The quantification of DNA – traces in the Deventer Murder Case

Deemzet Sep 3rd 2019

1. Introduction

At the revision trial in Den Bosch 2003/4, new evidence was introduced to the detriment of the applicant, mr. Louwes: traces of DNA were found. At first look, this finding was of little significance, because it was accepted by the investigating team, that mr. Louwes had visited the later victim, mrs. Wittenberg in the morning of September 23rd 1999 (she was found dead at noon September 25th). At the occasion, Louwes spoke to the later victim about arrangements concerning her last will and collected a document, which was considered very important by mrs. Wittenberg, in order to put it on file.

The Dutch Forensic Institute (NFI) stated, that the traces could not be explained this way. A part of the argument was founded on the supposed large amount of DNA found on the victim's clothing, notably a white cotton blouse. Their arguments were put forward rather offhand, giving every reason here to scrutinise the available evidence, in particular with regard to the quantitative properties of the findings under investigation, notwithstanding the fact, that the used technology was not devised to that goal. Otherwise stated, the NFI itself made this approach necessarily by introducing the quantity argument. This is the place to make a puzzling observation: all the reasoning by the NFI about the amounts of found DNA was brought forward by an assistant investigator of the NFI, mr. R. Eikelenboom (chemical analyst without training on DNA), but was nowhere mentioned in the report of the chief investigator himself: Dr. A.D. Kloosterman. He did not even co-sign the report of the aforementioned.

2. Abstract

From the peak heights in DNA-profiles, you can get a fair indication of the amount of DNA in the corresponding samples. From photographic material of the samples collected on the blouse, it is possible to measure the dimensions of the samples. From the relationship between those two measurements it is possible to evaluate the DNA intensity in the samples.

Trace #10 shows a blood trace of minor dimensions in comparison to the whole cut out sample, which was analysed. The DNA intensity calculated from the blood trace as source of DNA would be excessive in comparison to other blood traces investigated in the same series, whereas the DNA intensity in relation to the whole sample reaches a normal height. In the latter case, the sample must be considered as being formed as a saliva deposition.

The minor contributions (four) of a second donor could be well explained as being caused by the blood trace. They all fit in with the DNA-profile of the victim. This possibility was not even considered during the trial, because the DNA-results were not specified to such a level in the reports that were presented at the trial.

The NFI presented the mixed profile of victim and applicant in sample #20 as a typical trace of violent aggression. The amounts of DNA were presented as being very high. The NFI showed no interest in the most eligible samples with indication of violence: #7 and #12. These profiles even run off scale! The contributions of the victim were very high and the contributions of the applicant almost zero. On the body just beneath the traces on the blouse, there were markings indicating grabbing fingers.

The NFI claimed the source of the applicant's DNA in #20 to be skin cells transferred during violent action. The claim is not supported by scientific findings, now or then.

The pattern of DNA intensity in samples and controls around trace #9 are consistent with a saliva deposition caused by a conversation between the victim and the applicant under the specific circumstances, as existed in the morning of September 23rd.

3. Conclusions

In the revision trial, the DNA results were offered in two sessions; first introducing the traces #1 - #9 and later on the traces #10 - #20, selected with the purpose to bolster the results of the first series.

Only in 2006, a more systematic procedure was followed, regrettably only focusing on bloodstains and ignoring the already in 2003 observed crime scope traces on the backside of the blouse, which would exonerate mr. Louwes if his DNA were found here.
On basis of observations made by studying the blouse in 2003, the traces were placed in a number of categories, without much considering the (lack of) homogeneity of the categories formed. A number of significant traces were ignored. This caused a large number of anomalies. Here follows a summary:

- Sample #10 contains 'added manually' DNA peaks which coincide with the highest DNA peaks (twice homozygote) belonging to the profile of the victim. This information was withheld during the trial aforementioned.
- Sample #10, considered in relation to the size of the blood trace, would show an excessive height of DNA content; in relation to the whole sample area, the result looks quiet normal. In that case we have to assume that the source of DNA is mostly invisible as in saliva.
- Trace #10 is situated in a location, where you could expect the occurrence of saliva in concordance with observed crime scope traces in the immediate vicinity, which were not sampled for DNA.
- Trace #9 is regarded as a model for the traces associated with light red depositions, but trace #18 from this group contains far more DNA. No explanation is offered for this discrepancy.
- Trace#9 shows far more resemblance to the traces #15 and #17 than to #18. According to the hypothesis of the NFI, this should not be the case.
- Trace #9 nicely fits in a pattern of decreasing DNA intensity from back to front, as shown in figure 33 and in accordance with the hypothesis that transfer of saliva during a conversation was the source of DNA
- The most prominent traces with light red depositions were not sampled until 2006, but then categorized as blood stains. They did not contain any DNA traces of the applicant. Therefore, the light red stains do not form a homogenous group.
- Trace #20 was categorized as one of the light red stains. According to the NFI it is - also - a trace of violence (grabbing), because significant amounts of DNA of the victim were present. The NFI totally ignored the far higher amounts of DNA in the traces #7 and #12, which are in the same location as visible traces of grabbing on the victims body. These traces do not contain significant amounts of DNA of the applicant.
- The absence of a crime-scope signal in certain traces was used as part of the justification of the NFI hypothesis without an appropriate analysis of the phenomenon of photoluminescence.
- In 2003 the NFI stated the minimal amount of traceable DNA to be 200 cells in discordant with the value, mentioned in a validation by the NFI itself (Dr. A. D. Kloosterman notably) of the used apparatus. In this validation the minimal amount was stated to be under 0.2 ng (being 200 pg thus 30 cells).
- In the analysis of trace #20, the NFI theorised about DNA degradation during the forming of skin cells without any scientific foundation.
- The location of trace #20 varies between right and left on the blouse in subsequent publications.

4. Obtaining DNA-profiles

The foundation of every DNA-analysis is formed by the so-called profiles (more appropriate electropherograms), a graphical representation of observed DNA-fragments (see figure 8 for an example). The acquisition of those profiles is prone to a number of influences, caused by the apparatus in use, the procedures followed and the history of the traces:

4.1. Interference through the used technology of capillary electrophoresis (CE)

4.1.1. Injection procedure: electrokinetic injection

This procedure amounts to a technique using the same physical phenomenon as the electrophoresis itself; the ions are electrically pulled up from a sample vial into the capillary. As a result, the introduction of short fragments is more successful than the introduction of longer fragments. Not all fragments are injected; the time frame used is the restricting factor. The results are hampered by other ions present in the PCR mixture e.g. ionized nucleotide bases.

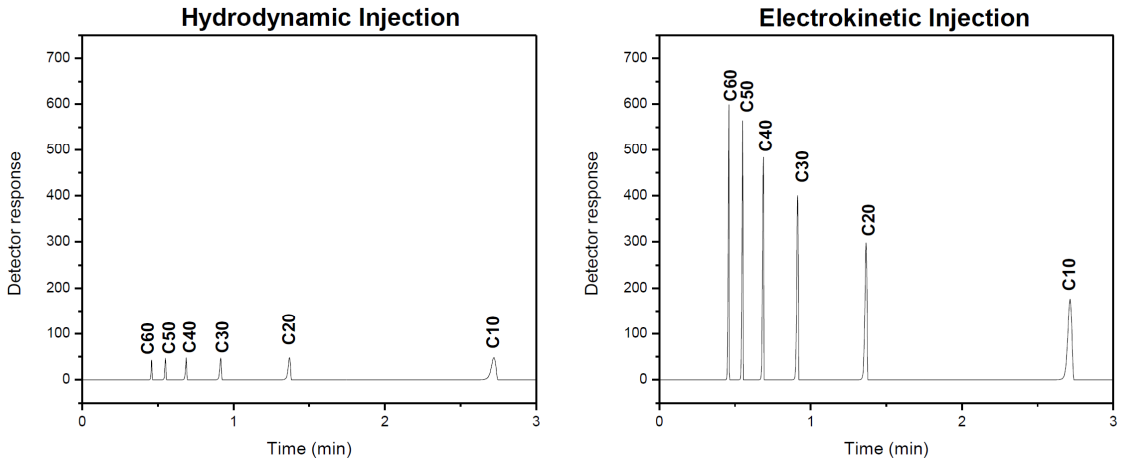


Figure 1. The effect of electrokinetic injection (right) on a DNA profile compared to a 'mechanical' injection (left). The correlation to fragment size is evident (Breadmore 2009[1]). In both cases, a peak broadening is visible (see also figure 2 & 3).

4.1.2. Diffusion

The electrophoresis is carried out with an elevated temperature, about 60°C. Therefore, the movement of the fragments is not restricted to dragging by the electric field, but also influenced by the so-called Brownian movement, which is random in direction. This implicates a diversifying of the speed of the fragments leading to a change in the observed peaks. When a fragment is larger, it will spend more time in the capillary before getting detected, resulting in broader and lower peaks, compared to smaller fragments.

4.1.3. Laser detection

The registration of passing fragments at the end of the capillary is achieved by radiation of attached chemical groups with laser energy, leading to excitation and consequently fluorescence in one of three possible colours. The fluorescence signals are measured by CCD's and translated into electrical signals. Larger fragments migrate more slowly, so the group of identical fragments will take more time to pass the end control, so in the end causing lower en broader signals.

4.1.4. Increasing resistance

Bilenko et al. 2003[2] reported an increasing resistance during CE, notably in the vicinity of the detector, so again slowing the speed during detection of larger fragments.

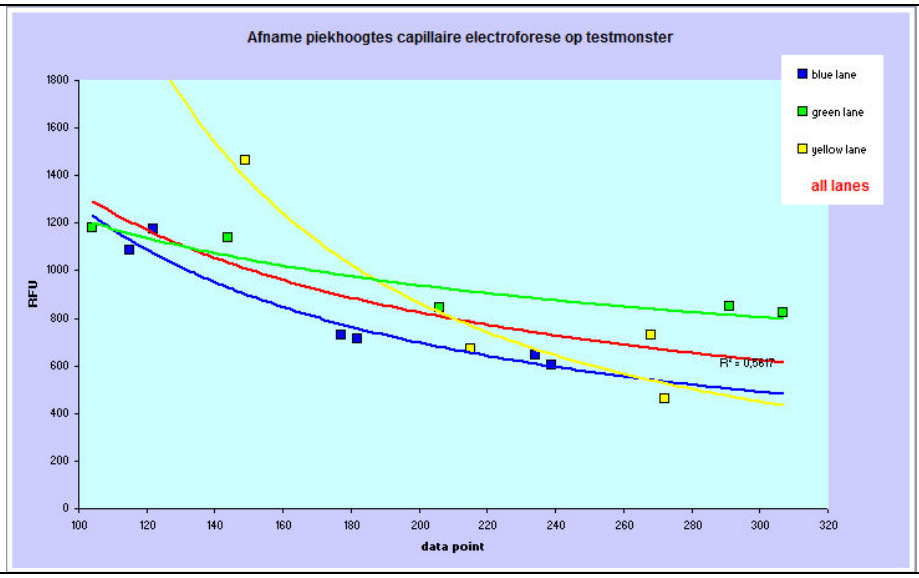


Figure 2. A number of laboratory samples used in CE. Detector response vs. fragment size. Because these are fresh samples, all change is due to systematic errors in the procedure.

4.2. Interference through PCR

4.2.1. PCR is a chemical reaction

As encountered in every chemical reaction, the PCR reaction is hampered by competitive processes. Processes with low change in entropy, meaning high probability, will compete. The more complicated the process - as with longer fragments to be copied - the more competition.

An example is the so-called stutter effect. As consequence of a competing reaction, a copy in loss of four base pairs is formed. Validation experiments have demonstrated the occurrence of more stutter products when larger fragments are processed.[6]

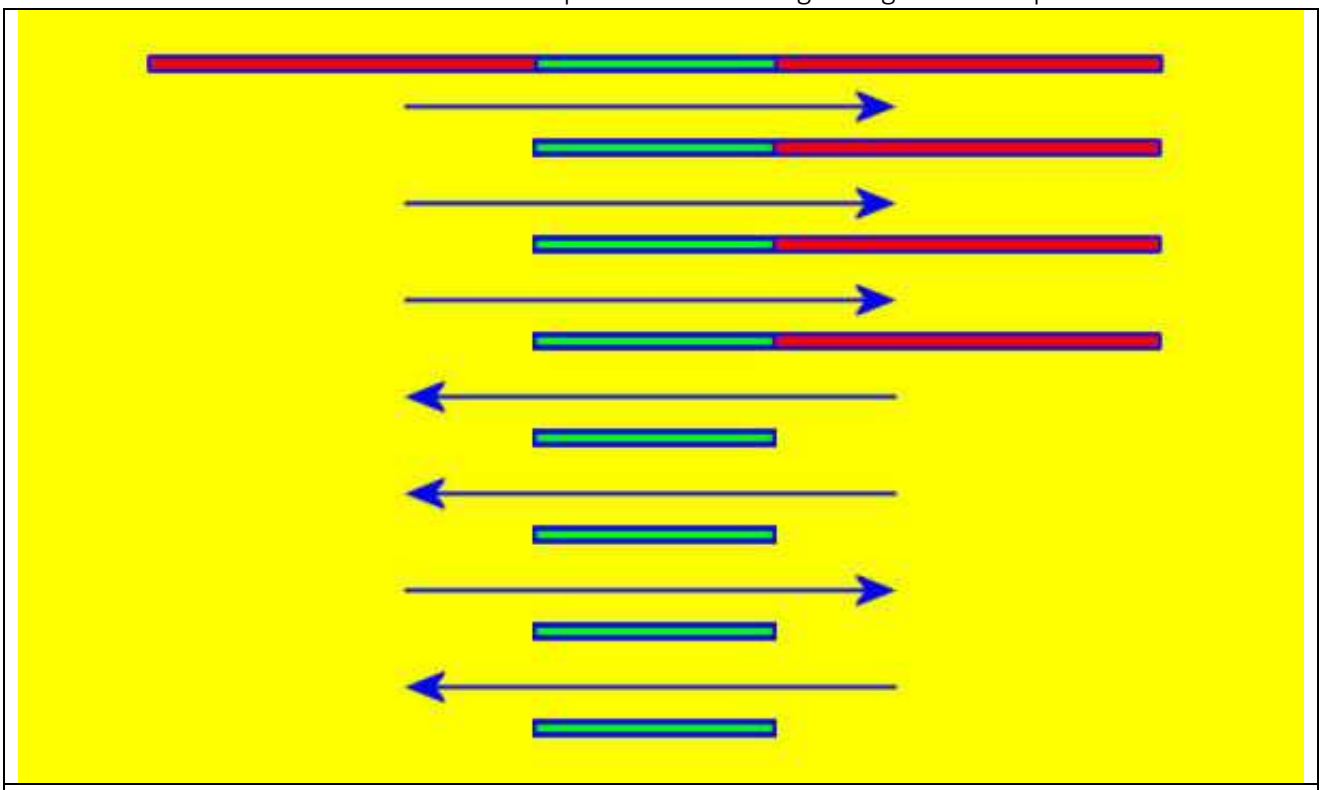


Figure 3. As long as the direction of replication stays the same, it will generate copies that are not available to CE (far too long). Only the occurrence of a replication in the opposite direction (50% chance in every cycle), delivers a copy - restricted on both sides - necessary for the ultimate forensic DNA analysis.

4.2.2. Starting PCR

To obtain a complete copy of a DNA fragment from a relevant location, the PCR process has to be successful twice in regard to that specific location - once on the original location and once on the first copy. That second copy must be carried out in the

opposite direction in order to deliver a product, usable for further processing and CE in the end. If the original sample is poor in DNA, random effects play an important role. The effect will be that a number of PCR cycles might be lost, each lost cycle halving the final amount of copies.

4.3. Distortion of samples

4.3.1. Degradation

Before sampling and the execution of PCR, the DNA has endured a number of environmental influences for some time, including cosmic radiance, UV-radiance and bacterial growth. Because of the random character of these processes, the odds are that long fragments suffer more than shorter ones. Damaged fragments will be included in the replication process in unforeseen ways. The biased caused in this way is convincingly shown by Schneider et al. 2004[3].

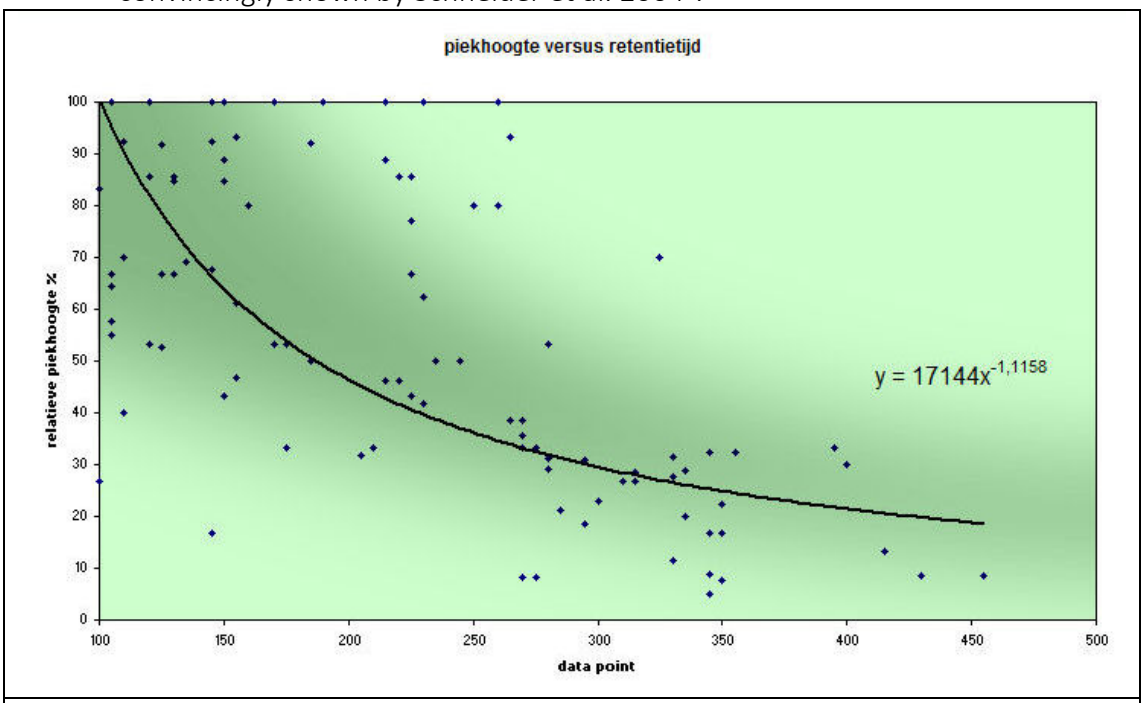


Figure 4. A number of samples randomly chosen from online collections. Fluorescence responses are normalised to 100% maximum for each sample and plotted according to fragment length.

Concluding: in assessing the amount of DNA in a DNA-sample, one should account for the disappearance of peaks at the far end of the DNA-profiles, as demonstrated in figure 4. The assessment of the amount of DNA in a sample should be carried out using peaks at the short (left) end of the DNA-profile. The absence of peaks at the far end in the profiles (especially mixed profiles!) should not lead to conclusions about the absence or presence of a DNA-donor, when the presence of peaks at the short side indicate otherwise.

4.4. Biases by procedure

Researchers have a number of options to influence the result of the DNA-analysis; for instance the choice of the sampling method, the amount van extract used, the amount of used PCR-product, duration of the injection of the PCR-product and the electric potential during the CE.

4.4.1. Extraction

The first 10 samples were extracted using Chelex, the next 10 using Qiamp. This might have generated differences, but the results do not show this to be a major problem, see the similar samples #7 and #12 in an analysis later on. Other groups of similar samples (bloodstains, light red stains and the controls) are fortunately usually extracted using the same method (#9 is an exemption).

4.4.2. The amount of injected PCR-product

This potential bias asks for a further discussion, because it seems to be a source of variance. For the moment, we will limit ourselves to point out that literature (Butler[4]) shows this variation to have minimal impact.

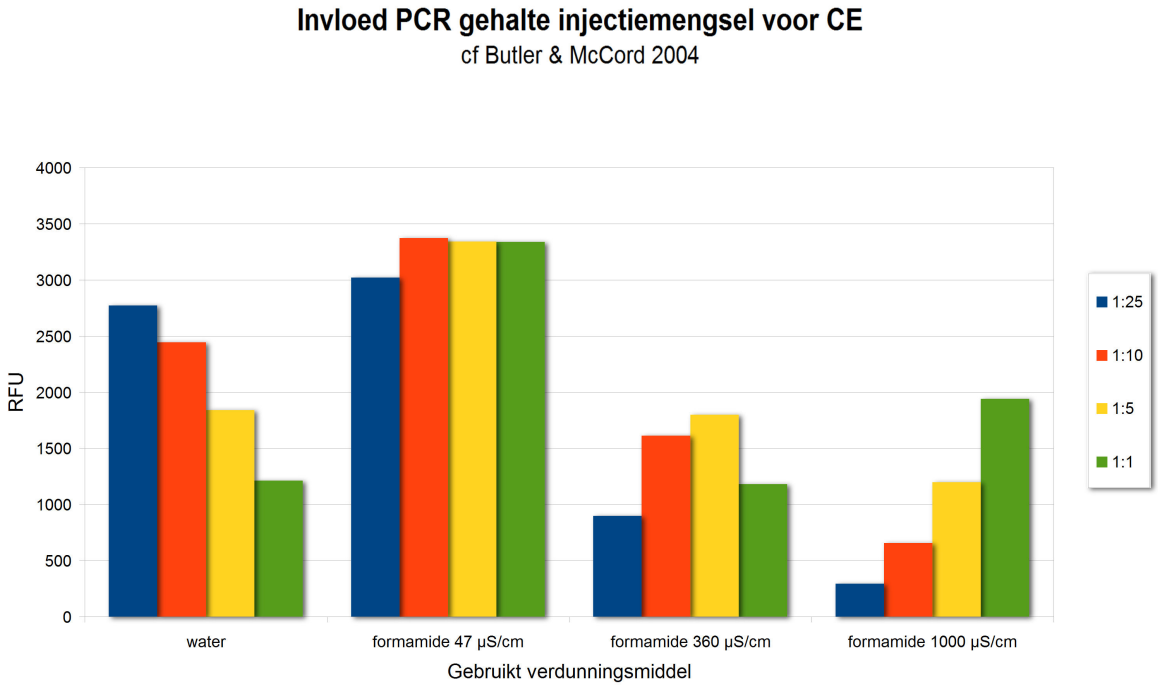


Figure 5. Impact of the increase of the amount of PCR-product in the injected mixture starting a CE. Adapted from Butler & McCord 2004[5]. When a suitable diluent (pure water or deionised formamide) is used, no effect or even a negative effect is to be expected (as shown in the left side of the chart).

