

The DNA Y-STRs Profile of Louis XVI (1754-1793)

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Abstract: We have obtained, for the first time, the Y-STRs profile of the King of France Louis XVI (1754-1793). His genomic DNA was extracted from his authentic hairs, that were studied by optic and electronic microscopy. Louis XVI's Y-STRs profile is very similar to those of three living Bourbons previously published, differing from them by three Y-STRs allele values only. Dating estimates of the divergence time of the common ancestor (Louis XIII) between Louis XVI and the living Bourbons correspond to the observed genealogical time. K.W. Naundorff, the famous pretender, is certainly not a Louis XVI's natural son.

Keywords: Y-chromosome STRs profile ; Louis XVI ; dating of the divergence time since Louis XIII.

INTRODUCTION

In 1793, during the French Revolution, the King of France Louis XVI (1754-1793) and Queen Marie-Antoinette (1755-1793) were beheaded. Before their deaths, both the King and the Queen remained imprisoned in the Temple (in Paris). The only surviving son of Louis XVI and Marie-Antoinette, Louis-Charles (born in 1785), remained also imprisoned in the Temple where he survived to the death of his parents ; he (officially) died in the Temple in 1795.

So, the establishment of the Y-STRs profile (profile of the Y-Chromosome based on the DNA genetic markers named Short Tandem Repeats) of Louis XVI remains a difficult work, depending crucially on the authenticity of the starting material. The first attempt to obtain Louis XVI's Y-STRs profile (1) by the Lalueza-Fox group was quickly contradicted by a further study (2) based on Y-STRs profiles established on three living Bourbons (TLB) relatives of Louis XVI (Figure 1). Soon after that (3), Lalueza-Fox et al. recognized their error.

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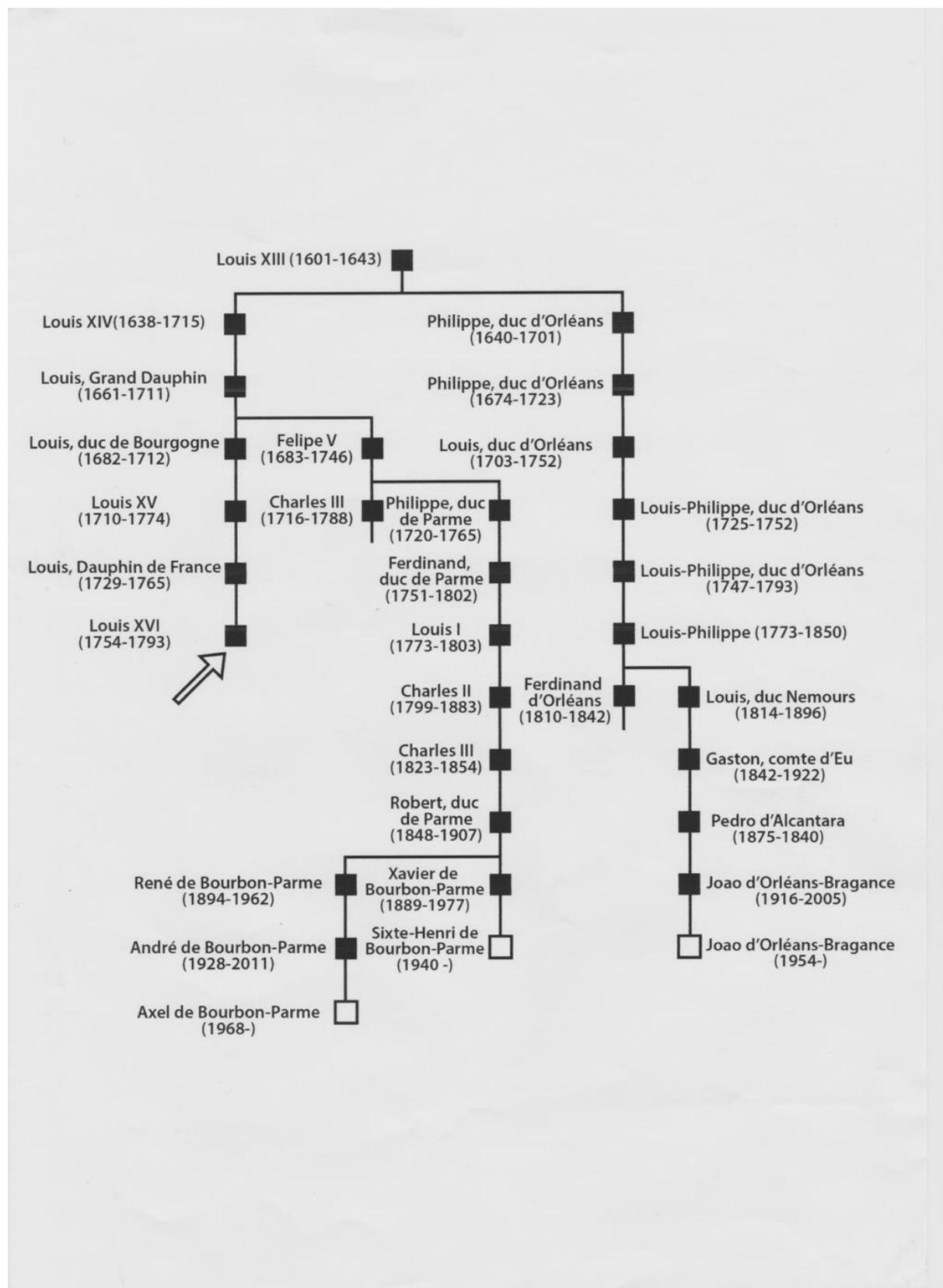


