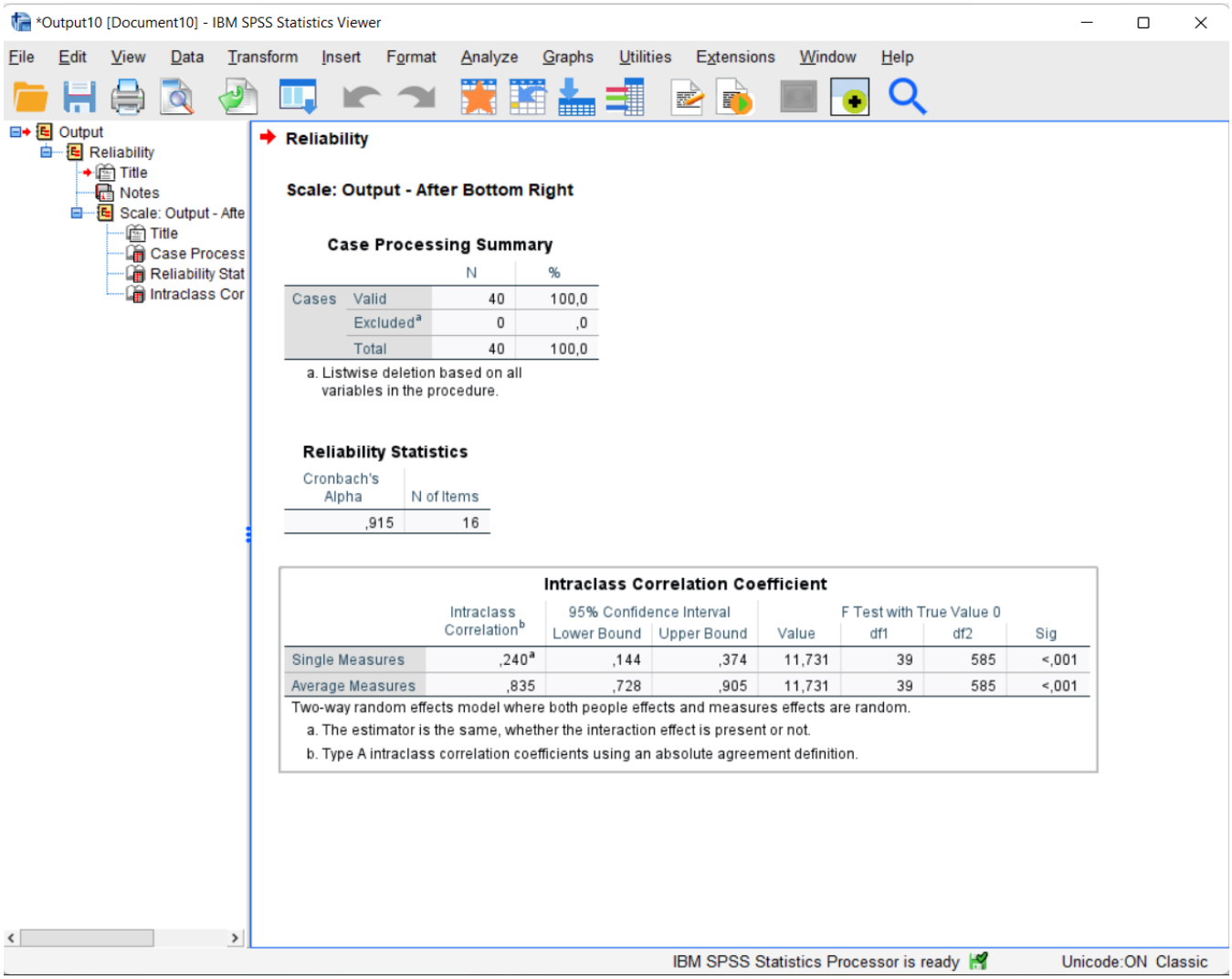


Figure 37: Output of the SPSS reliability analysis based on the shoeprint grading data of the bottom right print, after treatment. It contains the corresponding two-way random intraclass correlation coefficient with absolute agreement for multiple evaluators, for single and average measures, with a 95% confidence interval