During the NFI-investigations in the Deventer Murder Case (DMC), the investigators experimented twice (samples #1 and #9) with higher amounts of PCR- product in the injected mixtures with results, strongly resembling Butler & McCord 2004.

When surveying this phenomenon from a theoretical point of view, this outcome was to be expected. The electrodynamical injection is subject to only three variables; the electric potential, the electrical resistance and the duration of the process are the factors to determine the amount of ions that are introduced into the capillary (coulometry). Of these, the variation of duration of the injection was applied, so corrections were made to normalise the relevant results.

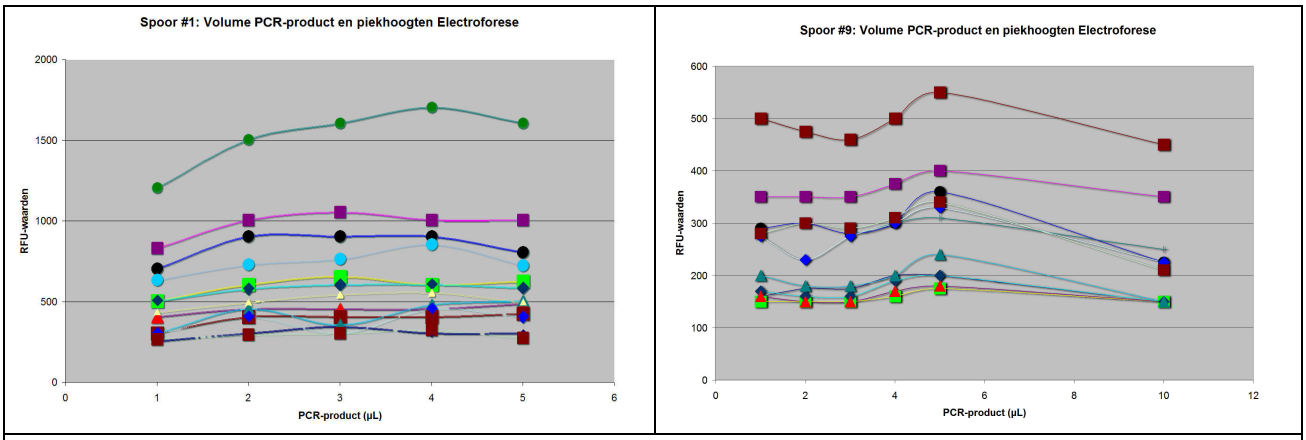


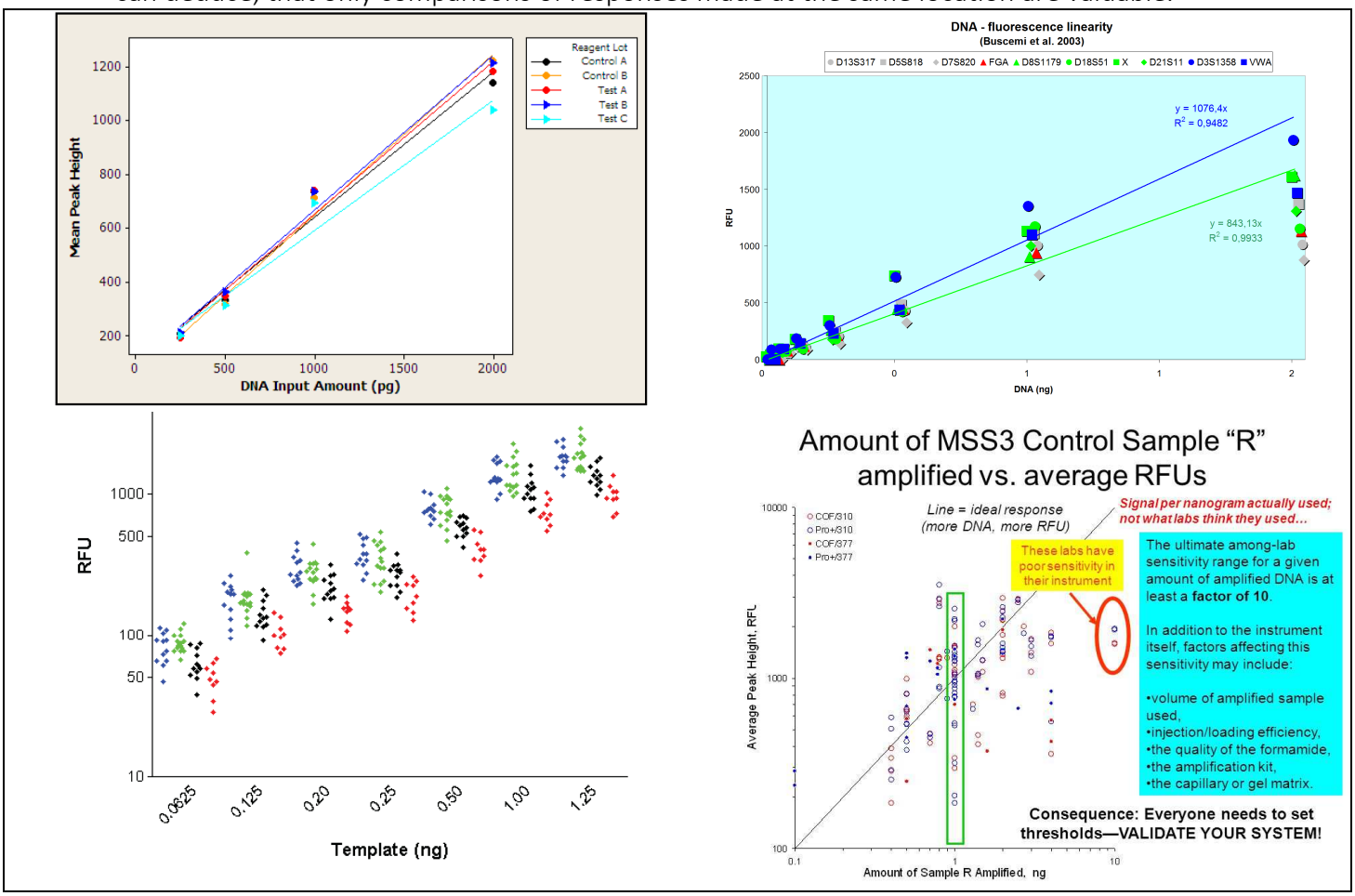
Figure 6. Comparison of peak heights in a number of experiments at the NFI with varying amounts of PCR-product of the same sample, added to the injection mixture before CE.

Concluding: When evaluating the amount of DNA in a sample one should take in account the decrease of peak height at the far end of the profiles. So the amount of DNA should be evaluated using the fluorescence responses at the left side of the profiles.

5. Applicability DNA-fluorescence values

On the left side of each profile (electropherogram), we find the peaks, which are the best, suited to use in an evaluation of the amount of DNA in a DNA-sample. However, to what extent can we trust the result? In literature we only find a few studies concerning the height of DNA-peaks in selected locations in relation to the amount of DNA analysed before replication by PCR. Those studies made use of different DNA-kits, but supplied by the same manufacturer (Applied Biosystems), while using the same apparatus. Moreover, there is a diagram in the user guide of the kit, used by the NFI (SGM-plus), plotting the mean peak heights against amount of analysed DNA, showing a high correlation (figure 7a.).

Taken on face value, the results shown in figure 7 confirm the notion that fluorescence responses carry an indication of the amounts of analysed DNA. Up to 1 - 2 ng applied DNA - amounts often encountered in forensic practices - the correlation is linear. From 1 ng upwards, the curve tends to flatten. From this follows, that responses above 1000 RFU are an indication for even higher amounts of DNA then follows from mere extrapolation. From the diagrams shown in literature one can deduce, that only comparisons of responses made at the same location are valuable.



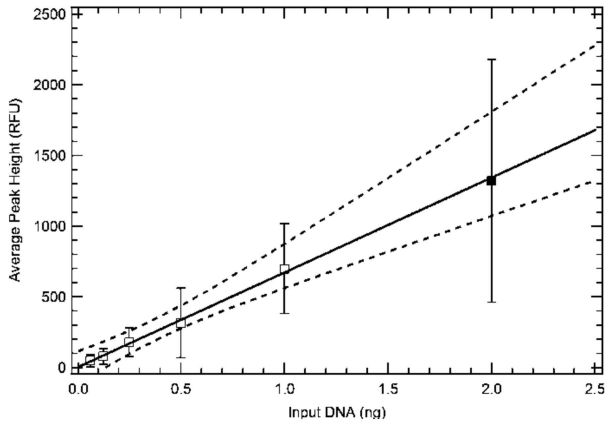
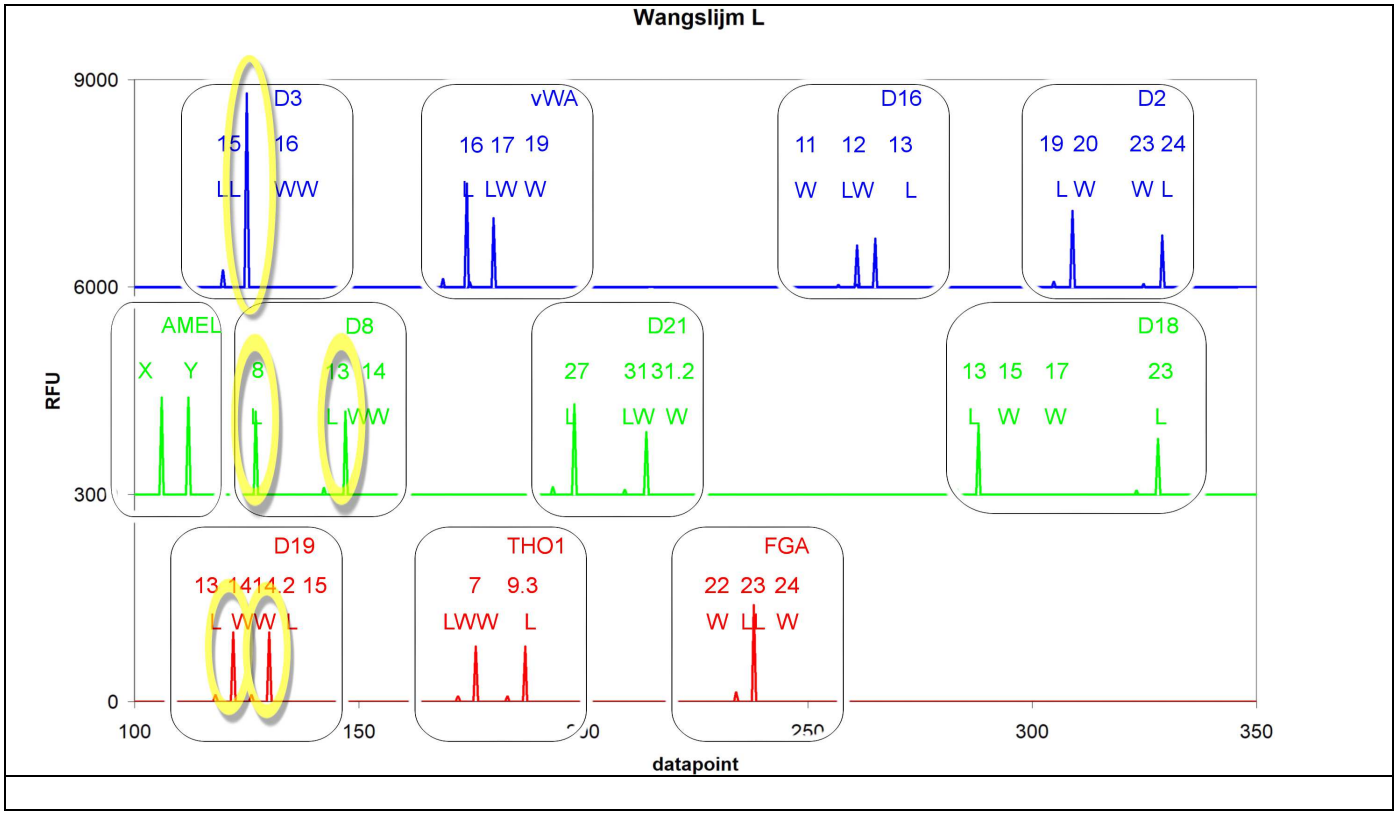


Figure 7. Clockwise: (a)sensitivity study of the SGM-kit of Applied Biosystems[6]; (b)validation van Profiler Plus (after Buscemi et al 2003[7]); (c)validation of Identifiler (Collins et al. 2003[8]). Figure (d) demonstrates the necessity to compare the results of the same laboratory[9]. Figure (e) shows the typical variance in the mean values (dotted lines show the 95% interval). [10]

6. Application in the Deventer Murder Case (DMC)

6.1. Electrophoresis

To analyse the profiles in detail, the loci most left in the electropherograms are most suitable: D3S1358 in the blue lane, D8S1179 in the green lane and D19S433 in the yellow lane. D3 is homozygote for the applicant, mr. Louwes (L) at 15 bps and for the victim mrs. Wittenberg (W) at 16 bps. D8 is also homozygote for W at 14 bps, for L, we have to combine 8 bps and 13 bps. D19 is heterozygote for both; 13 and 15 bps for L and a kind of doublet (14 and 14.2 bps) for W.



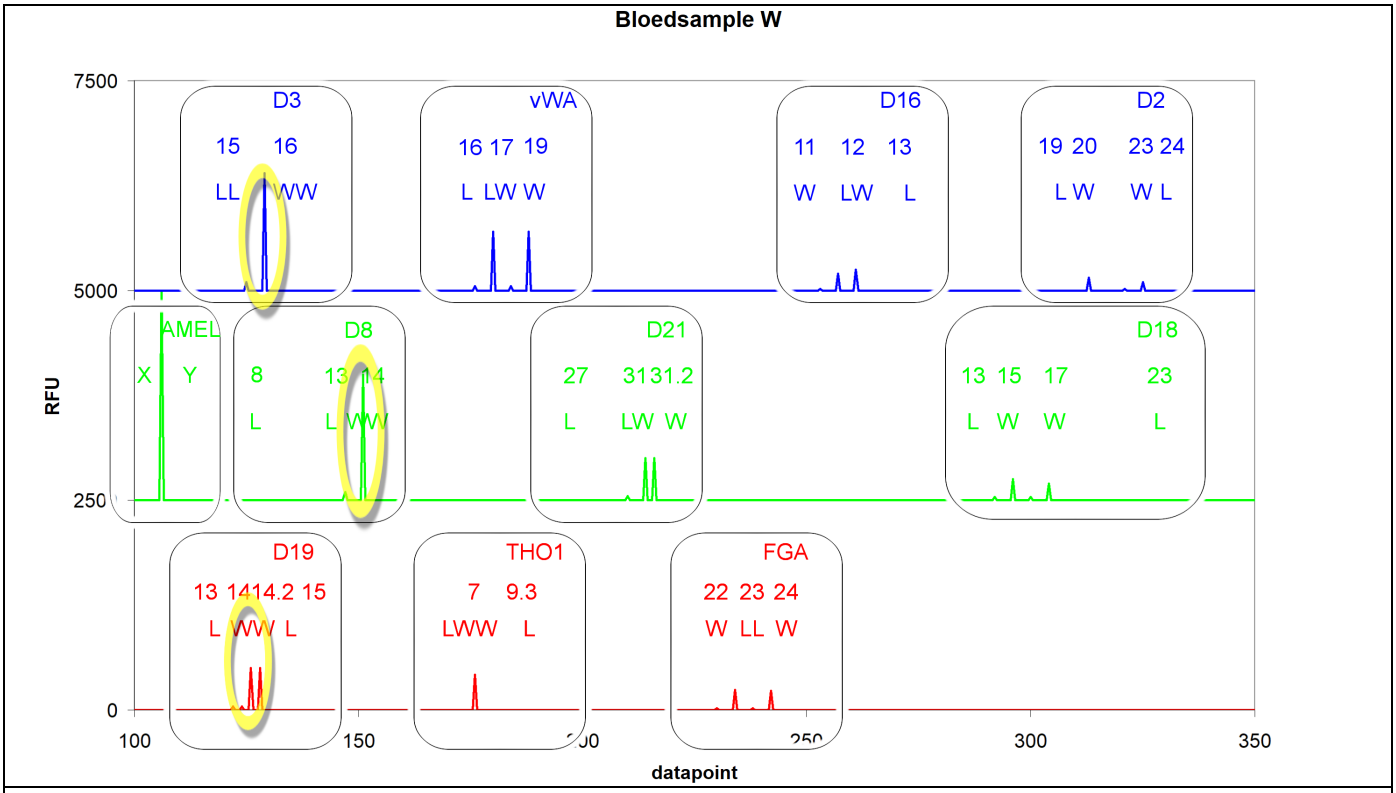


Figure 8. The reference profiles of L and W. In yellow the used peaks to quantify the DNA content of the samples.

The results of the measurements of the whole ensemble of 20 traces as measured in 2003 - at the three locations aforementioned - clearly show the mutual consistency:

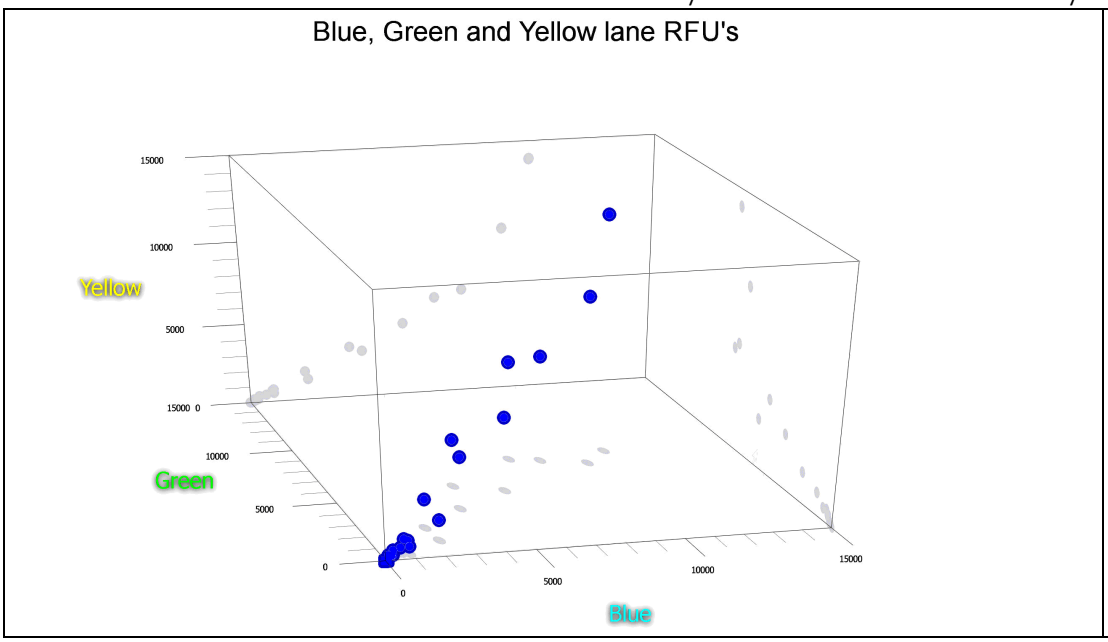


Figure 9. Correlation between the fluorescence responses in the three locations aforementioned in the DMC.

6.2. Quantiblot measurements

Between sampling and PCR, the NFI ran a provisionally quantitative measurement for each sample. The results were reported in a table with other characteristics (DNA labels and PCR labels). The method used, tentatively recognised as Quantiblot, only permits a restricted number of results, so these results are not very exact. Where appropriate I used these results as some sort of extra control. Those controls raise additional questions, for instance trace #8 shows an unexplained discrepancy in comparison with the produced profile. Trace #20, having a very low score in Quantiblot, also raises questions, in surplus with the uncertainty about the original location, which varies between reports (several texts locate the trace at the right side of the blouse and an accompanying figure by contrast at the left side of the blouse).

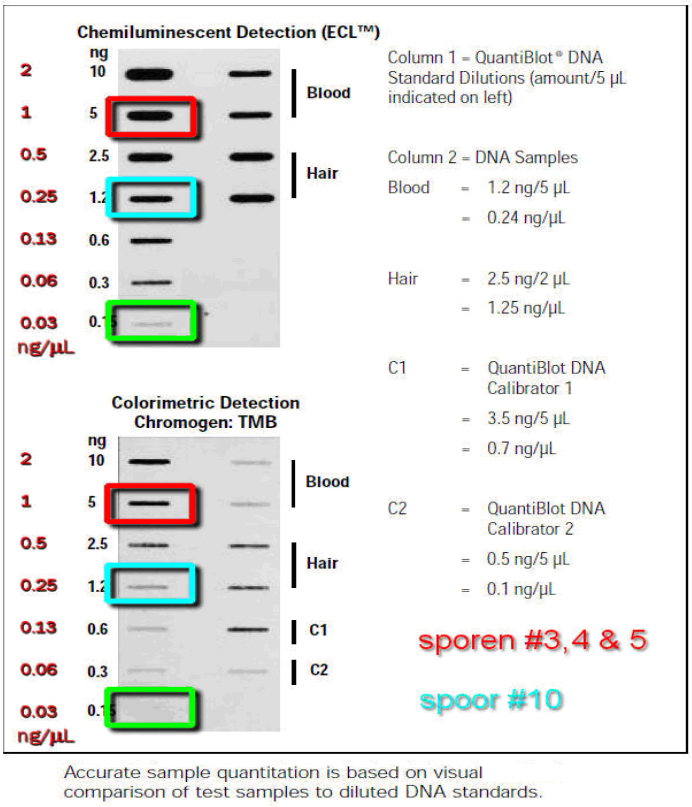


Figure 10. Quantiblot in action. As is demonstrated, the number of potential readings is restricted. A lot of readings in the DMC show extreme values (1 ng/µL respectively 0.03 ng/µL).