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Figure 1. A simplified masculine-genealogy of the Bourbons and relatives descending from the common male ancestor Louis XIII (1601-1643). The *propositus* Louis XVI is arrowed. The three living Bourbons (TLV) Axel de Bourbon-Parme, Sixte-Henri de Bourbon-Parme and Joao d’Orléans Bragance are represented as open squares.



But the real Y-STRs profile of Louis XVI remains unknown until now. In the present study we find the Y-STRs profile of Louis XVI, based on his authentic hairs ; the methodology used here is similar to the one we adopted recently (4) for the study of K.W.

Naundorff (1785?-1845), who was the most famous pretender to Louis XVI's throne. Table 1 summarizes datas about all Bourbons subjects and allied (and about the pretender) under study.

Table 1. The five Bourbons under study.

Names	Starting material	References
The three living Bourbons (TLB)		
. Axel de Bourbon-Parme, born in 1968 ; son of André de Bourbon-Parme (1928-2011).	Buccal epithelial cells	2
. Sixte-Henri de Bourbon-Parme, born in 1940 ; son of Xavier de Bourbon-Parme (1889-1977).	“	“
. Joao d'Orléans-Bragance, born in 1954 ; son of Joao d'Orléans-Bragance (1916-2005).	“	“
Karl Wilhelm Naundorff de Bourbon, (1785?-1845).	conserved hairs	4
Louis XVI (1754-1793) ; son of Louis, Dauphin de France (1729-1765)	“	present study

MATERIAL AND METHODS

The hairs

The lock of hairs (designated as number 2) is attached to a cardboard located in a glass-frame, comprising from top to bottom (Figure 2) : a drawing (designated as number 1) showing the left profiles of Louis XVI, Marie-Antoinette and the Dauphin ; a small envelope (designated as number 3) ; a folded portion of the tie (designated as number 4) ; a large envelope (designated as number 5) ; a red seal of sealing wax (designated as number 6) representing Louis XVI's right profile, and located (designated as number 7) on another portion of the tie.

The small envelope contained the lock of hair, because it is written on its surface : “*cheveus du Roi et de la reine*” (see Figure 20). The large envelope contained both the other portion of the tie and the small envelope, because it is written on its surface, on five lines : “*doné à toulan – cravate du Roi – Louis XVI – et cheveus du Roi – et de la reine.*”

The texts are drawn up in old french, because there is one n in the word *donné* (given) and a s, instead of a x, at the end of the word *cheveus* (hairs). In both texts the word *Roi* (King) is written with the letter r in capital, while the word *reine* (Queen) – that is not named – is written with the letter r in small letter ; that corresponds to some conventions used at that time.