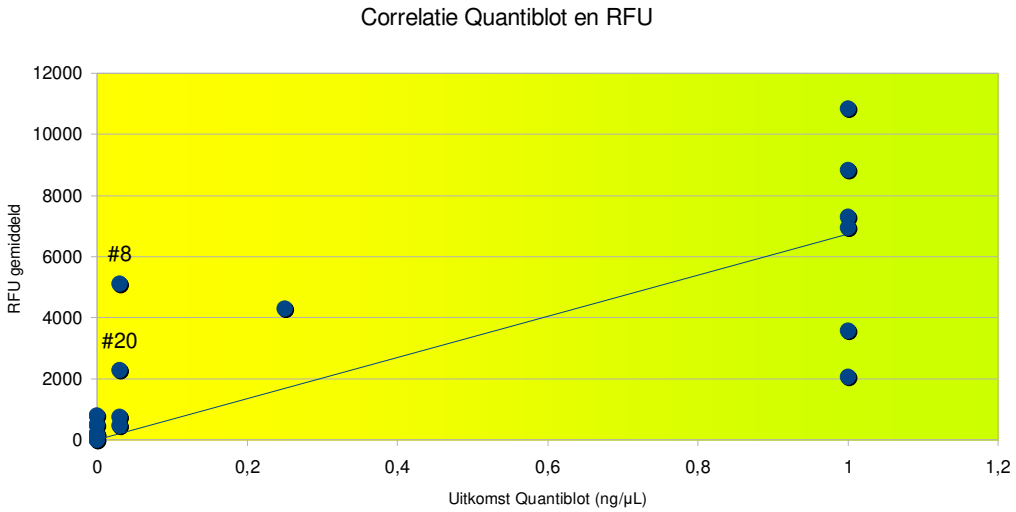


Figure 11. The correlation Quantiblot readings - fluorescence responses has to deal with the lack of precision in the Quantiblot method. Still there a number of striking outliers, in particular the samples #20 and #8 with high fluorescence responses and minimal Quantiblot-scores.

6.3. Corrections

Two kinds of corrections are needed:

6.3.1. Variation in taking of samples:

The DNA samples were cut from the fabric of the blouse en subsequently extracted. The sizes of the samples vary. Using the particularity of a hexagonal decoration of the fabric of the blouse, one can trace a surface of reference on every picture of every sample and count the number of pixels on it. In the same way, one can establish the number of pixels in the sample of interest and use the value to calculate the size of the sample in cm^2. The next step is calculating the strength of the DNA - signals per cm^2 as a measure of DNA - intensity.

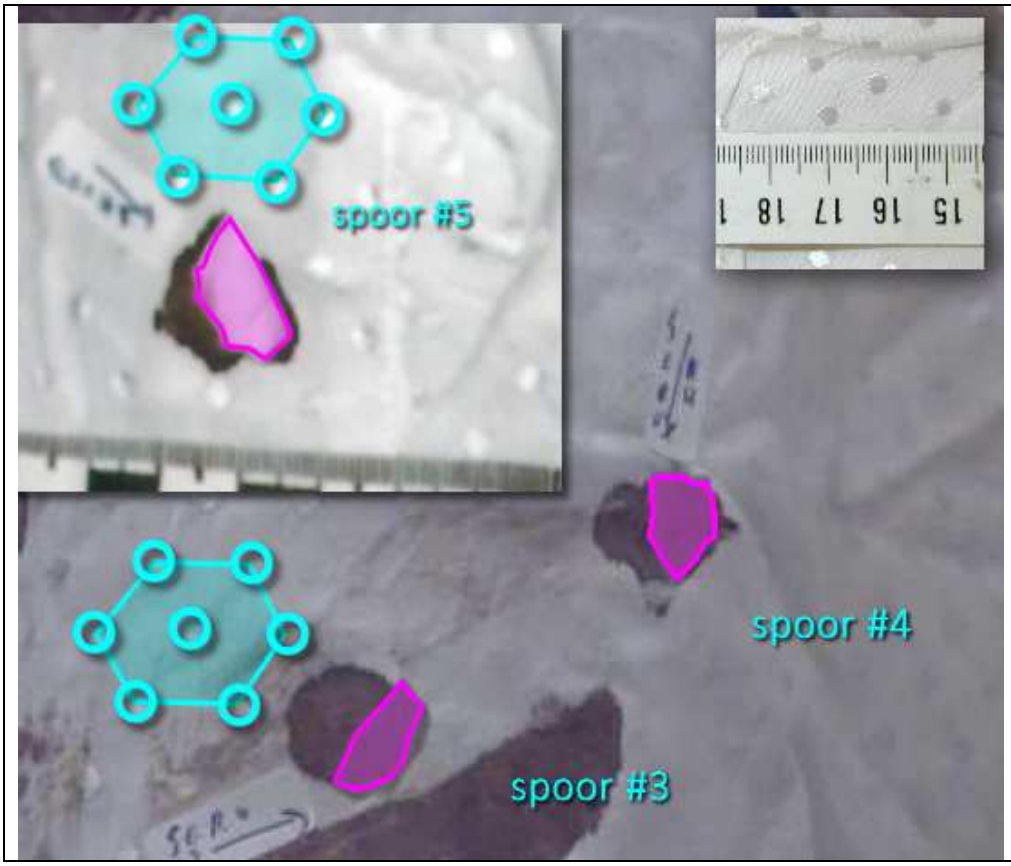


Figure 12. Example illustrating the method to calculate the size of the cut samples. The size of the magenta structure equals 4.06 cm^2, as is calculated from a picture, containing a tape measure. Therefore, by comparing the number of pixels in a cutting, filled in with cyan pixels with the number of pixels in the magenta structure, one obtains the size of the cutting.

6.3.2. Variation in the duration of injection

The duration of injection determines 1:1 the number of ions, available for CE. To correct for the variation in duration of injection, all fluorescence response values are recalculated to the value for 15s injection time (only the samples #1 and #9 used 25 s).

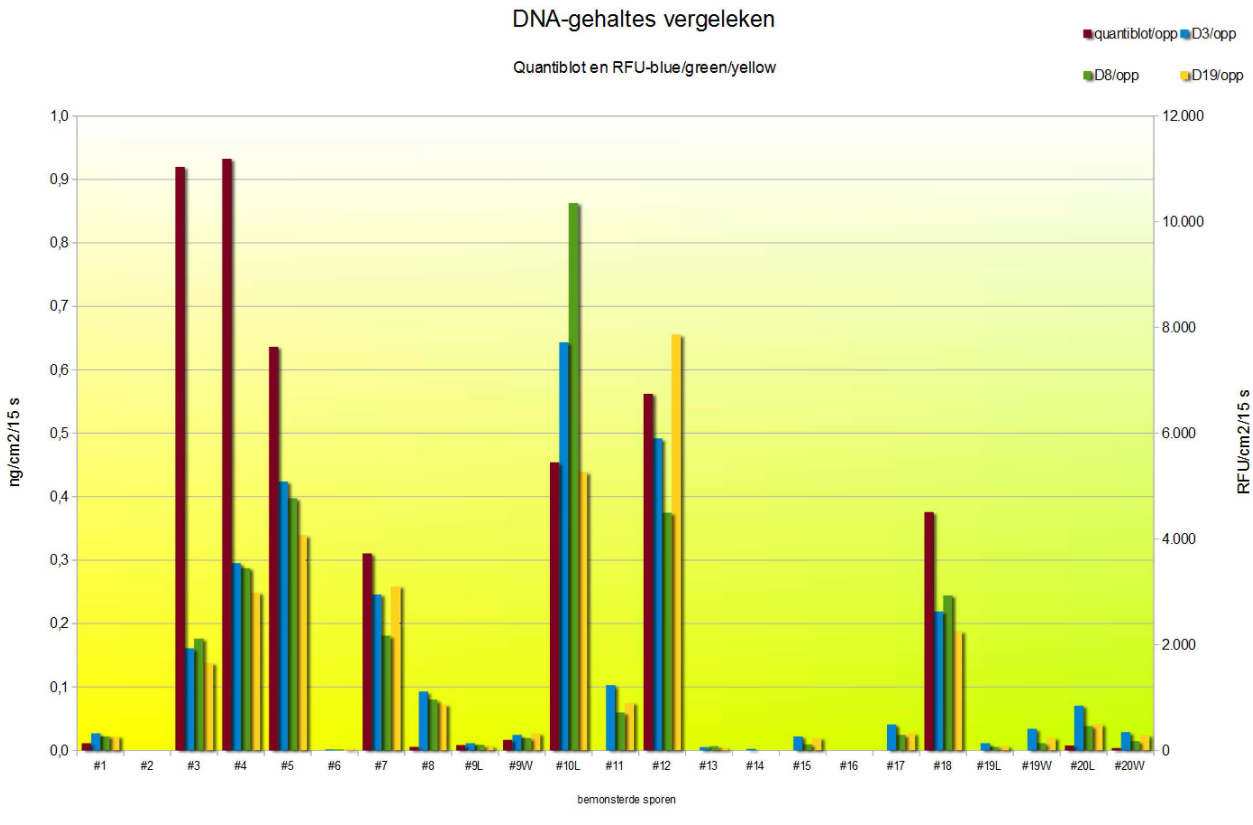


Figure 13. Overview of all - normalised - results. In the relevant cases, the samples are differentiated to the contributions of L and W. The Quantiblot results (brown) are plotted using an alternative Y - axis. The other plots are calculated using fluorescence response values.

7. Results and discussion

The comparison of trace values as shown in figure 13 gains in relevance, if we compare them within the categories of traces as defined by the NFI and adapted here. To begin with, I want to emphasise, that at several occasions, the NFI made declarations about the strength of certain DNA traces, without considering the strength of other traces (in particular regarding the traces #10 and #20). In the following discussions those ignored traces will be included and compared.

7.1. Blood traces

Samples #3, #4 and #5 consist of blood traces, caused by falling - so called passive - blood drops from blood of the victim W. The DNA intensities of these traces (fluorescent responses per 1 cm^2) are comparable to those of trace #10, which was attributed to L. However, there are important differences in the method of sampling, which were not addressed adequately up until now.

7.1.1. The size of the sampling.

The samples of the bloodstains #3, #4 and #5 varied between 1 and 1.5 cm^2, whereas sample #10 measures just over 0.5 cm^2. The amount of DNA response was about the same, so after normalising the result, the response of #10 appeared as the largest (figure 15). Only the Quantiblot result is trailing, but the crude reading of Quantiblot results can be held accountable for that result.

When comparing the sizes and intensities of all the bloodstains (see figure 14), one would expect to find far less DNA in trace #10 than in the other bloodstains, because this stain is so tiny.

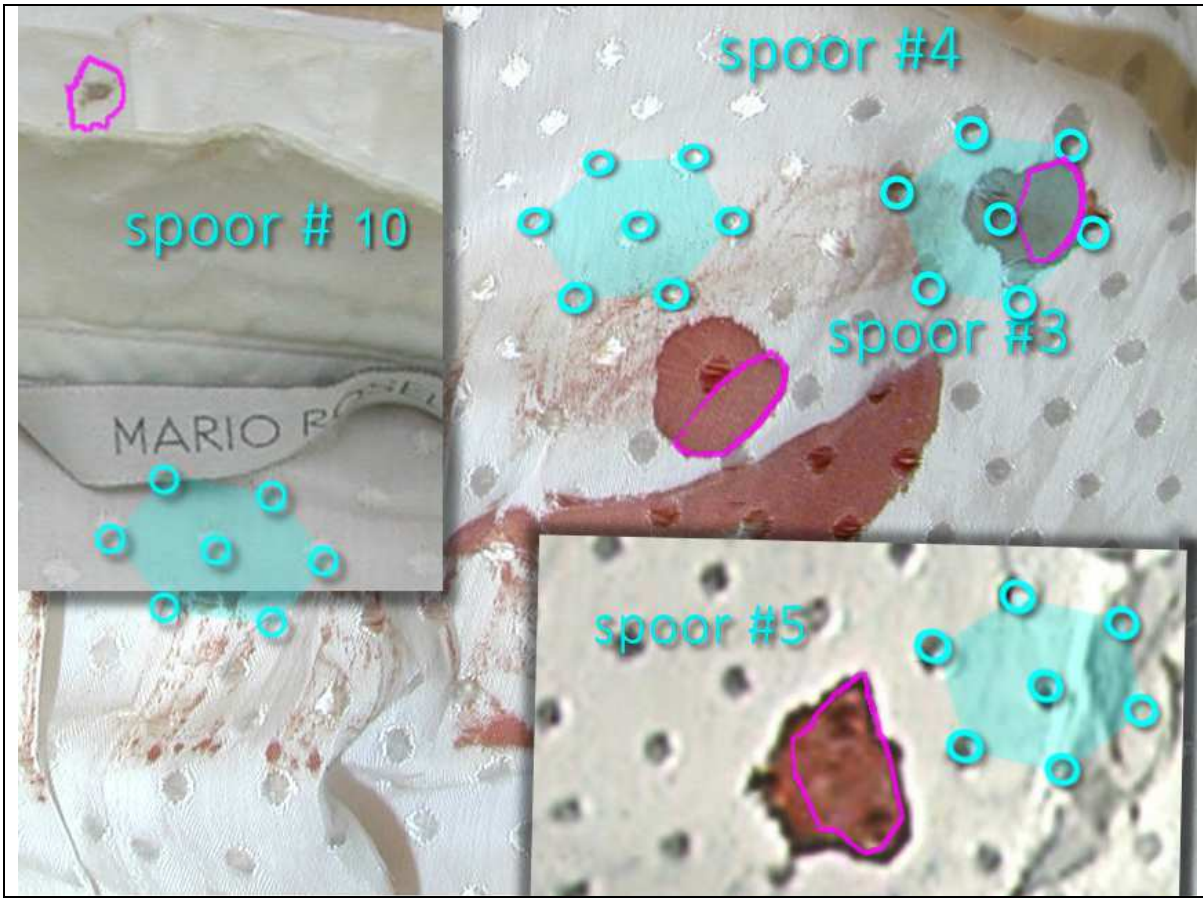


Figure 14. The mentioned traces with used references to compare the sizes. The DNA fluorescence response of trace #10 is comparable with those from the traces #3, #4 and #5. However, the bloodstain in #10 is much tinier.

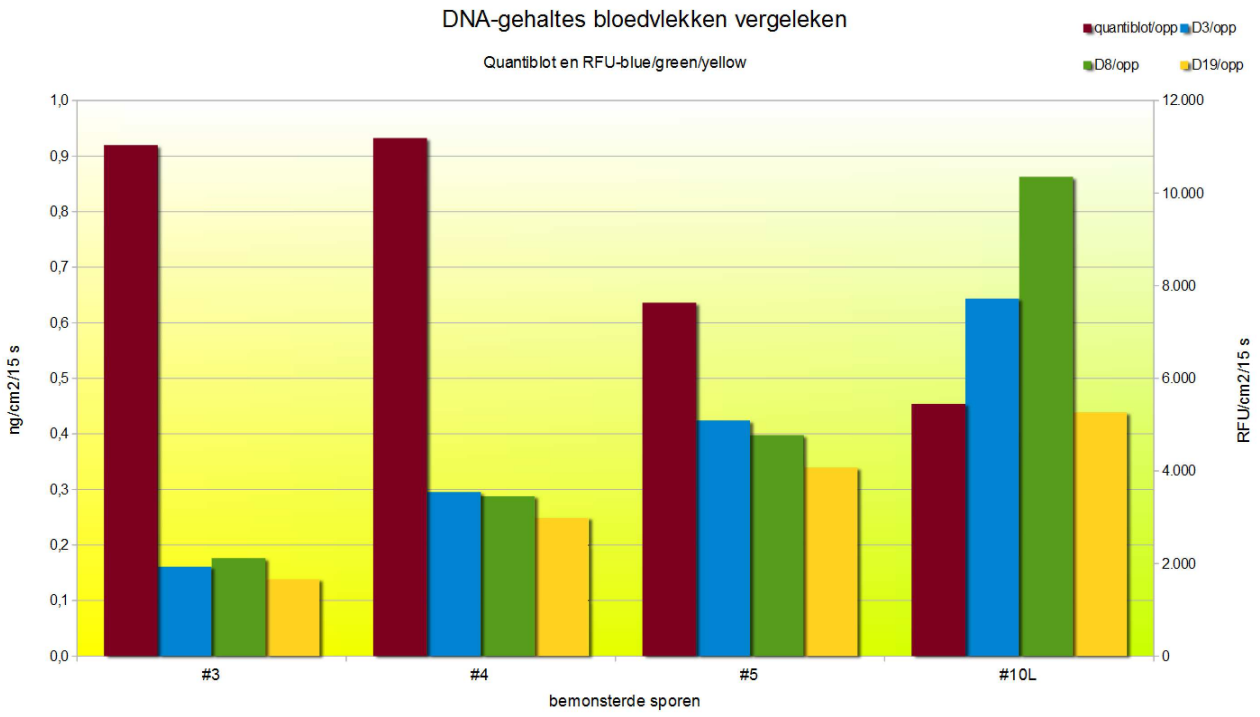


Figure 15. The intensities of the so-called bloodstains compared to each other on base of size of the cuttings. The cutting of trace #10 shows a DNA intensity comparable to those of the traces #3, #4 and #5, but those stains are completely filled with blood, whereas trace #10 contains only traces of blood.

7.1.2. Cutting of samples

The way the samples were cut from the blouse is significantly different between them. The samples #3, #4 and # 5 were cut in such way that practically the whole cutting consisted of blood residue. Sample #10 shows a totally different picture. Whereas the cutting is 0.55 cm^2 in size, the bloodstain in it is only 0.08 cm^2 in size. If we follow the hypothesis of the NFI, which links the DNA content of #10 to the blood of L, then we have to recalculate the DNA intensity using the size of the *actual* bloodstain.

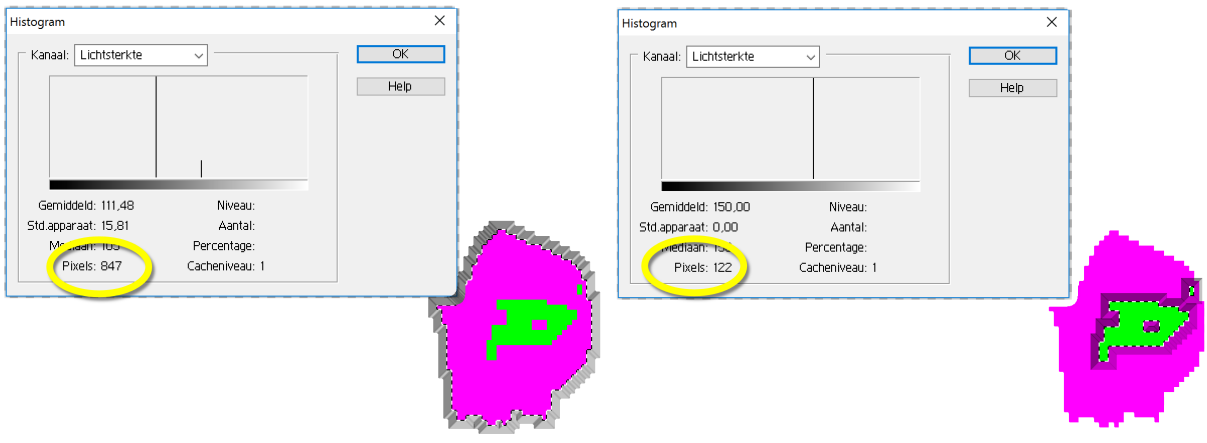


Figure 16. Method to calculate sizes of sample cuttings: after selecting the size of a certain stain or structure in Adobe Photoshop Elements and choosing the option 'Histogram', the program immediately displays the number of the selected pixels. This has been done for the stain and the reference structure to be able to calculate the actual size of a stain.

The result is used to complete the diagram with the value #10bloed in figure 17. It is immediately clear, that this result - based on the NFI hypothesis - is completely unrealistic. Stated otherwise: such a small bloodstain will never contain so much DNA.

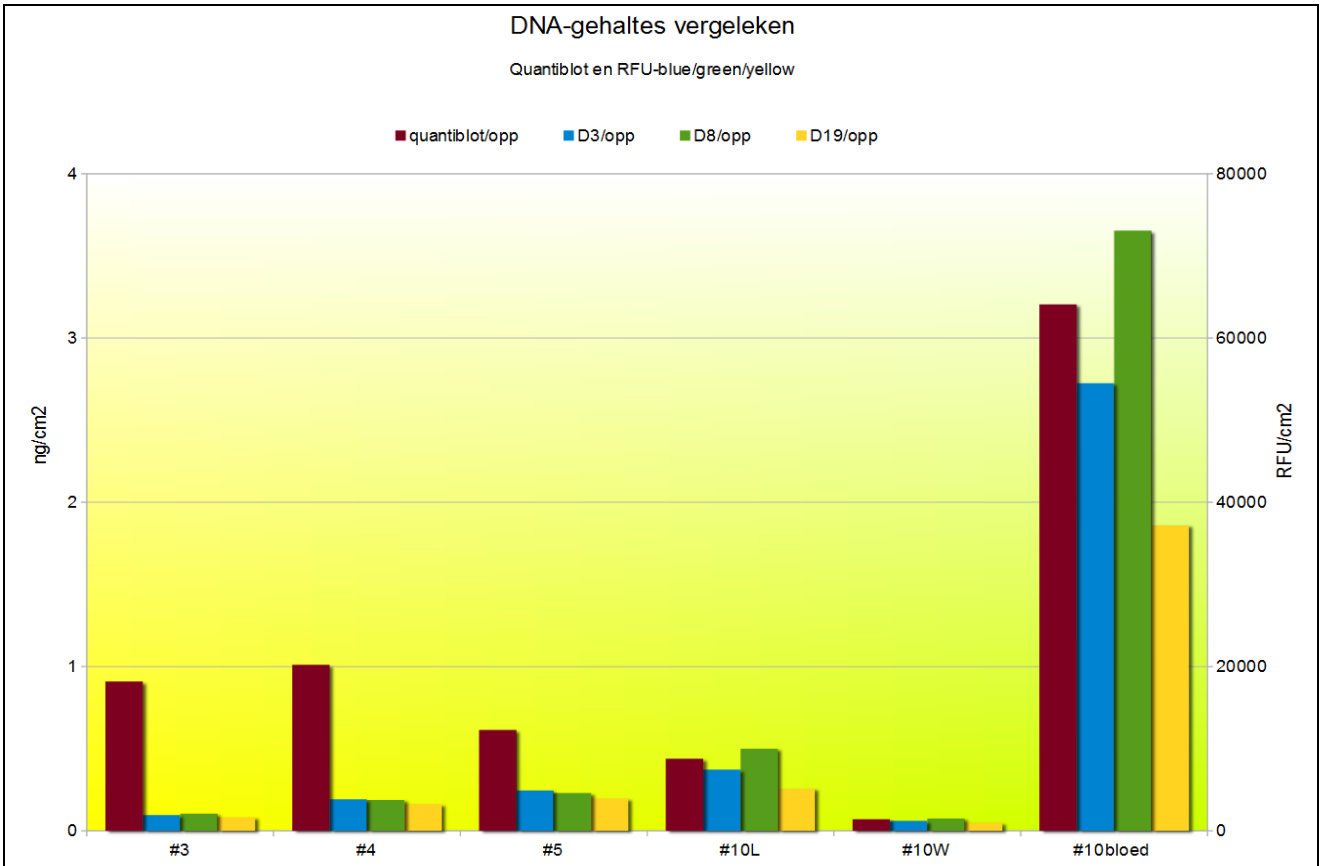


Figure 17. Strengths of the DNA intensities compared to each other on basis of the sizes of the actual bloodstains (except #10L, where the whole cutting is used as base). The anomaly of #10bloed (based on the containing *visible* trace) can be explained only by accepting, that most of the DNA in stain #10 sits outside the visible bloodstain, as in saliva. If the whole sample is used, the result (#10L) becomes again comparable with the other values.

Then, the so called am-peaks, tiny peaks in the DNA-profile #10, which exactly coincide with the three most left located peaks in profile of W,(see figure 8), are brought into focus. In the same manner as before, the DNA responses in those peaks were calculated in relation to the size of the actual bloodstain and added to figure 17 as #10W.[11]
As can be seen, the results are of the same order as the responses in stain #3. This is even more relevant, if one realises that stain #3 originated from a passive drop of blood and bloodstain #10 originated from another kind of blood deposition, a so-called transferred bloodstain (also characterised as such by the NFI). Such transfer would lead to reduction of DNA, if the blood were transferred from a bloodstain on the cotton of the blouse, as is hypothesized in this article. DNA in the white cells containing DNA show a very distinct adhesion to cotton - actually applied in medicine for filtering -, much stronger then the red cells of blood[12].
To conclude: cutting #10 displays a high DNA intensity, compatible to passive bloodstains. At the same time the major part of the cutting (85%) is free of any visible deposit. Assuming the DNA is uniformly spread in the trace, the greatest part of the DNA is part of a transparent deposit, as in saliva.
If the DNA was to concentrated in the visible bloodstain, its intensity would be about 10 times as high as in the bloodstains #3, #4 and #5. Justification for this anomaly is lacking. Considering a number of observed facts (creation of new bloodstains on the blouse in the aftermath of the discovery of the body, similar characteristics of the those bloodstains and the bloodstain in #10) and an alternative explanation is easily found. When the height of the DNA peaks of the victim W in trace #10 is related to the size of the bloodstain in #10, the relation between the this DNA response and the size of the bloodstain appears to be quite 'normal'.

What should be done: obtain similar analyses of the bloodstains #29 and #42. Trace #29, because it is a 'twin' of trace #10 and evidently created after the crime. Trace #42, because it is yet smaller then #10, whereas it seems to be connected somehow to #10 in several ways (size and location).

7.1.3. The low bloodstain intensity in #10

The graphical procedure to measure surfaces (figure 16) of stains and structures on the blouse, revealed an important bonus. After selecting a surface, Photoshop also reported the (actual and mean) luminosity of the pixels of the spot. Through comparison of the luminosity of the stain in question with the luminosity of a 'clean' part of the blouse, one can calculate the 'optical density' (more in general: absorption A) of the stain:

$$A = \log_{10} (I_{in}/I_{uit})$$

The proportion Iin/Iuit is used to compensate for differences in exposure values in the pictures. If a stain is black, A=∞. If a stain is fully transparent, A=0. The optical density is a measure for the intensity of the bloodstain. For all passive bloodstains A shows a value between 0.24 and 0.35. The variance can be explained by the age of the bloodstains shown, so of the pictures. We have pictures from the crime scene, from the mortuary and from the NFI laboratory (years later). Pictures, made during testing of blood drops on the blouse, made at the NFI, were also included. Only the values of the bloodstains in the cuttings of #10 and #42 (transferred stains) differ significantly with values of 0,16 and 0,13 respectively.

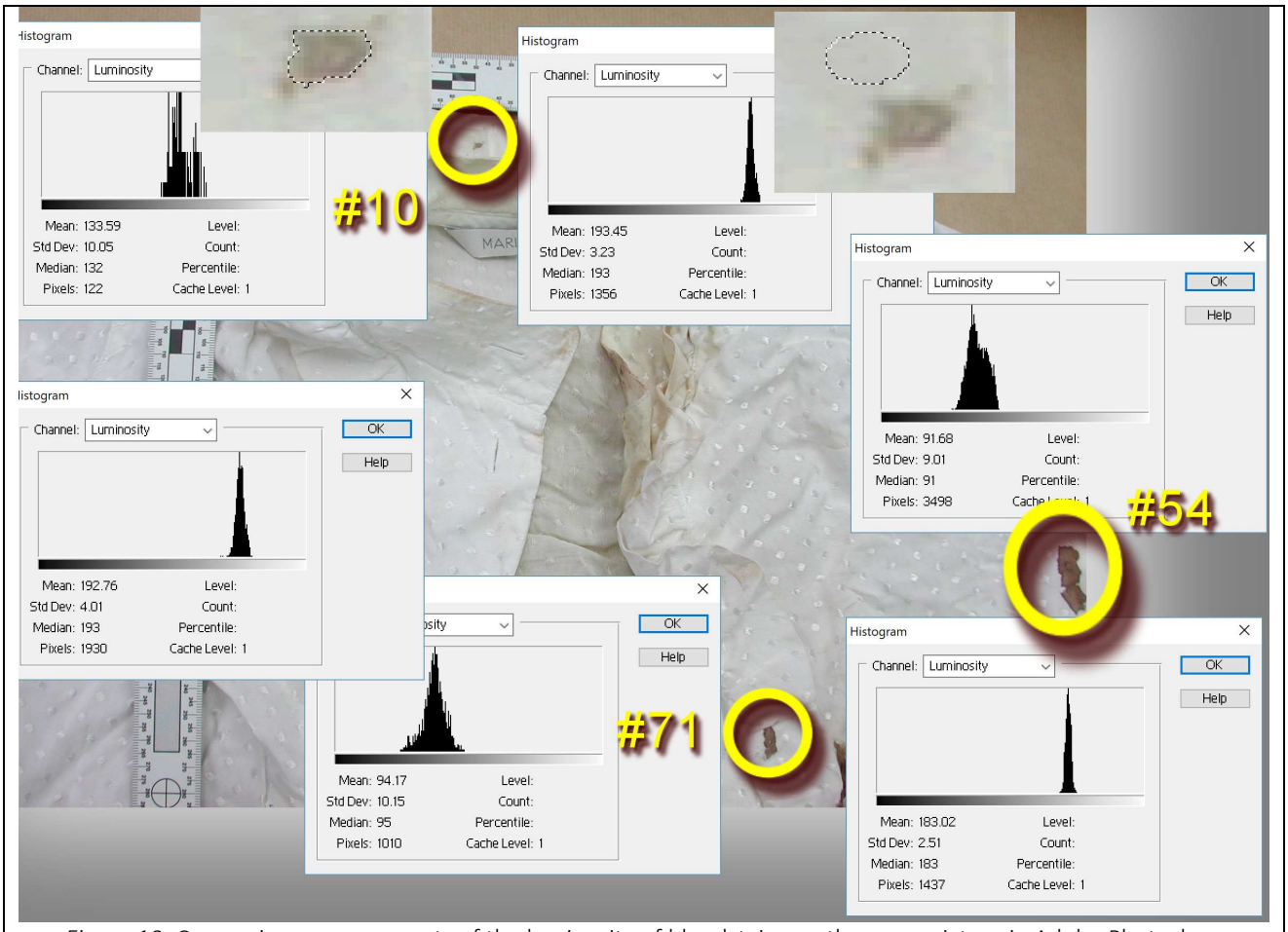


Figure 18. Comparing measurements of the luminosity of bloodstains on the same picture in Adobe Photoshop Elements.

From the measurements - in the same picture - of the optical density of stain #10 and of the passive bloodstains #54 and #71, showing values of respectively 0.16, 0.30 and 0,31 (figure 18), it is conservatively estimated, that the intensity of the bloodstain in #10 amounts to about 60% of a passive bloodstain.
Using these results, new calculations were made of DNA intensities based on size and optical densities of the traces considered. In figure 19, the outcomes are displayed as #10L/bloed* and #10W/bloed*; they show the consequences of the hypothesis of the NFI and the hypothesis put forward here (the blood in trace #10 being blood of the victim).
Now it is shown once again, that the NFI hypothesis, stating the blood of Louwes is the cause of the DNA profile #10, is completely out perspective. At the same time, it is shown, that the strength of the DNA peaks, corresponding to the profile of the victim, after correction for the size of the stain and the lower optical density, fits comfortably between the corresponding values in the stains #3 and #4, stains undoubtedly formed from passive bloodstains with blood of the victim. This again strongly supports the hypothesis, put forward here, that the visible part of stain #10 is produced from blood from the victim during uncontrolled - as being not recorded - manipulations of the blouse in the evening of September 25th 1999. Manipulations revealed by comparing pictures of the blouse at the crime scene and at the start of the autopsy 24 hours later (see figure 20).
To bolster this hypothesis, figure 22 shows a profile, calculated from the profile of L, based on his cheek swab, combined with 10% of the profile of W, based on a blood sample. Every detail of trace #10 can be found in this reconstruction (compare figures 21 and 22)!
Later on, a very weak bloodstain, #42, was considered to be caused by blood from Louwes. However, size and optical density of the stain are so small, that the corresponding DNA responses would be as high as 1% of those of the stains #3 and #4. The highest DNA responses in #4 are around 4000 RFU, so the highest peaks would attain 40 RFU, so lower than the threshold of 50 RFU. This would be true only for the highest peaks, as a consequence the profile would not be attributable! So again, the DNA profile in #42 should be considered as to be caused by a transparent deposit.
After the NFI reported their results, the FLDO (Forensisch Laboratorium voor DNA Onderzoek) checked the samples for Y-str profiles and found one in sample #42. From there they checked the autosomal DNA in the sample. The NFI only reported Louwes' DNA in sample #42, similar to the way, they reported on sample #10. But the FLDO not only found markers of Louwes (17) but also 6 markers of the victim and 6 markers, attributable to them both. So it matters, which laboratory investigates. Moreover this result completely facilitates the possibility, that the visible blood trace in trace #42 caused the match with the victim, whereas a not visible saliva residue caused the match with Louwes. The sample of the twin trace #10 was never analyzed by the FLDO, although it was ready to be sent off. A contra-expertise would have prevented a lot of trouble (likewise as with trace #20).

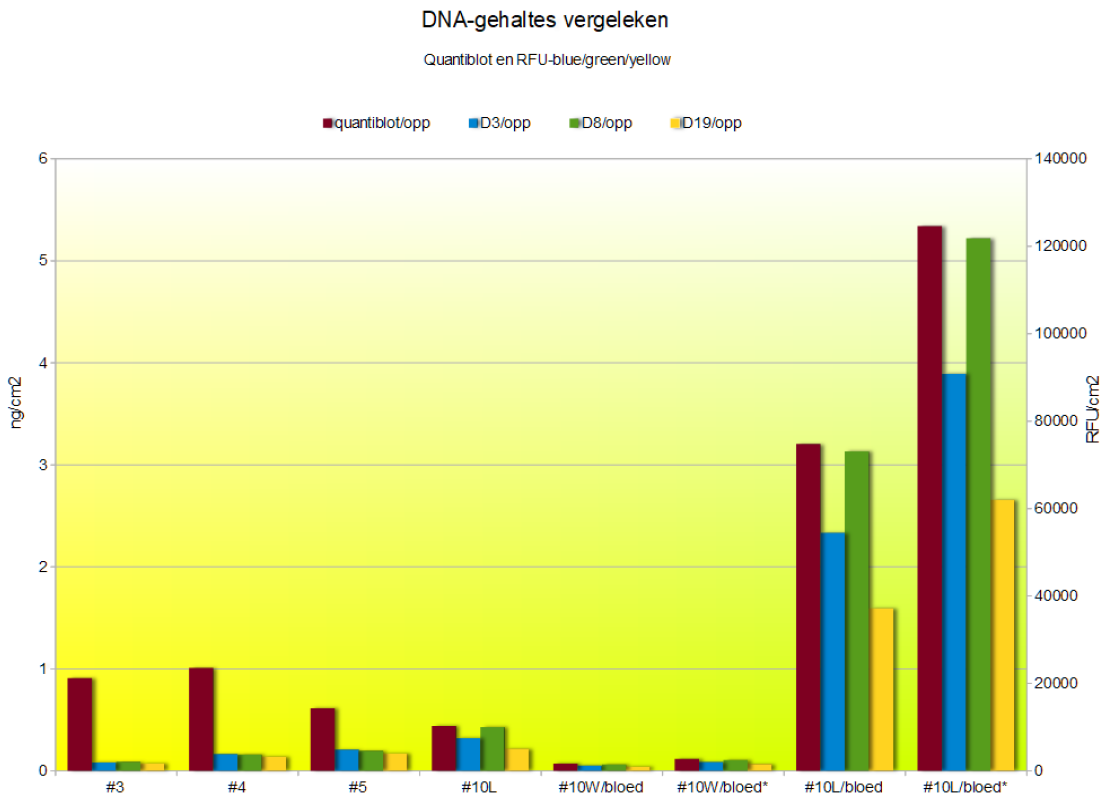


Figure 19. As in figure 17, but now including the correction from the lower optical density of the bloodstain in #10 (indicated with *). To explain the excessive high value for #10L/bloed* in comparison with the other values, one has to assume, that the bulk of the DNA of Louwes is situated outside the bloodstain as a transparent deposit, in this case saliva (as displayed in the already high value of #10L). The value of the DNA-traces of the victim (#10W*) are more then ever in concordance with the values of the blood traces of the victim, in this case #3 and #4.

Figure 20A. Left-under: blouse of the victim 24 hours *after* recovering the victim, showing fresh transferred blood traces after discovery of the victim (red). Other displayed pictures from the crime scene September 25th 1999. In green corresponding blood stains. Note the deterioration of the condition of the blouse.

Figure 20B. The picture, shown in 20A only shows a part of the transferred blood stains. These two pictures show the collection of blood stains not present at the crime scene (1999), but still visible at the NFI in 2003.

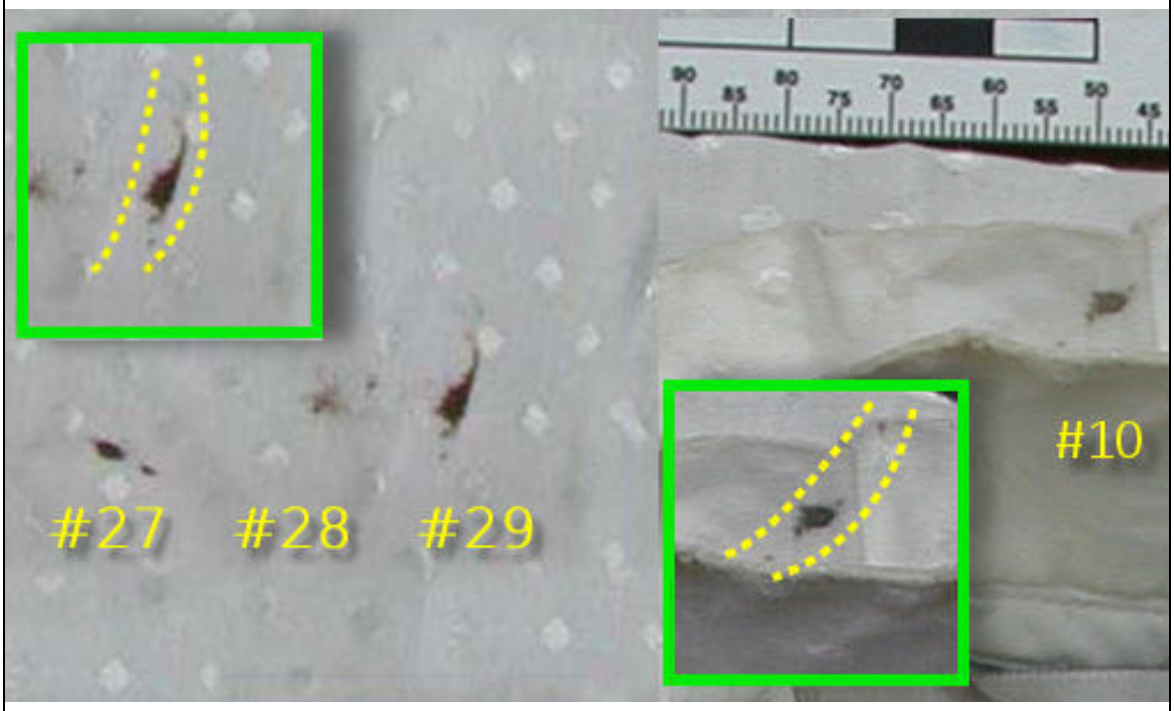


Figure 20C. Demonstration of the similarity of the stains #10 and #29. The latter was deposited on the blouse by investigators after the fact = between September 25th and 26th 1999 (see figure 20A) . Stain #10 was discovered only in 2003.

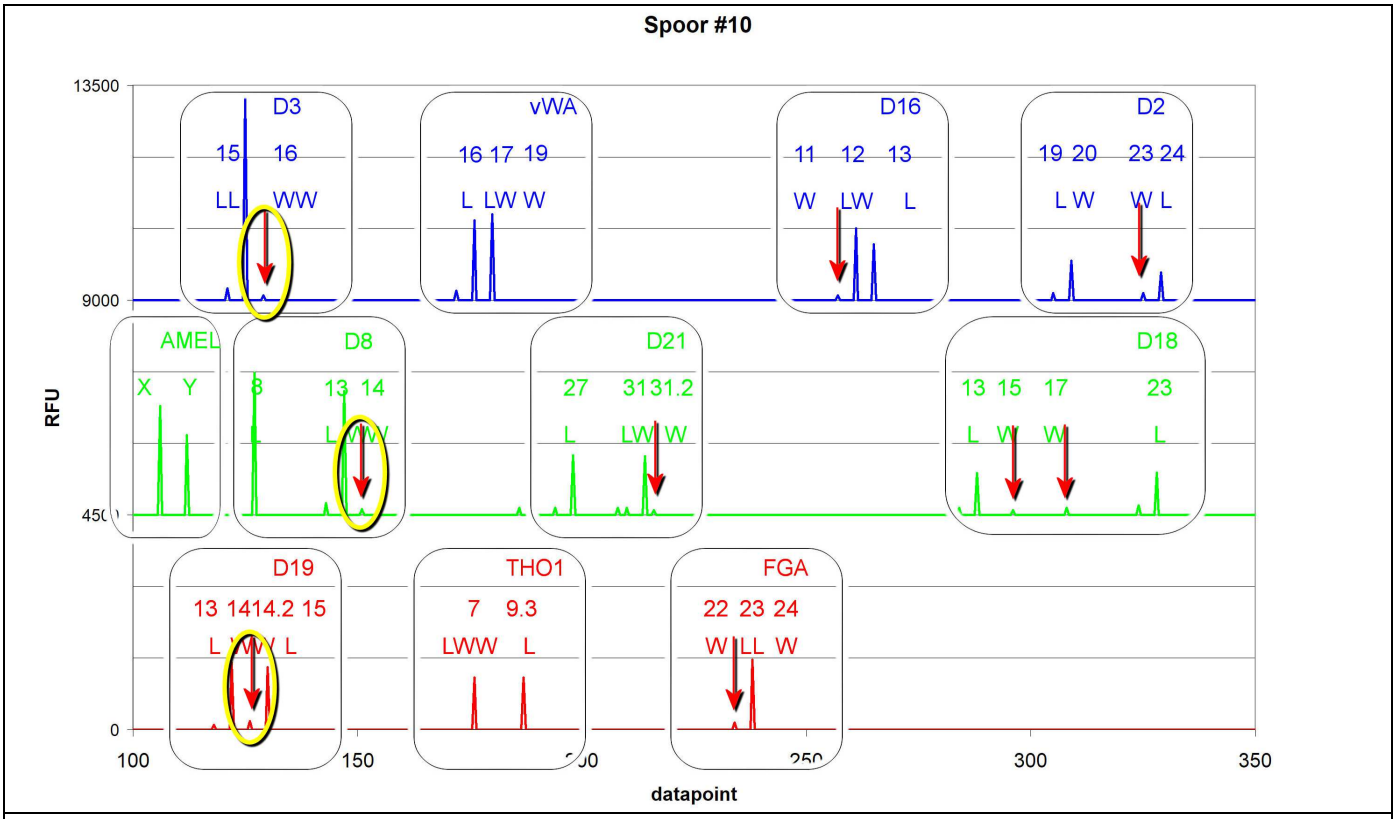


Figure 21. Profile of trace #10. Red arrows indicate positions corresponding to the profile of W.

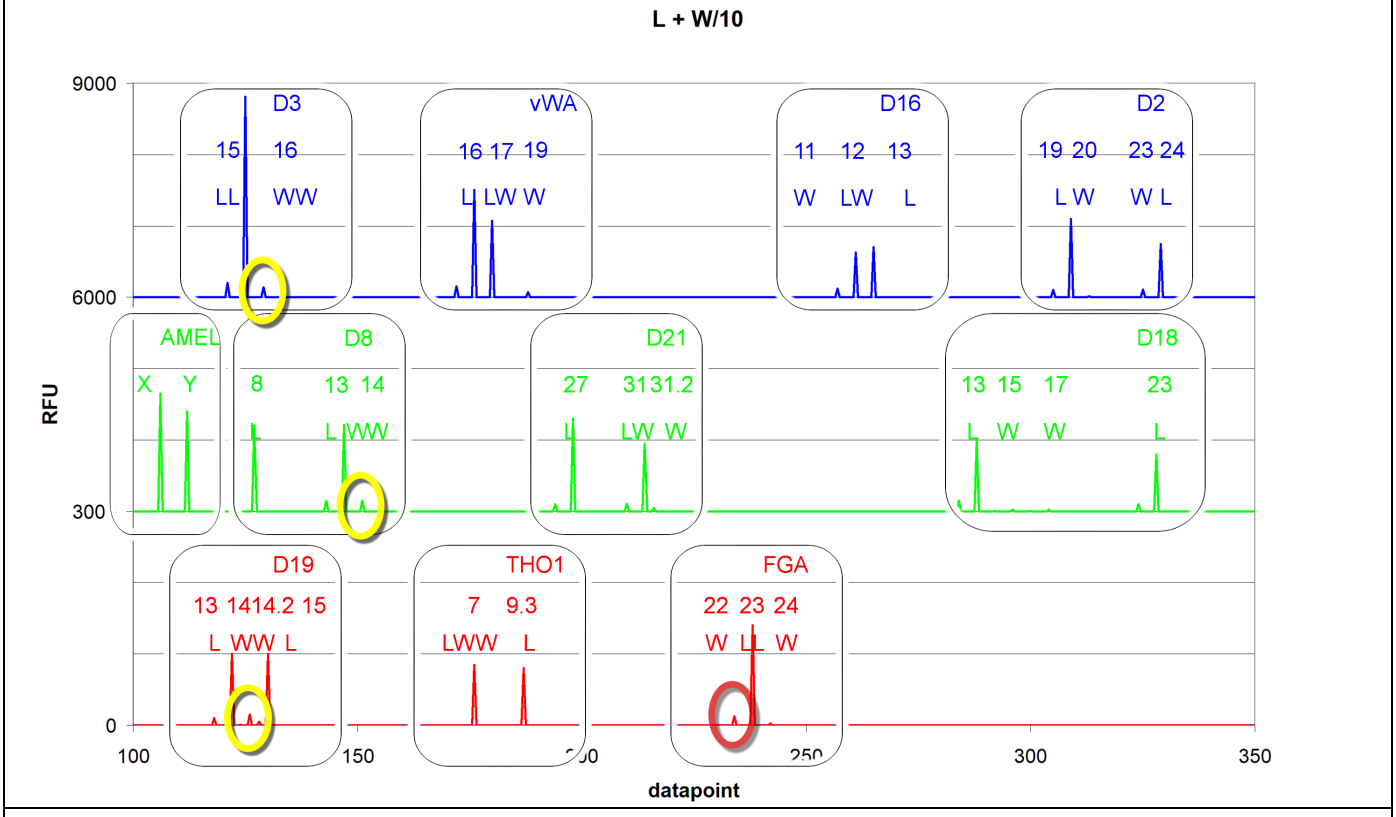


Figure 22. Reconstruction of a profile, using the profile of L from a cheek swab, combined with 10% of the profile of W from a blood sample (see figure 8). Compare to figure 21 to see the striking resemblance.

7.2. Traces caused by grabbing

The NFI categorised trace #20 as a grabbing trace, a trace caused by the violent attack of the victim, during which the perpetrator ('s fingers) first got contaminated with the victim's make-up at her neck and subsequently touched the blouse with much impact. In this way - the NFI explained - the trace combined a 'high' content of DNA of the victim with an even higher content of DNA of Louwes.
The presence of DNA of the victim was also explained by the presence of blood in the immediate vicinity of trace #20. Pictures made before sampling did not show any blood at the location of the trace itself, only a vague light red stain is visible there, hypothetically formed by traces of make-up. Furthermore the Court ruled, that the knife stabbings, causing the spill of blood on the blouse, occurred later. The contribution of Louwes to the trace - twice as high as the victim's - was attributed by the NFI to the transfer of skin cells.
The NFI examined 20 traces, but totally ignored the significance of the traces #7 and #12. These traces proved to contain the highest DNA values by far (about 10.000 rfu), so high, that anomalies related to overloading of the capillary (and PCR procedure?) are visible (colour bleeding and cut off peaks notably). Because the values exceed the 'limit of linearity', one can expect the values measured to be significantly too low, possibly with a factor two. These excessive values all concerned peaks in the profile of the victim. At the locations of these traces, crime-scope signals were seen, invisible to the naked eye in the pictures of the blouse. The - adjacent - locations of these traces suggest, that they form a part of a larger collection of such traces, containing a huge amount of the victim's DNA.

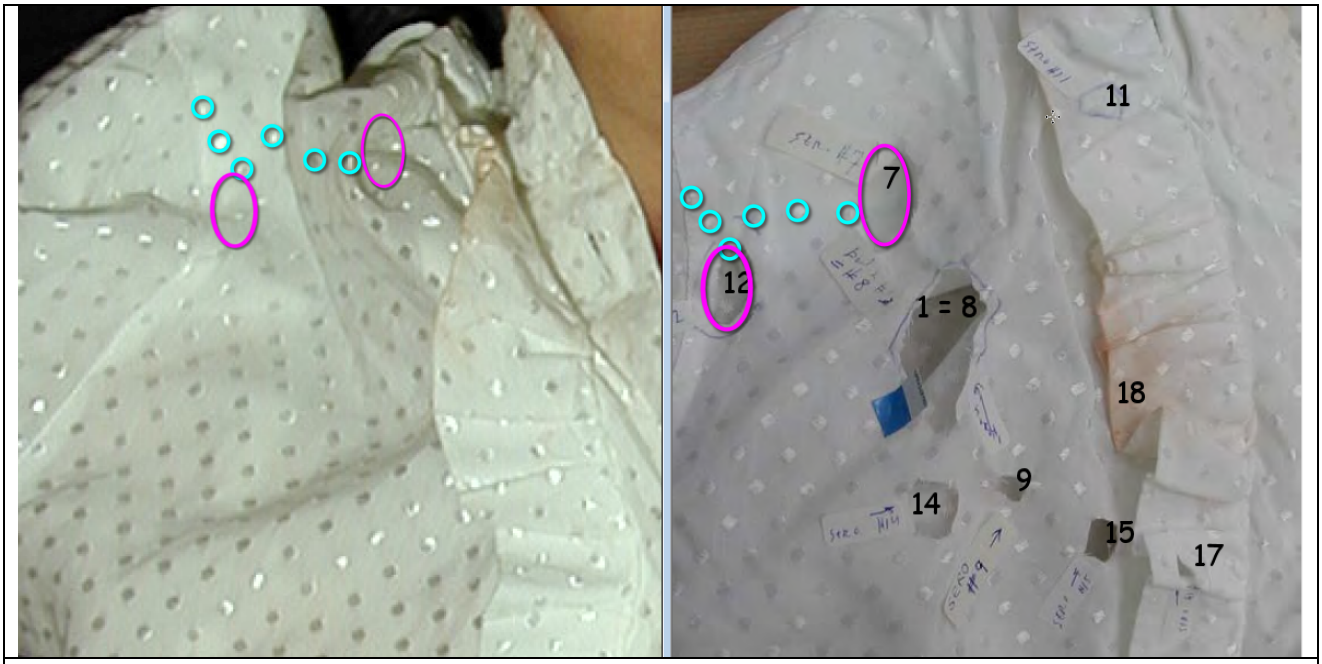


Figure 23. Locations of traces #7 and #12 on the blouse at September 25th 1999 and the end of December 2003. The position of these traces makes it plausible, that they form a part of a larger deposit of DNA of the victim. Between them, no other samples were taken.

From the hypothesis of trace #20 being caused by violent action, it is logical to apply this hypothesis to the traces #7 and #12. The autopsy report contains indications to justify this (figure 25). A further complication must be considered: in traces #7 and #12 only a few cells of Louwes were found, as the result of Y-str profiling. Put otherwise, the perpetrator had sufficient protection at his (her) disposition while leaving these traces, but this protection failed in the case of trace #20. Logic fails here.

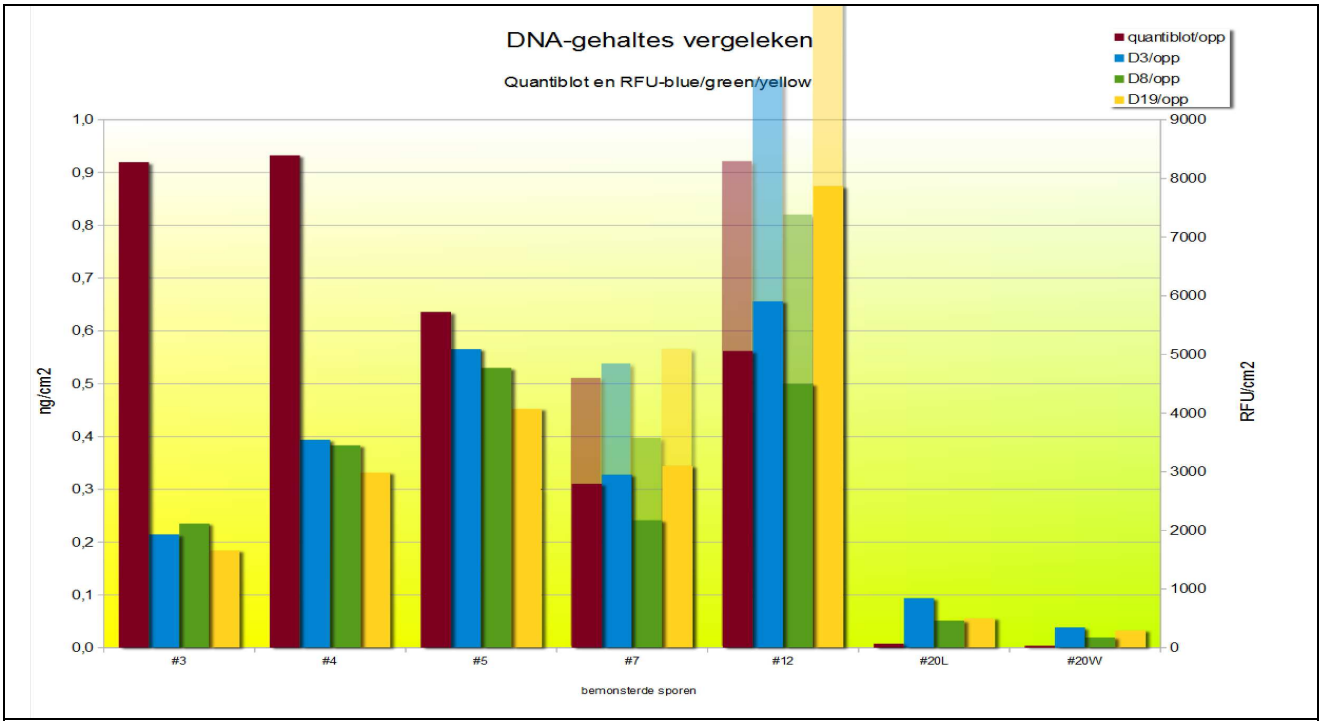


Figure 24. DNA intensities in traces #7 and #12 compared to those in the bloodstains #3, #4 and #5. Also shown are the DNA intensities in trace #20, specified to the contributions of Louwes (L) and the victim (W). The effect of the transgression of the 'limit of linearity' in traces #7 and #12 is shown as indication.

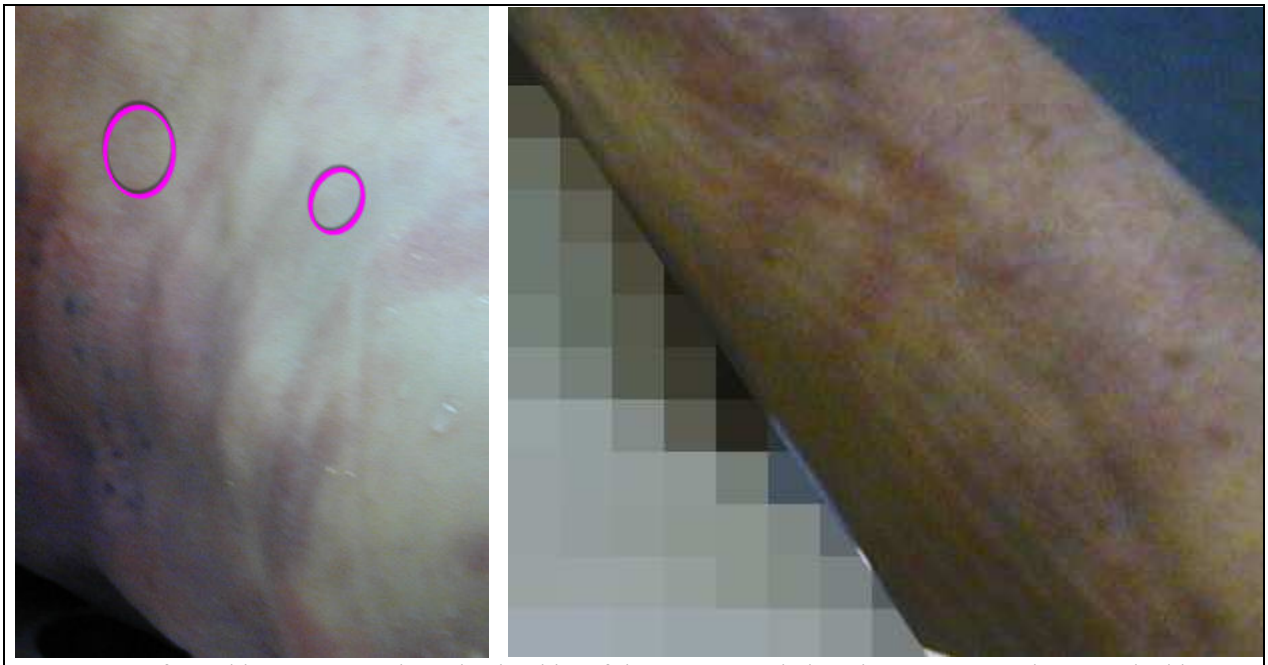


Figure 25. Left: Grabbing trace on the right shoulder of the victim, just below the traces #7 and #12 on the blouse. The significance of these markings was never brought to attention, especially not by the NFI, being responsible for the autopsy report. Right: A similar trace on the right forearm of the victim.

In figure 24 it is demonstrated, that trace #20 is far too weak to be regarded as the result of violent action. In addition, the fact that the NFI also categorised #20 as a DNA-trace related to the light red deposits - as discussed in the next paragraph - shows the uncertainty in the theorizing by the NFI.

7.3. Light red stains

From the beginning this group of stains was presented as a homogeneous category of traces with the same mode of origin. In the hypothesis of the NFI, it was make-up, with the perpetrator had transferred during the aggression from somewhere on the victim's body to

the blouse. There was no consideration for the fact that the same colour was visible at other locations on the blouse. Later on (2006) those other traces were investigated with negative results, without raising any alarm within the NFI.
Of the traces, investigated in 2003, one can only recognise trace #18 in the original pictures of the blouse at the crime scene. So colour hue and intensity of the whole group cannot be evaluated properly. As visible in later pictures, trace #18 shows a strong similarity with a number of traces elsewhere on the blouse, the major example being a large spot at the left sleeve, which was categorised in 2006 as a bloodstain (showing a DNA profile of the victim exclusively). Another prominent stain, just near the central button of the blouse can be shown to originate from some moment after the crime was committed. A picture made in the evening of September 25th 1999 shows the location still without any staining; the button mentioned beforehand was still in place, whereas at the autopsy September 26th, the blouse arrived totally unbuttoned. The right tip of the collar is visible in the first pictures as being light red, but was not sampled for DNA. Yet it was cut off (for chemical analysis that did not take place or was not reported).

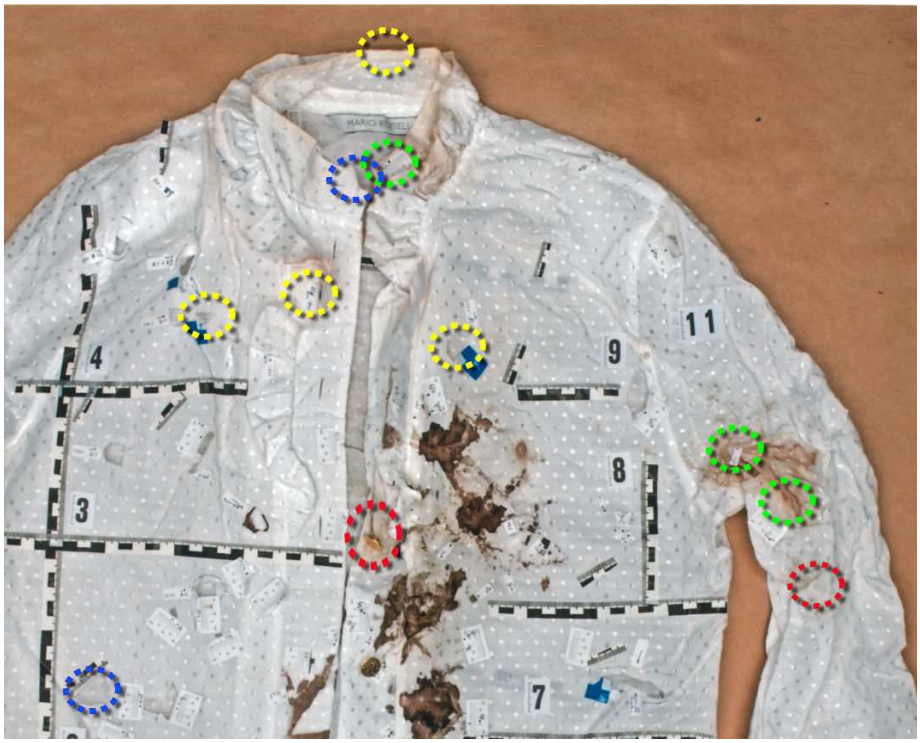


Figure 26. Upper. All traces marked in yellow were sampled in 2003/4 as belonging to the category of light red stains and hypothetical specified as make-up stains. The traces marked in green were sampled in 2006 as bloodstains. The traces marked red were not sampled at all. In the middle, around the button in the centre sits a light red stain that was not there when the victim was found. Neither was it there in the mortuary that evening. Therefore, it was left there in between by the investigators. This is also a possible in the case of the other stains, not visible in the crime scene pictures (#9, #19 and #20).

Below. In UV light, a wealth of new details is revealed. But none of it was addressed properly during trial.

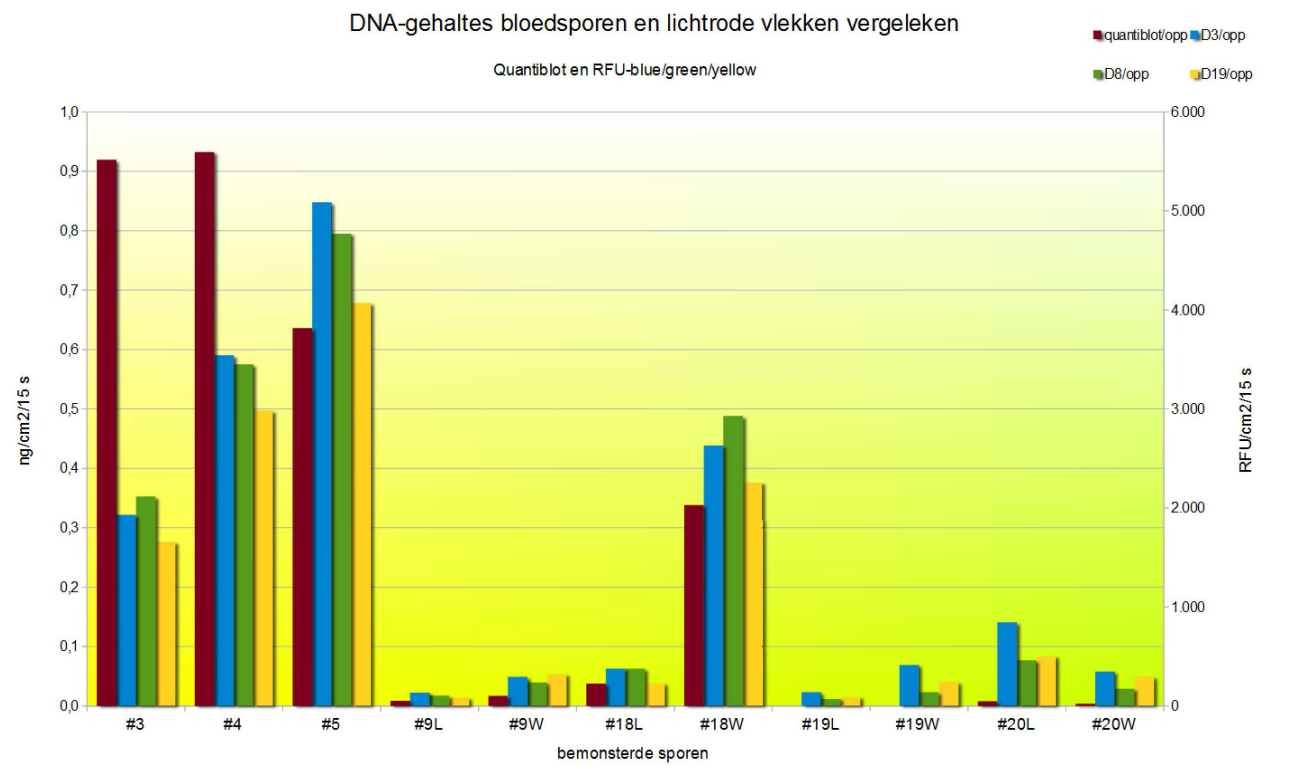


Figure 27. DNA intensities in the light red stains (#9, #18, #19 and #20) compared to these values in the bloodstains #3, #4 and #5. The DNA intensities are divided in contributions of Louwes (L) and the victim (W).

As follows from figure 27, this category does not contain stains of similar characteristics. Especially #18 deviates far too much. This stain shows more similarities with the bloodstains of the victim when considering the DNA intensities. There is not any resemblance with #9, not in the size of the stain (#9 is very tiny in comparison with #18), nor in its DNA content (which is approximately tenfold in strength in #18). The combination of the size of the stains (in stead of cut out samples) and intrinsic DNA content leads - estimated as total DNA content - to a ratio in the order of one to hundred!.

Figure 28. Trace #18 left to right: September 25th 1999 and end of the year 2003 (2x), out- and inside.

Here we put forward the hypothesis that trace #18 is formed by blood, which underwent a chemical modification, in which process it lost most of its colour. This because the

perpetrator tried to remove the blood, spilled during the first phase of the crime using some cleaning agent. Thus leading to the formation of chemical substances that did not test for blood with the much-used tetra base test. This hypothesis is supported by a number of generally ignored clues:

- After the victim died, she was during many hours left in a position, deviating from the position she was found in later on.
- There were liquid residues in the corridor and the living room. indicating a displacement. Contrary to court order, those residues were not investigated for DNA.
- The garments (blouse, cardigan) were pulled upwards, as if the victim was dragged by pulling her feet.
- Traces on the blouse indicate, that the cardigan, the victim wore, absorbed significant quantities of a red brownish liquid.

Figure 29. All stains from light red to brown and yellow shown on the blouse, are compatible in colour with the great number of variations known of ammonium ferric citrate.

Therefore, the hypothesis put forward here states that the colour of #18 is caused by ferric-ions, bound by ammonium- and citrate-ions or similar components from a cleaning agent. Because of this new bonding, the ferric haemoglobin complex disintegrates, loosing its original almost black colour, being replaced by complexes far more colourless. In some configurations ferric citrate is just yellow. Because the ferric-ions are able to partner up with citrate- and ammonium-ions as well with water molecules in many ratios, a lot of colour hues are possible. Additionally all these complexes are soluble in water very well.
The presence of iron in the light red stains was only reported by the NFI in 2006, while mentioning another component, being titanium. In this context it is important to remember, that the blouse was made of cotton and viscose, whereas the latter product is often whitened by administering titanium oxide. Through measuring the ratio of isotopes in the iron, the NFI good have lifted the cloud of doubt about the origin of the iron trace (mineral or biological e.g.).

7.3.1. Control samples

To bolster the hypothesis about the origin (make-up) and cause (violent action) of the light red stains, the NFI took a number of control samples:
"In order to precise the type of cell material responsible for the contribution of male DNA markers in the found mixed DNA profiles of the sample [ARA952]#9, the light red substance, four control samples where taken on the blouse S12 in the vicinity of this of stain. In this case we meant to take samples at locations, where no traces were visible, nor in visible light, nor with the use of the crime scope. The control samples are marked

in picture I [see figure 30 - author] *with red circles. In the DNA profiles of these samples, we didn't find DNA marks of a male individual."*

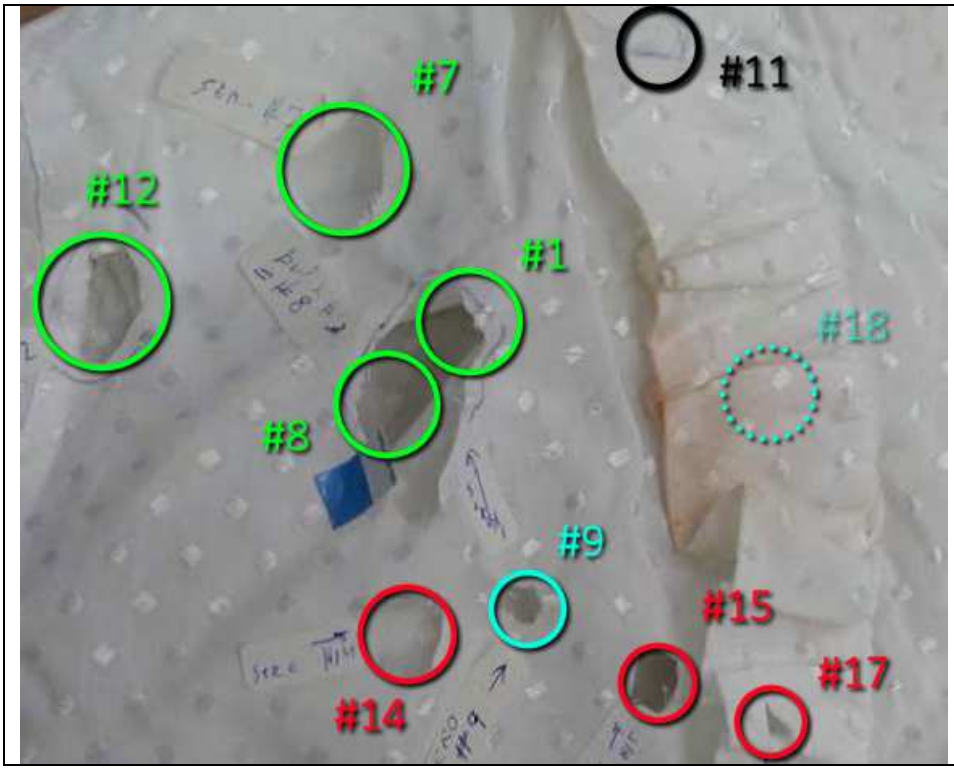


Figure 30. The immediate vicinity of trace #9. Only trace #11 is completely devoid of any DNA trace, connectable to Louwes. The traces marked green were not considered to be control samples, the red ones were. Note, the controls were not randomly taken, as opposed to proper scientific procedure.

The significance of using the term 'crime scope' is incomprehensible in the concept of taking controls, but understandable on the base of the sought-after effect: a number of traces in the immediate vicinity of #9 and visible under the crime scope were already sampled and found to contain markers of Louwes. Now the hunt was loose to find traces devoid of markers of Louwes. Also striking is the search of negative controls only around trace #9, the most insignificant light red trace in the category under scrutiny, see figure 27. The ultimate success of this manoeuvre came to its pinnacle in the conclusion of Mr. Machielse during the revision application 2006/8:
"The light red stains being crime related, is utter plausible. If the DNA of the convict is found in the light red stains and not or in significant smaller quantities in other instances, this is an important indication of his quilt, even if the DNA traces were connected to saliva or sweat." (Conclusion of the General Prosecutor at the occasion of the revision application of the Deventer Murder Case March 20th 2007).
Note, that the set up was such, that the chance to find DNA of Louwes in the controls was minimised at forehand. In the analysis of these samples, the injection times were not extended (in contrast to the analysis of other weak samples) and the cuttings were all small, twice smaller than 1 cm^2. Trace #18, a light red stain in the immediate vicinity measured a mere 2.66 (or more) cm^2!

However, were those control samples really devoid of DNA of Louwes? Figure 31 gives the answer.

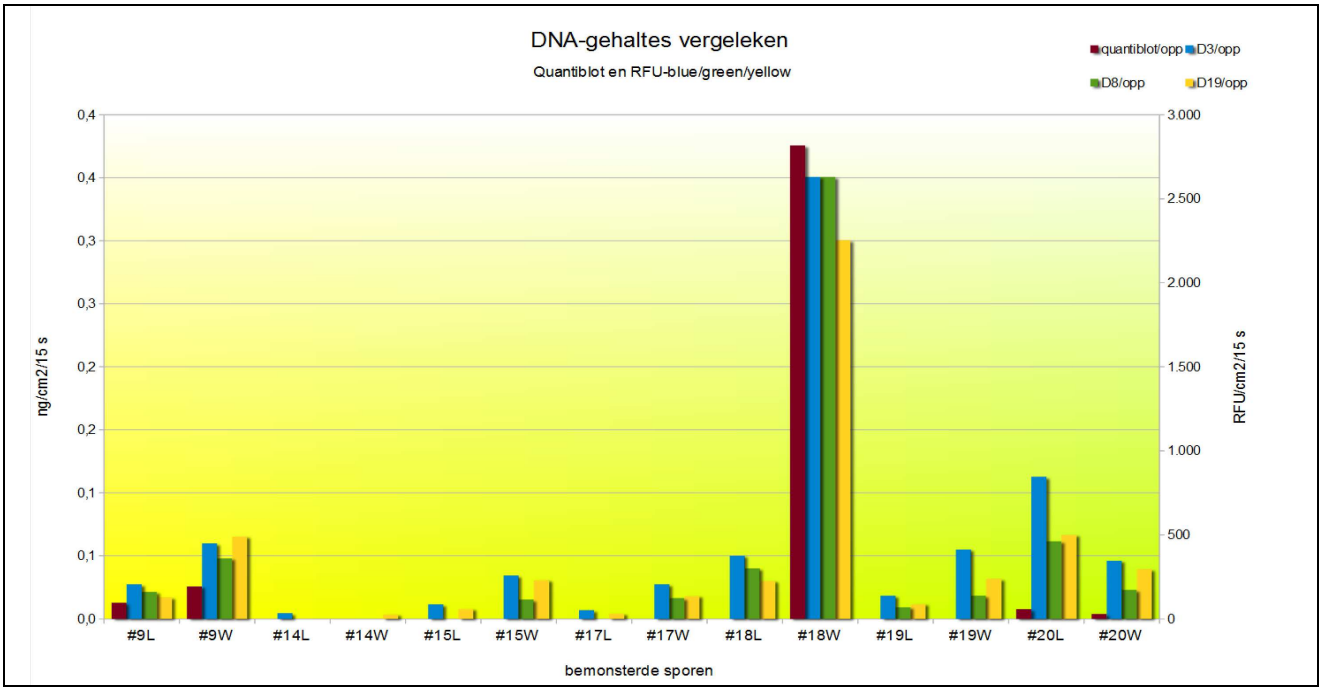


Figure 31. The control samples #14L, #15L and #17L are not completely devoid of DNA markers of Louwes. The 'target' of the controls, #9 has greater resemblance to the controls #15 and #17 then to trace #18, supposedly belonging to the same category as trace #9.

This raised question calls for a further analysis: in par. 4.1.1 it was demonstrated that peak heights decrease strongly in the first minutes of a capillary electrophoresis. The smallest DNA fragments are capable to compete with a variance of small ions present, the bulkier ones are in a wrong spot. Soon, the accompanying peaks drown in the always-present background noise. In the case of the traces #14, #15 and #17, only D3 survives, being the best visible marker in town. This marker is present in every control sample. This is not enough to justify a positive identification but it does falsify the claim of absence (The quote *'absence of proof is not the proof of absence'* was seldom more appropriate).

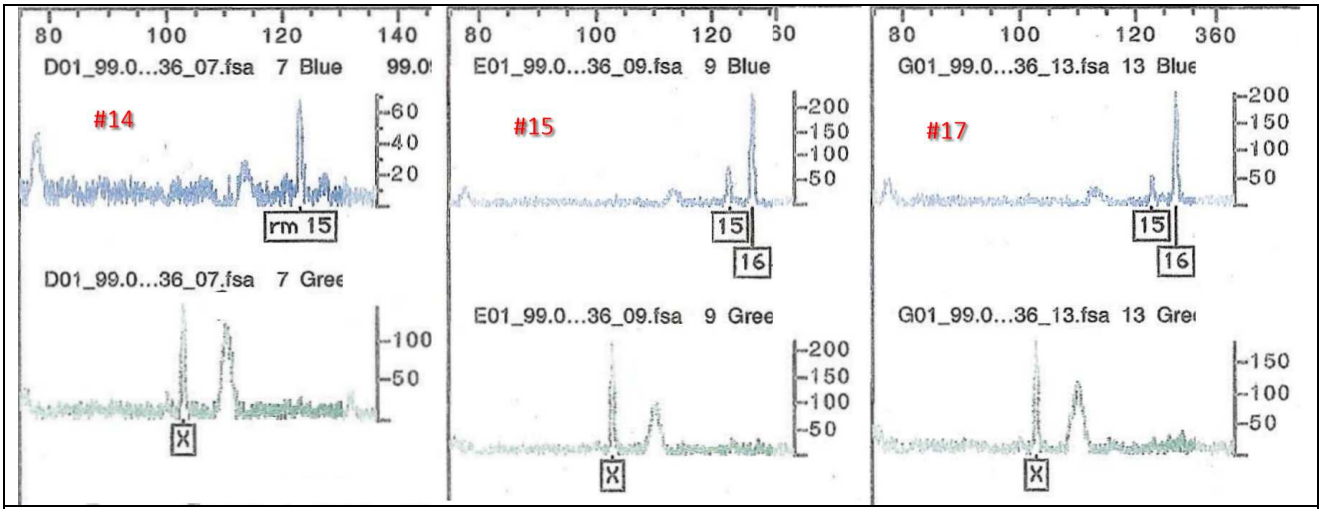


Figure 32. Marker 15D3, connected to Louwes is present in every control sample. In #15 and #17, it exceeds the stutter value connected to 16D3. In #14, this peak is even the only one in this location. It was removed manually by the NFI! In addition, the X-amel is in all instances accompanied by a marker at the proper location of the Y-amel. In #14, it is just as high as the X-amel, in concordance with the situation in D3. The Y-amel did not receive a label but looks the same as the marker in #13, where is actually labelled 'Y'.

On top of this, we will combine the results of the control samples with the other samples in the vicinity to bolster the hypothesis that Louwes was looking over the

shoulder of the later victim to a document, she showed him, while asking him for advice.

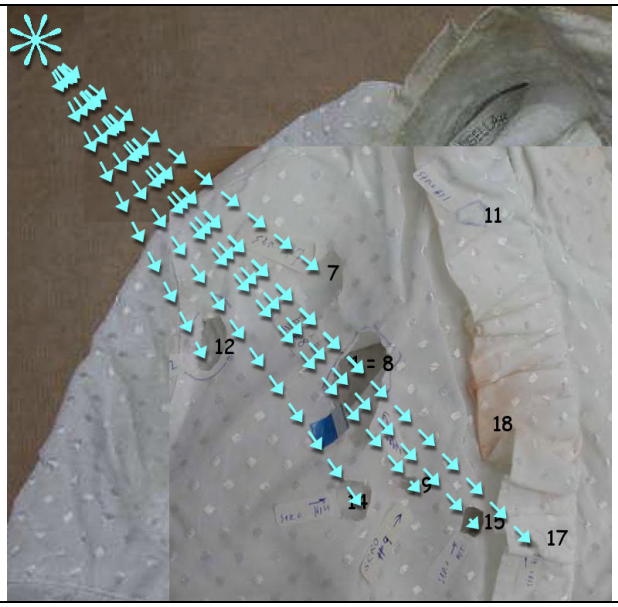


Figure 33. Trajectory of saliva particles, left behind by someone, looking over the right shoulder and delivering comments.

Using figure 33, it is possible to correlate the distance the saliva particles travelled with the strength of the DNA-markers in the samples. Two types of values are displayed. Firstly the DNA intensities, calculated as before on base of the NFI results. Secondly Dr. P. de Knijff of the FLDO performed a so-called Y-str analysis. Those measurements are primarily qualitative in nature, giving the number of matches within the profile of each sample. Therefore, this again yields quantitative data, which can be displayed.

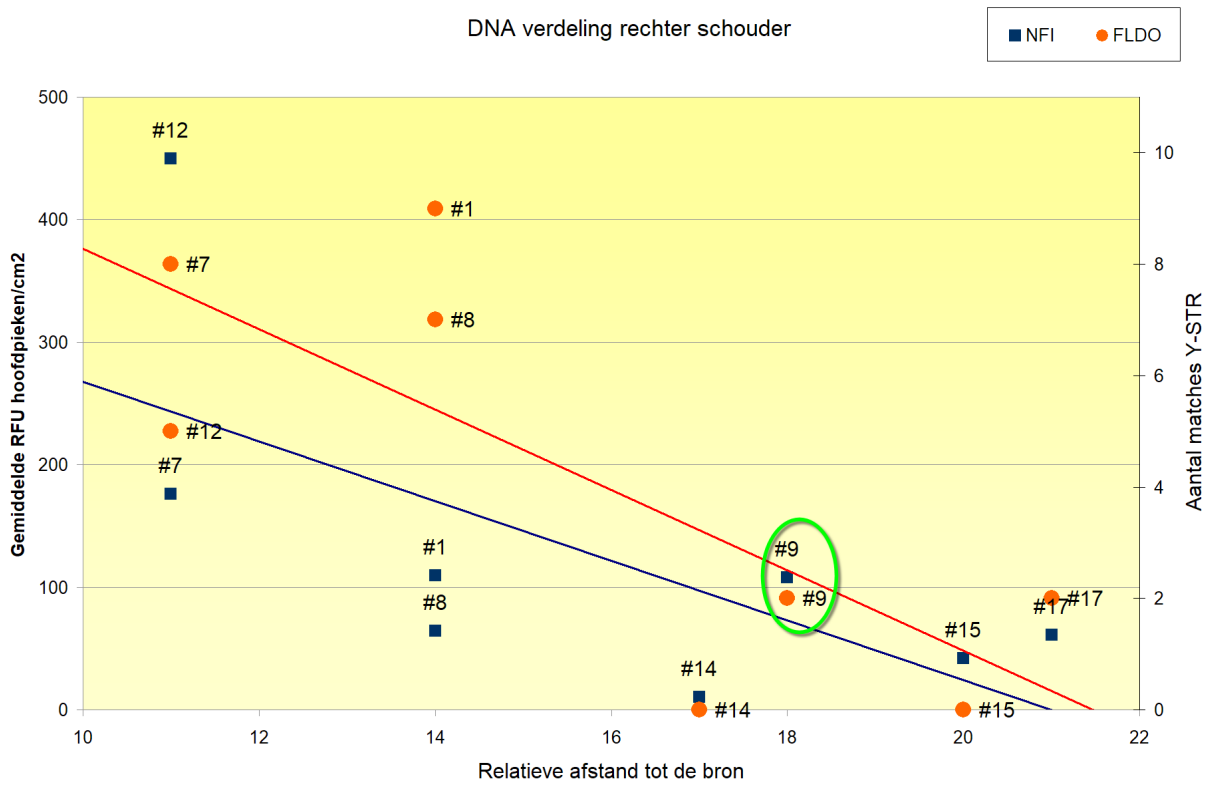


Figure 34. The strength of the DNA presence of Louwes in the traces around #9, #9 inclusive, correlates with a growing (relative) distance from the source as hypothesised in figure 33.

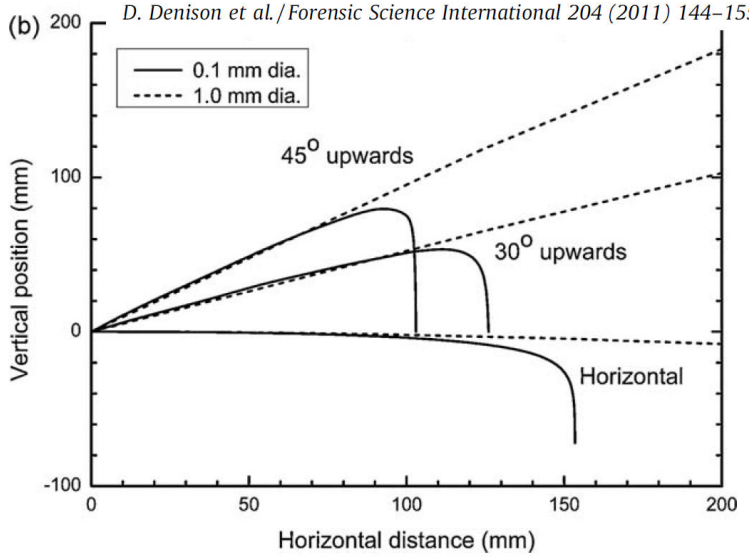


Figure 35. Examples of trajectories of saliva particles, in dependence of particle size. Reference in the figure.

7.3.2. The crime scope

During the trial of Den Bosch, Mr. R. Eikelenboom argued, that the light red stains would not probably contain saliva, because the stains did not respond to the light from the crime scope:
"The locations in the red circles did not react under the crime scope, so did not indicate the presence of body fluids. Therefore, it is utterly improbable, there is any saliva present. It might be possible, that a very small quantity of saliva goes undetected by the crime scope."
In another report, I argued, that such a statement could not be uphold under physical, chemical and biological scrutiny. The light red stains contain ferric ions that counteract the mechanism of crime scope reflection of light in many ways.[13]

However, there is more to it. The amount of DNA, present in saliva varies enormously per individual. Taking an average as 100%, the minimum and maximum values are 1,3 and 560% respectively[14], as plotted in the left frame of figure 36. Otherwise put, the amount of saliva might be quite small, whereas the DNA results might be quite high in the same sample.

Looking from the other side, we observe the content of fluorescent substances (tryptophan and tyrosine) to be variable too. The most important substance (tryptophan[13]) varies between 24% and 206% per individual around an average of 100%[15]. Most likely this variation is the direct consequence of the variation in protein content (especially amylase content) in saliva, of which values are plotted in figure 36 in the right frame. Important is the finding that a high DNA content can coincide with low protein content, because the source of protein in saliva differs from the source of DNA. Hedman et al 2011[16] reached the conclusion that there is no correlation between the two variables. Therefore, it quite normal to find saliva with maximum DNA content being invisible under crime scope light because it hardly contains any fluorescent substances.

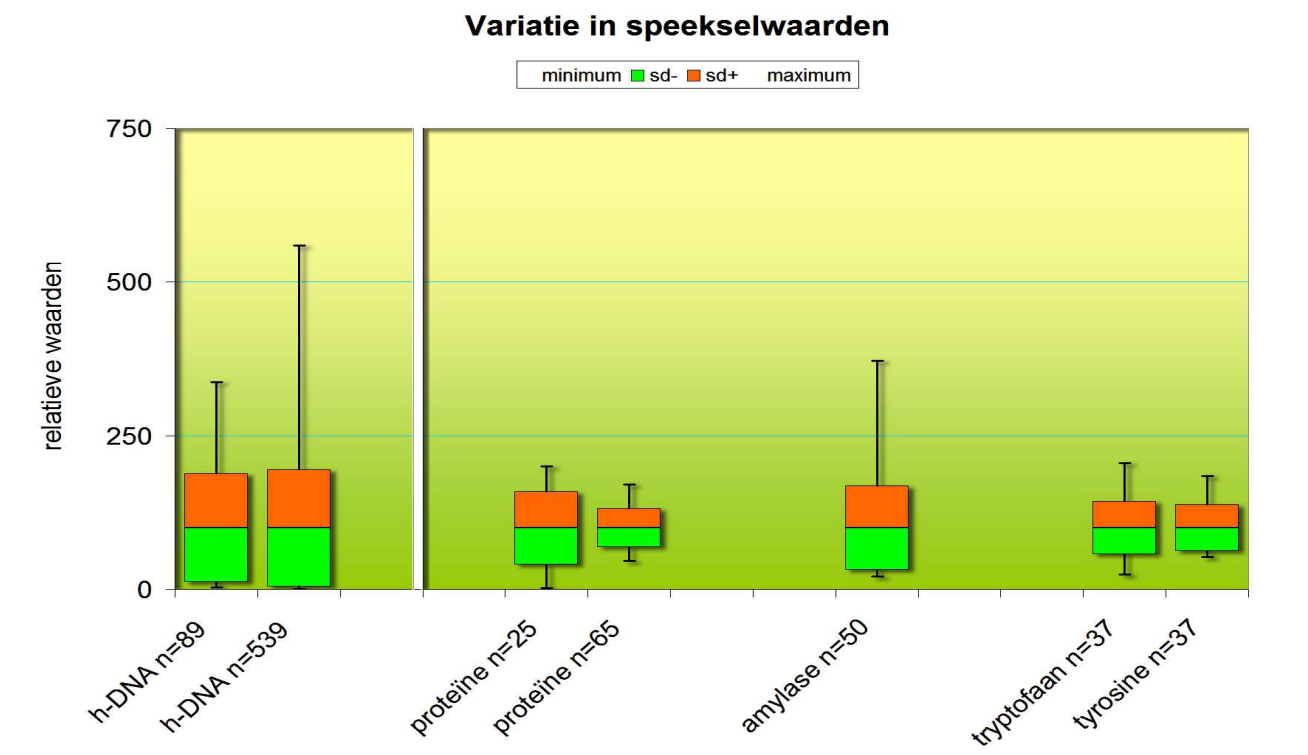


Figure 36. In the frame to the left the results of two investigations into the DNA-content of saliva with emphasis on the specification of human DNA (to exclude the effects of bacterial DNA)[17].In the frame to the right a number of investigations with indications of the presence of fluorescence in saliva.[18] Averages all normalized to 100%. The red and green boxes indicate the results within the standard deviation, the whiskers extend of the extreme values. When combining the variance of the DNA content (1:430) with those of tryptophan (1:8,6), the theoretical variance amounts to 1:3700.

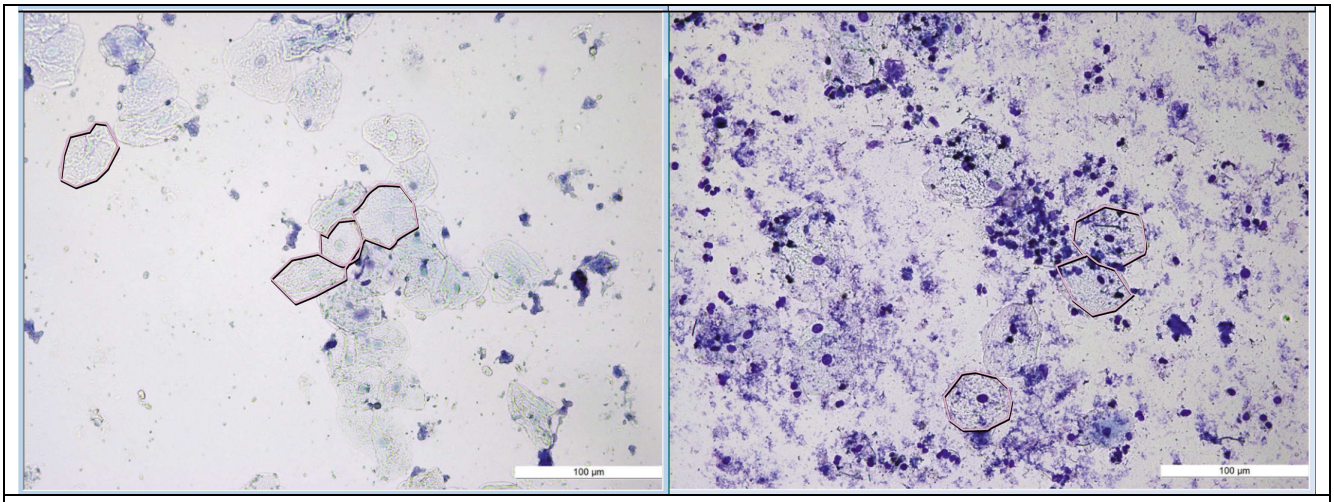


Figure 37. Saliva under the microscope. At the left with very low content of leukocytes, the main source of DNA. At the right the opposite, more typical by the way.[19]

7.4. Traces of saliva?

If all traces of Louwes were caused by saliva, wasn't his saliva production incredible high in such case?

An example:

If a healthy subject counts from 1 to 100, an average of 50 mm^3 saliva is expelled from his mouth (Xie 2009)[20]. So in about two minutes. The average subject carries 12.5 ng/mm^3 DNA in his or hers saliva (Sun en Reichenberger 2014)[21]. So in two minutes 625 ng DNA is expelled on average. Using the equipment of Dr. A. D. Kloosterman at the NFI since 2000, you would need 0.2 to 1.0 ng to produce a complete DNA profile. [22] Therefore, in theory an average

subject might produce somewhere between 600 and 3000 complete profiles in a mere two minutes. Given the conversation, Louwes and the victim allegedly had, the duration would have been between 10 and 20 minutes, so by far exceeding the theoretical values above-mentioned.
Not to mention the variation in DNA content as discussed in the preceding paragraph. Only a handful of complete profiles of Louwes were recovered from the victim's clothes, so what?

7.5. Trace #20 and apoptosis

When arguing about the significance of trace #20 during the revision trial of Den Bosch 2003/4, Mr. R. Eikelenboom referred - knowingly or not, to the phenomenon of programmed cell death (apoptosis), as might be connected to the cornification of skin:

"The chance of achieving a DNA profile is also relevant. From blood, semen or saliva, in general a DNA profile is extracted rather easily. With touch traces or skin cells, this opportunity is slim. Although the skin continuously releases cells, those cells contain little DNA substance, because the cells involved are dying. If the perpetrator exercised some force, the chance to obtain a DNA profile increases."

And:

"Skin cells being released, are dead. The DNA in such a cell is largely fragmented. When using the Low Copy Number method, only a few cells are required to get a useful DNA profile. To get a useful DNA profile from skin cells with a standard procedure, as used by the NFI in case of DNA analysis, at least 200 cells must be transferred. In normal businesslike and standoffish meetings, such as handshaking or having a direct conversation between participants, less substance is transferred."

The Court complied, reading the following:

"(Chemical analyst, Dutch title: ing.) Eikelenboom has in this matter stated in the first place, that the DNA profiles, obtained in the investigation under review are yielded using standard procedures, as the NFI applies normally. In general such procedures will not yield profiles from material transferred during businesslike standoffish meetings, such as handshaking or having a direct conversation between participants. During the session of January 26th 2004, ing. Eikelenboom declared, that in order to obtain an useful DNA profile from skin cells, a minimum of 200 cells must be transferred and that in general during the beforehand mentioned businesslike standoffish meetings less substance will be transferred.(..)."

Possibly ing. Eikelenboom reasoned in his discussion about the DNA content, that the low content of DNA in skin cells gives rise to the need of a tenfold rise of the threshold of required DNA (200 in stead of 20 cells) he referred to. The DNA expert of the NFI (Dr. A. D. Kloosterman 2000) had established the optimal DNA-input - while introducing the new DNA procedures with the SGM-plus system – to range from 0.2 to 1.0 ng, being the content of 30 to 150 cells[22]. In 2003 a trainee wrote a report under auspice of Dr. Kloosterman, mentioning a threshold of 100 picogram, so 18 cells (Emily Besselink, UvA/NFI 2003)[23]. None of the participants - so neither Dr. Kloosterman - made a note of this before the Court .
Another possibility should be considered, simply the possibility that ing. Eikelenboom confused the notions of numbers of cells and picograms. The detection threshold in the period was often found to be 100 to 200 picogram, so about 20 cells. A reference introduced by ing. Eikelenboom himself (sic) mentioned the crucial values:

" Currently, multiplex PCR DNA type profiling routinely produces full profiles at or below 100 picograms of purified DNA. Therefore, as few as 20 cells will be sufficient to produce a DNA type profile. The skin surface represents a large potential for a source of DNA profiles." (R. A. Wickenheiser 2002).

Probably, none of the participants in the deliberations read this passage.
To bolster his claims, ing. R. Eikelenboom added a curious remark in his report:

" On request, Dr. A Linacre states, 200 cells are required to obtain a DNA-profile with standard methods. According to him, a transfer of this amount of cells is not possible during incidental contacts."

The remark was followed by a referral, but the text belonging to it is missing. I contacted Dr. Linacre and got the following answer (mine emphasis):

" "On request, Dr. A Linacre states, 200 cells are ***required*** *to obtain a DNA-profile with standard methods."*[:] *This is a* ***misunderstanding*** *as 200 cells was equivalent to the* ***optimum*** *amount of starting template. (..) In my opinion 190 cells should generate a full profile but 50 pg of DNA* [equivalent to 8 cells – author] *is highly unlikely to do so.".*

In the meantime, some sort of crossfire was laid; was there a threshold of 200 cells, or was the threshold 20 cells, but because of the low quality of skin cells, still 200 cells were needed to compensate this low quality? The whole situation is utterly unclear, if you read the NFI-reports, the expert statements or the arrest of the Court. Therefore, we have to study the reasonability of a multiplying factor of 10, when discussing DNA profiles from skin cells.

7.5.1. State of science at the moment of giving evidence.
Every reference about cornification (origin of skin cells) in relation to programmed death of cells mentions an incomplete understanding of the mechanism or mechanisms (cf. Eckhart et al. 2013[24]). This is true before and at the time of giving expertise. And it is true today, cf. Clare Rogerson et al. 2018[25].
The idea, put forward by ing. Eikelenboom that the DNA is fragmented and lost is not confirmed by scientific testing..

7.5.2. Cornification causes nuclei to get invisible.
The above differs from the loss of DNA. DNA constitutes 10% of the mass of a nucleus. Nuclei are - view under the microscope - visible structures, constructed of millions of molecules. DNA molecules on their own are often invisible and are relatively stable, as we know from detecting DNA in fossils of thousands of years old, such as the DNA of Neanderthals.
There are indications of DNA being released from the skin and so being transferred more easily (Kita et al. 2008)[26]. The investigators coupled individual DNA molecules to antibodies and so made them visible. Analysing the found DNA they made clear, that in some participants almost complete DNA dominates. The profiles, found by Kita et al. through sampling the skin (foils and cotton swabs) are well comparable with those collected from #20. But nowhere the investigators seem to have used violence to obtain those results.
The proposed mechanism of apoptosis involves the removal of the products of fragmentation by phagocytes and so the DNA stays available for analysis (during forensic typing the phagocytes are destructed and the DNA will be released). In the meanwhile the question if apoptosis is part of cornification stays unsolved, cf. Lippens et al. 2005[27]. Fischer et al. 2007[28] conclude, that at least a similar process is responsible.

7.5.3. The loss of nuclei during cornification is not universal.

If the involved person suffers from parakeratosis (dandruff and psoriasis e.g.) the nuclei are not lost during cornification (cf. Fischer et al. 2017[29]) . In combination with the release of skin flakes (not beyond imagination, Louwes is bald headed), the transfer of DNA could be enhanced instead of suppressed. This possibility was completely ignored at the time (it should have been a part of the speculations).

7.5.4. Programmed cell death.

Fragmentation of DNA is considered a part of programmed cell death. The fragmentation does not occur - judging by the current state of science - at randomly chosen sites, but at selected sites in the DNA, the so called restriction sites. The enzyme DNase1L2 is often thought to be involved. A consequence would be, that the DNA gets fragmented in lumps of 180 base pairs or - more frequently - lumps of multiples of 180 base pairs (see figure 38). The results of Kita et al.[26] show, that the size of the found DNA fragments indeed start a value of 180 base pairs as minimum. The fragments isolated by PCR range from 100 to 340 bp, so many fragments can be retrieved and are available for successful CE. The fragments used in the quantitative analysis here range from 110 to 150 bp, so are hardly hampered by the fragmentation.

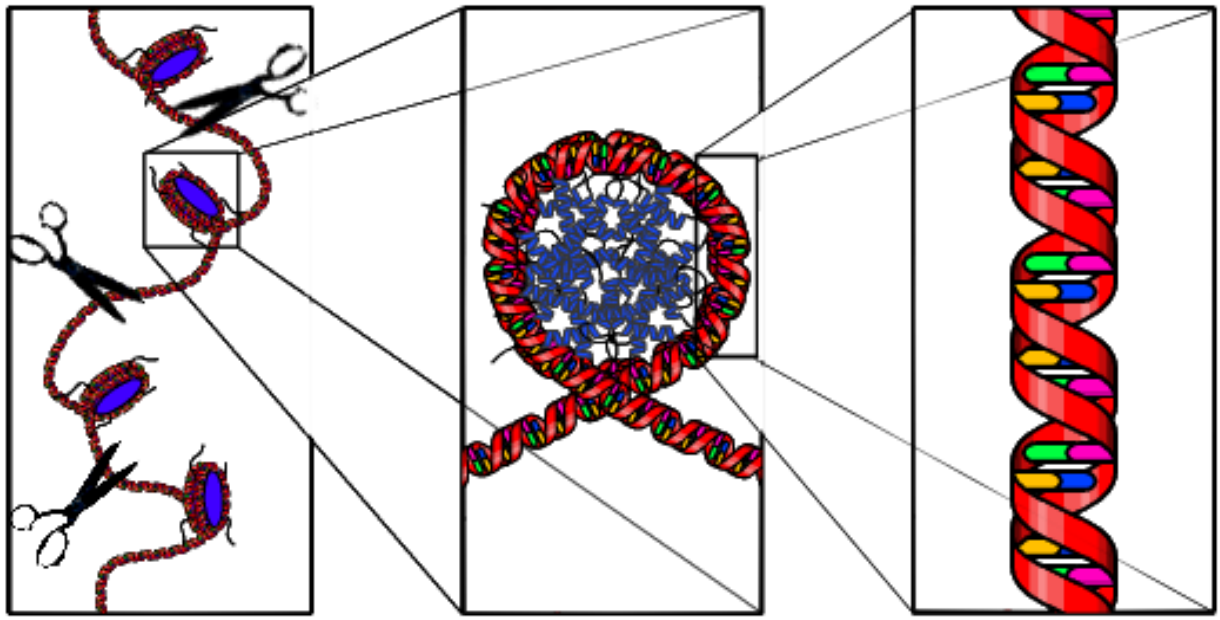


Figure 38. The DNA is safely stored in the nucleus because large parts are periodically winded around so called histones forming so called nucleosomes (in the middle). This leads to a string of beads. The string between the beads is called 'linker DNA'. Linker DNA is sensible to enzymes that are able to cut DNA (DNase). In this way, lumps of 180 bp or longer are formed.

Concluding. Firstly no indication let alone proof is offered, that #20 originated from skin cells. Secondly there is no justification for the need to transfer an abnormal high number of cells in order to collect a profile.

To conclude with a tale telling particularity: the NFI report of January 19th 2004 mentions #20 twice as positioned on the *right* side of the blouse. In the NFI report January 22nd 2004, again the position is described as at the *right* side of the blouse. In the same report, a figure places #20 at the *left* side. In the arrest of the Court, it has returned to the *right* side, although it is also positioned just above the upper stab wound (which was a stab wound just above the heart).
Where or which was the trace?

8. DNA on the nails

Already in 2003/4 conserved clipped nails of the victim were checked for autosomal DNA. Only autosomal DNA of the victim was found. Later on (2006, so of no importance to the Den Bosch trial), the FLDO (Forensisch Laboratorium voor DNA Onderzoek) investigated the nails for Y-str DNA and found partial matches with the profile of Louwes. At the occasion, the matching samples were checked for autosomal DNA, again with negative results regarding Louwes.
To start with here a table of results, where all data refer to the victim, with the exception of the column with Y-str data (* = not investigated):

nail samples	position	NFI					FLDO	
		Qantiblot	autosomal				autosomal	Y-str
		score	RFU			matches	matches	matches
			D316	D814	D19	(17)	(26)	(17)
AFZ648#1	R under	1	8000	7500	6300	17	25	8
AFZ648#2	R upper	0.5	1750	1900	1710	9	24	4
AFZ948#1	L under	0.13	4700	3500	2950	10	*	0
AFZ948#2	L upper	0.03	0	60	50	1	26	6

The included values of the RFU's are absolute values. If the estimated surface of the combined clippings amounts to 0,5 cm^2 (5 nail clippings, 1 cm wide and 1mm across) then the scores for DNA-density in the autosomal samples is twice as high (figure 39), placing them on top of all the RFU's reported in this survey. Where the number of matches is mentioned, the corresponding maximum in matches is found in the header. Yellow figures relate to the victim, green figures relate to Louwes.

8.1. Autosomal results by the NFI and FLDO

During the revision trial 2003 Den Bosch two jars with nail clippings were retrieved from a storage space, each containing five clippings . These clippings where sampled separately left and right and on both sides (under and upper), after which the samples were extracted. No details about the methodology were revealed. It seems reasonable to presume, that the two sets of clippings were swiped with four cotton swabs. At the other hand, later on the term *'nail scraping'* was used, indicating the use of a spatula, although this method is more appropriate when the nails are still part of the victim. It is hard to imagine sampling the upper side of a nail clipping using a spatula.
Possibly, the use of this terminology is once again a *'cock up'* . There are more to follow.
After extraction, the result was tested by a semi-quantitative method, once again probably Quantiblot. The approximate value is in the table (ng/µL). The scores for right under and right upper are very high. Because the source of these scores were in the same container (only left and right clippings were separated), one can imagine the DNA was spread around. Dried skin flakes can be expected to emigrate to the walls of the polymer jar (electrostatic) and back.

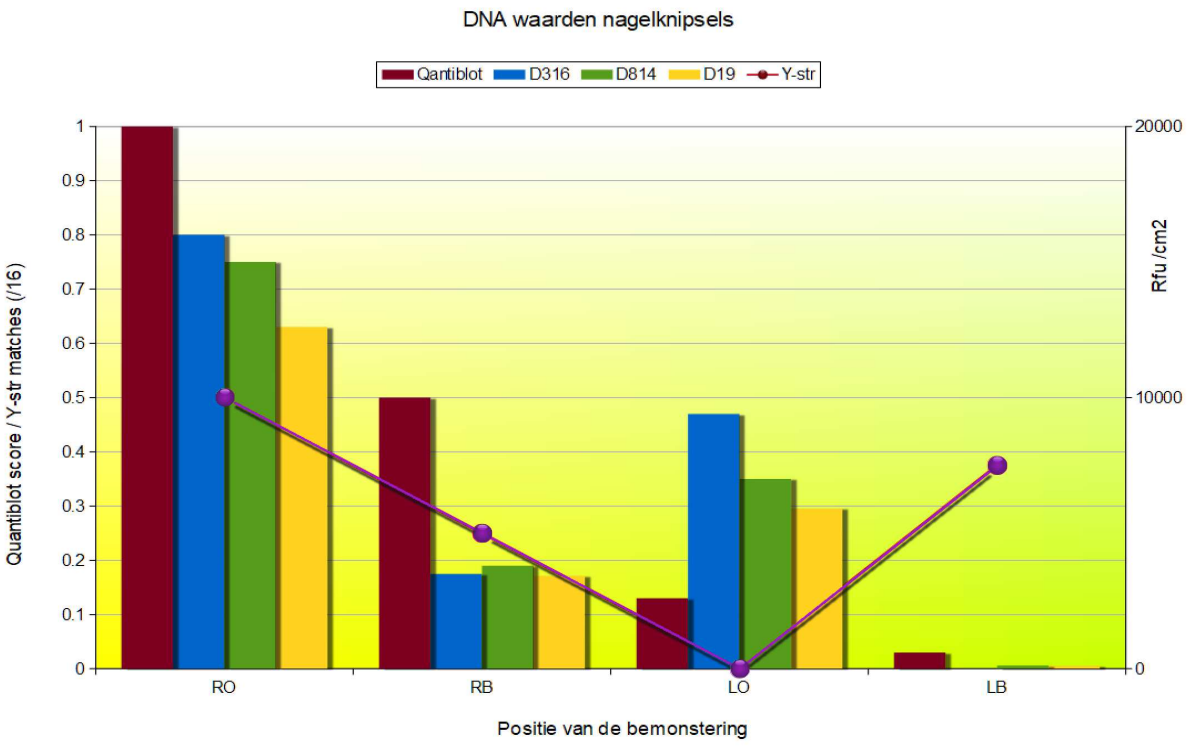


Figure 39. DNA on the nail clippings. Bars: the results of the Quantiblot measurements and the DNA-intensity based on RFU's with a nail surface of 0,5 cm^2(NFI). Only matches to the DNA of the victim were found. Superimposed is the number of Y-str matches with the DNA of Louwes according to the FLDO. RO = right under, RB = right upper, LO = left under, LB = left upper.

On the other hand, the under sides of the nails are coarser, so a better adhesion there seems logical.
In the next columns, the highest RFU-scores are recorded as usual. Once again, they refer to matches with the profile of the victim. The result left-under was characterized as absence of DNA.

Nowhere there is a match to the profile of Louwes!

This emphasis is necessary, because an error popped-up later on. The autosomal results show a problem in relation to the Quantiblot results. Now, right under and left under have the highest scores and right upper is unexpectedly low. The discrepancy left under vs. right upper is unacceptable, especially since the RFU scores are so high and so well correlated.

Maybe there is a cock up here also. This cock up starts in the report NFI January 19th 2004 (Dr. A. D. Kloosterman), page 5/10:
"The nail clippings of the right hand [AFZ648] and left hand [AFZ948] of the victim were investigated to the presence of blood. No blood was found. The nails are sampled for a DNA-investigation."

This sentence matches with the listing of submitted traces. However, at page 6/10, we read:

"At the occasion, the under sides of the nails of the left and right hands ([AFZ648]#1, [AFZ948]#1) and the upper sides of the nails of the left and right hands ([AFZ648]#2, [AFZ948]#2) were sampled."

This might seem to be without consequences - you have to read twice to see the difference - , but further on, this might have created serious problems, as Kloosterman writes the FSS a letter summing up various details to enable the FSS to give a second opinion:

43	AFZ648#1	Nail scraping victim #1 (right hand) (10 ul input; T=15 sec)
44	AFZ648#2	Nail scraping victim #2 (right hand) (10 ul input; T=15 sec)
45	AFZ648#1	Nail scraping victim #1 (left hand) (10 ul input; T=15 sec)
46	AFZ648#1	Nail scraping victim #2 (left hand) (10 ul input; T=15 sec)

Of course, it is annoying to lack any certainty about which samples carry which code, but more serious is the display of lack of accuracy and carefulness at the NFI. In addition, the values for input do not match the values written on the electropherograms themselves (resp. 2, 8, 10, 10).
The increased values are not justified by the Quantiblot results by the way. The rise in values has no relevance to the measurements as argued in par. 4.4.2. Possibly, the NFI tried to force an outcome that included a mach to the profile of Louwes.

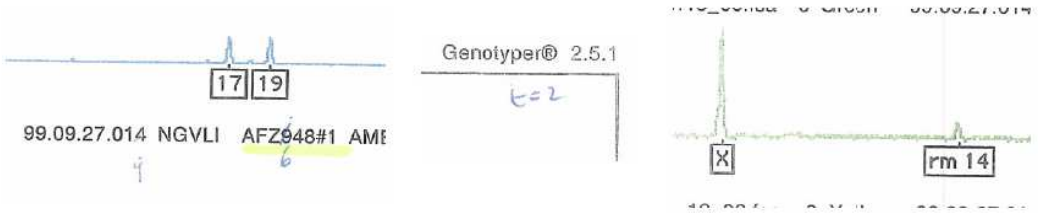


Figure 40. At left a number of corrections, as found more then once on the DNA plots of the nail clippings. In the middle is noted an injection value of 2 µL. At the right is shown the occurrence of DNA left-under (X-amel is clearly present at RFU=400), but other matches failed except one. This measurement was not repeated, unlike was done in other cases.

The discrepancies continue. Another forensic institute, the FLDO repeated a number of investigations for autosomal DNA and indeed only found DNA of the victim (2006). The FLDO found almost complete profiles of the victim in the samples AFZ648#1, AFZ648#2 and AFZ948#2, whereas AFZ948#1 was not investigated. So there was a major disagreement with the NFI, reporting absence of any DNA in sample AFZ948#2 whereas the FLDO reported a complete profile just and only there!

Nevertheless, no harm to Louwes; any trace of his DNA was absent in all autosomal investigations.

But the confusion was there, even though the NFI had the opportunity to comment the results of the FLDO. They did not offer any explanation for the risen discrepancies in the traces ARA852#42 (par. 7.1.3) and AFZ948#2.

8.2. The Y-chromosome investigation at the FLDO

8.2.1. Fingernail dirt

Louwes stayed in the clear until NFI was questioned in 2006. In response to the questions, A.D. Kloosterman (May 19th 2006) volunteered a suggestion (mine emphasis everywhere):

"From the nail clippings of the right and left hands [AFZ648 en AFZ848] (author: codes are OK here) a DNA-profile was retrieved, matching the profile of the victim (see expertise report January 19th 2004). Investigation of DNA in ***fingernail dirt*** *might yield results of high criminalistic relevance, if DNA-markers of another individual beside the victim are found in the sampled* ***fingernail dirt.*** *Generally sampled* ***fingernail dirt*** *will contain high quantities of DNA of the victim. In such case, this DNA will mask DNA markers of a second individual, if present. In cases as under scrutiny, with relatively high levels of DNA of the female victim present, a potential presence of male DNA in the sampled* ***fingernail dirt*** *can be detected by practicing a Y-chromosome specific DNA investigation. "*

As you can count, Dr. Kloosterman uses the term ***fingernail dirt*** four times in a case, where ***fingernail dirt*** was never collected. There were nail clippings, but that has other connotations (touch DNA). In addition, the NFI leaves unmentioned the fact, that one of the samples (left-under) was void of victim's DNA, so masking of male DNA could not occur there. Furthermore, the expert neglects mentioning the high sensibility of Y-chromosome testing, making the discrimination between traces of violence and simple innocent touch traces hazardous. The latter problem was even the trigger of the discussion about the interpretation of the NFI-results up to date.

Nevertheless , the recommendation of the NFI has been followed and the result - no surprise - was abused. The contract was awarded to the FLDO. The result was added to the table at the start of this chapter; partial profiles were obtained with 0 - 8 matches to the profile of Louwes, so 9 - 17 matches on a maximum of 17 were **lacking**.

The Attorney-General assisting in the revision procedure before the High Court, which was started in the mean time, confiscated the results:

"1.22. In response to appendix 50 I subpoenaed the FLDO-report under discussion dated September 11th 2006 of Dr. P. de Knijff with the Public Prosecutor. From this report, it appears that four samples, received by the FLDO, revealed a Y-chromosome DNA profile. In the other blood traces, no Y-chromosome DNA profile was found. The found profiles included blood stain#42, two samples from material, that was found on the underside and upper side of the nails of the left hand of the victim and a sample from material of the upper side of the nails of the right hand of the victim. [author: this listing is incorrect, left and right are switched, see the table.] (..) *Regarding the three* ***fingernail dirt traces,*** *De Knijff reaches the conclusion on the basis of Y-chromosome DNA-research, that the profile matches the profile of the applicant* [Louwes]."

Beware: one of the traces contained only a quarter of the maximum matches, the 'strongest' trace not even half. De Knijff stated that the markers in the profiles matched with the profile of Louwes. There is a delicate difference there.

In the follow-up, the poisonous term *'**fingernail dirt**'* went viral under the population of legal experts and researchers, e.g. De Knijff October 4th 2006:

*"From the fact that the Y-chromosome profiles in the **fingernail dirt** match with the Y-chromosome profile in ARA852#42 on can not reach the conclusion, that the male cell material in the **fingernail dirt** must have the same man as origin. Regarding the **fingernail dirt** samples, at least two possibilities (scenarios) must be considered:*
*A) The male cell material in the **fingernail dirt** originates from the suspect.*
*B) The male cell material in the **fingernail dirt** originates from another male, possessing the same Y-chromosome profile as the suspect by chance."*

Maybe, I must repeat:

No fingernail dirt was collected!

The damage, originating in 2006, is still visible today, reading the advice of the ACAS (Acceptatie Commissie Afgesloten Strafzaken) January 21st 2014:

*"For the sake of completeness the Commission points out the advice in the report by the NFI of May 19th 2006 to investigate the **fingernail dirt** of the nail clippings of the victim for Y-chromosome DNA. The result is included in the report of the FLDO N06-102 of September 11th 2006, to be discussed hereafter."*

In the wake thereof, the Attorney General, supervising the ongoing procedure regarding the Deventer Moordzaak, Mr. D.J.C. Aben stated:

*"The ACAS also draws particular attention to another research topic, the presence of some **cell material** at or under the nails of the victim, where **autosomal** and Y-chromosome DNA markers match those of* [Louwes]. *"*

In this statement, the expression ***fingernail dirt*** euphemistically replaced by the expression ***'cell material'***.

Therefore, I have to state a new warning:

No cell material was collected!

and in addition, repeat another:

Any autosomal match with Louwes was ever reported from the nail clippings!

8.2.2. Cell material

The moment to bring the expression 'cell material' into focus. You can see cells, look at your hand or your dog. Observing single cells is harder to do. You will need a microscope. Forensic investigators can find cell material, using a microscope, in particular when probing the nails of a victim. Such an action was not accomplished in de Deventer Moordzaak - you could guess already. Nevertheless, the nail clipping were secured and sampled in insecure - since not recorded - procedures. Our best guess is, they were sampled with cotton swabs. Another guess, the nail were all clipped with the same pair of scissors. Another certainty, the undersides of the clippings made contact with the uppersides of their siblings in the two containers.
Results were analyzed through PCR and electrophoreses and DNA-traces were found. Originated this DNA from cells? Certainly not, they originated from PCR. Originated the

templates that went into the PCR from cells? This is unclear and impossible to establish after the fact. Does extra cellular DNA exist? Sure! There are 'tags' to describe the corresponding phenomena: CNA, eDNA and fcDNA. Earlier on I referred to Kita et al. 2008[26]. In the meantime, a host of information about this CNA is published, long before Aben introduced the expression ***'cell material'*** in the pending case, cf. Suzanna Ryan 2012[30] and Vandewoestyne 2013[31]. One aspect surfacing is, that the used method of extraction -QIAamp - is relative effective in sampling this existence of DNA.

8.2.3. Quantitatively

As mentioned, the observed Y-str-profiles were incomplete, containing only 8 - 4 - 6 - 0 of 17 possible markers. What does this mean?
From the used markers mentioned in the FLDO-report, one can infer, that the used kit was the AmpFlSTR Yfiler kit. Sensibility tests using this kit were published by Johns et al. 2006[32], Joachimsson 2007[33], en Maintz-Press et al. 2007[34].

source	no profile	partial profile	(almost) complete profile
	pg DNA		
Johns et al.		62,5	125
Joachimsson			62,5
Maintz-Press et al.	10	40	45

All studies agree about the number of cycles to be optimal at 30. The results in the pending case (max. 50% of the possible markers) find themselves in the zone between no profile and partial profile, leading to the conclusion, that the amount of template of Louwes' DNA would lie between 0 and 40 pg, or after recalculation, 0 to 7 molecules of the Y-chromosome. The content of the four samples in discussion would have amounted to a grand total of 20 molecules.
Remember, during 10 minutes a healthy speaking subject, on average expels more than 3000 ng DNA, so about half a million sets of DNA molecules (par. 7.4), as one set weights 6.6 pg. So including half a million Y-chromosomes.

8.3. Sources?

Nevertheless, how did this DNA pop up in over here? Of course, there are two separate answers to this question. There was DNA of the victim and there was DNA of Louwes.

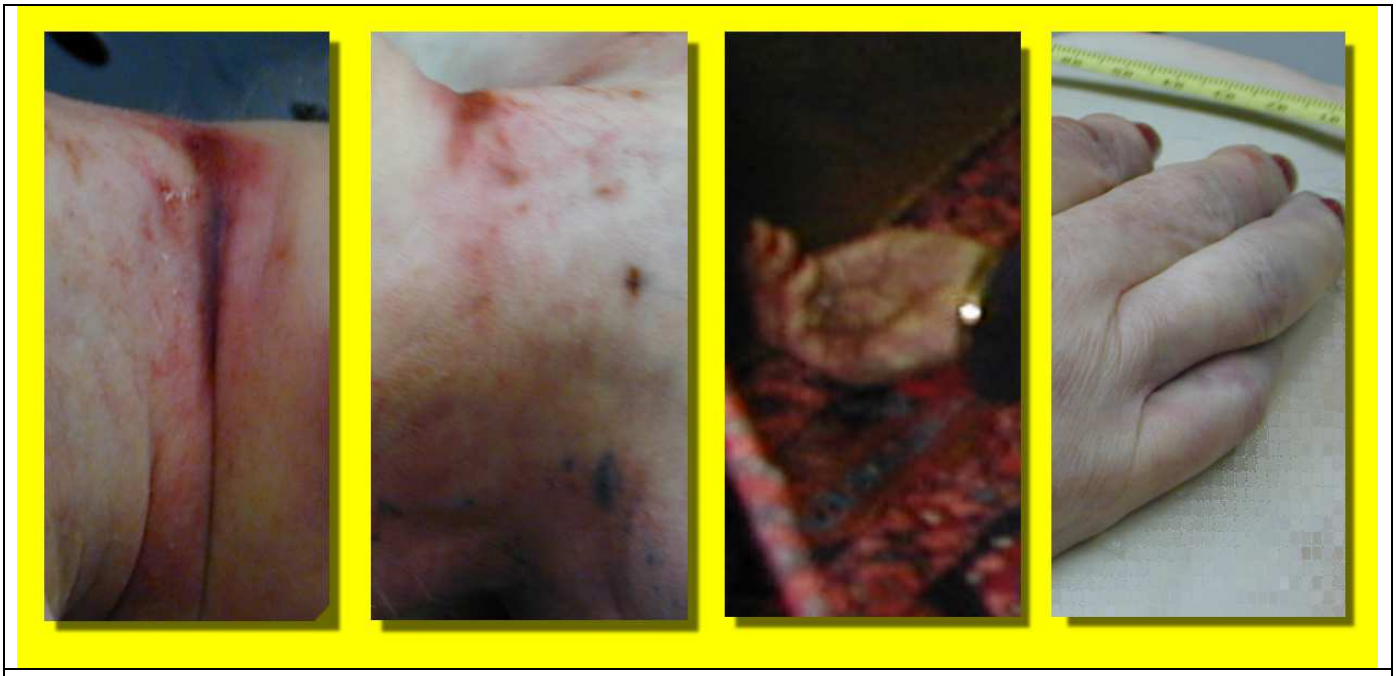


Figure 41. Left to right. Impression of the ligature at the front side, at the right side, the right hand at the crime scene and on the slab during section.

8.3.1. The autosomal profiles

At other occasions, I submitted evidence, leading to the conclusion, the victim was strangled, leading to her death. After the fact, her remains were displaced and handled by the perpetrator(s) and the crime scene investigators.

To evaluate the strangulation, you have to study pictures of the crime scene and the autopsy. Not very amusing but still very necessary. It is clearly visible, that the impression of the ligature is absent on the right side of the neck. This appears to be caused by a defensive action of the victim, forcing her right hand between neck and ligature. Hereby her right hand caught diagonal markings, as to be seen in the accompanying pictures, in particular her index and middle finger. At the neck, the action is visible in the form of scratch marks. For this reason, the load of the victim's DNA as measured by the Quantiblot method is particularly high. After the PCR, the high load of DNA is changed from right under en right upper to right under and left under, presumably by a slip of the pen (see also par. 8.1), one of many as demonstrated before.

8.3.2. The Y-chromosome profiles

Therefore, from this reconstruction follows that the victim only could defend herself from her attacker by using her left hand. If she (partially evidently) succeeded, there would be skin cells or blood of the attacker under the nails of the left hand, visible in the autosomal profiles. As already argued in par. 7.2, the attacker probably protected himself very effectively against transfer of substances. By the way, the nail clippings tested negative on blood.

As shown in figure 20, the condition of the blouse deteriorated notably between the condition at the crime scene and when photographed at the start of the autopsy. Bloodstains were proliferated on parts of the blouse, not in contact with the wounds at the body and many folds appeared, as if the blouse was taken off and subsequently put

on. Between the moment of taking crime scene pictures and the arrival at the autopsy, the blouse was fully unbuttoned (report autopsy and pictures taken at the mortuary). At the autopsy, the garment of the victim is rearranged. Possibly, the blouse was put on by the victim, or by the perpetrator. In any case, the blouse was taken off to start the autopsy. In the latter case, handling of the victim would be cumbersome as a consequence of settled rigor mortis, making it necessary to apply some force to pull the hands through the sleeves, including the parts attaching to the shoulder.

Figure 12. At left the blouse at noon September 25th 1999. At right in the evening of the same day in the mortuary. A new blood stain appeared after stretching the fabric and two buttons were loosened.

The defence of Louwes consisted of is claim, he visited mrs. Wittenberg in the morning of September 23rd , collecting a document. The nature of the document was such, that it would provoke a serious conversation between the two, with focus on that document. They were both standing. In line with those circumstances, we find DNA-traces of Louwes concentrated on the right shoulder of the blouse of mrs. Wittenberg. Figure 34 demonstrates the pattern in strength of the DNA-deposits in accordance with this reconstruction. Only one trace seems to deviate (sample #20) but this very sample was reported to be found at the *right* side of the blouse *twice*, before the NFI switched it to the *left* side in the ultimate report for unknown reasons (see figure 43).

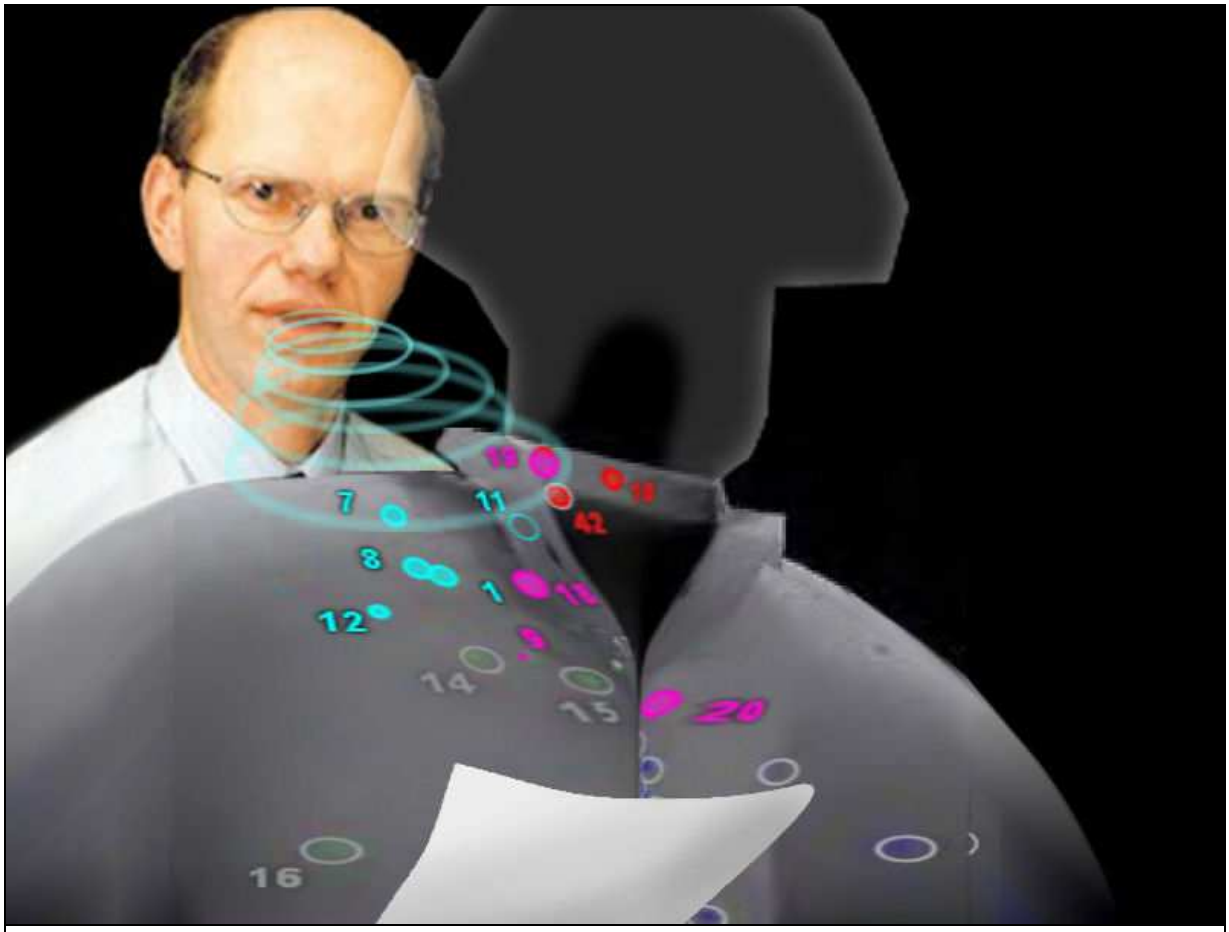


Figure 43. The defence of mr. Louwes includes DNA transfer by saliva on the right shoulder, when commenting on a document, he was about to archive.

In any case, figure 43 demonstrates the probability of a transfer to the right hand is higher by way of contact between hand and blouse, just as can be seen in figure 39 and the table in this chapter. As was shown before, the number of molecules, waiting to be transported in this way amounts to several hundreds of thousands. Only several dozens were enough to account for the measurements shown. Probably, some migrated during the sampling, if only one pair of scissors was used. In addition, during storage and transport. The circumstances of the actual nail clipping are utterly unclear. There are two separate police reports, each reporting another time and another place of the actual execution, both corresponding in the total lack of any detail.

9. References

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