Interestingly, we know that Toulan was a “Conventional”, who had the responsibility of the

surveillance of the members of the royal family during the imprisonment in the Temple ; he was very grown fond to the King and the Queen (and to the royal family in general) during this period.

Microscopy and elementary analysis

The hairs and other objects were examined and analysed in confocal stereoscopic micrography, and by SEM (Philips XL30 model, environmental version) – EDX (probe Bruker AXS energy dispersive X-ray ; PGT system analysis ; Spirit model, Princeton Gamma technology).

DNA extraction

Three cut portions of the brown hair number 1 containing dandruffs and the whole hair number 2 were submitted to DNA extraction to obtain genomic DNA. The genomic DNA of these hairs was extracted using a standard method (0.5M EDTA, sarcosyl 20% and proteinase K 10mg/ml), and purified using a commercial kit (NucleoSpin + Kit ; Macherey-Nagel, Duren, Germany) in accordance with the manufacturer's instructions (with some modifications)

Y-STRs amplification

From these DNAs, we amplified 15-Y-STRs by using the AmpFirst Identifier PCR amplification kit (Amp FIRSTY filer™, Applied Biosystems), according to the instructions given by the Company ; this amplification kit is specially adapted to the study of ancient DNA (a-DNA). The fifteen STRs studied are

the following : DYS19 (=DYS394), DYS385.a and .b, DYS389 I and II, DYS390 (=DYS708), DYS391, DYS392, DYS393 (= DYS395), DYS438, DYS439 (= Y-GATA-A4), DYS448, DYS456, DYS458 and DYS635 (= Y-GATA-C4) ; Y-GATA-H4 was detected in an independent reaction. To detect the longest STR alleles, we proceeded to three successive assays with progressive degrees of stringency.

The experimenter involved in molecular biology experimentations used protective clothing, sterile gloves and facemask, to prevent exogenous contamination. DNA extraction and purification were performed according to our previously protocol (5), in a dedicated laboratory. The laboratory performed DNA typing under strict precautions, following previously published criteria for a-DNA authentication (6). Only one of the authors of this paper (G.L.) proceeded to molecular biology experimentations, and his Y-STRs profile is known.

Dating

Time estimation for each chromosomal lineage was made using the ASD (Average Squared Distance) method of Goldstein et al. , 1995 (7) on the 15 Y-STRs (excluding DYS385. a and .b) ; this method is based on a strict single stepwise mutation model. The set of Y-STR mutation rates we applied in the estimations is that of Burgarella and Navascuès, 2011 (8). We used the generally accepted generation time of 25 years, that corresponds to that we observe in the tree (Figure 1), to produce a time estimate in years.

RESULTS

Figure 3 shows optical views of a cut up portion of the tie ; it is an “armure-simple” cloth, threads being disposed in the mode 1-2-1 relatively to weft threads. Both sorts of threads are composed of linen fibres, but there is evidence of some cotton fibres (Figure 4) disposed externally to linen threads. Morphologically, both sorts of fibres are easily distinguishable (Figure 5) : the linen fibres are cylindric, with nodes regularly spaced along the fibre length ; cotton fibres, flatter, are short with pointed extremities (and there are characteristic twists along the fibres). The same results were obtained for a cut up portion of the tissue (part 7, in Figure 2).

Figure 6 shows two photographs (in optical and in SEM) of the upper-left corner of the small envelope paper. As shown in Figure 7, this paper is a “papier-chiffon”, where we can distinguish linen and hemp fibres. This sort of luxurious paper was used in the past for the writing of official and fiduciary documents. The same results were obtained for a cut up portion of the large envelope (Figure 2).

Figure 8 shows optical views of two ink blots

(number 1 and 2), located on the top of the small envelope paper. Examined in retrodiffused SEM (Figure 9) the ink blot number 1 is constituted of a set of bright particles (this indicating that they are constituted of heavy elements). EDX analysis of one of them (Figure 10) shows that the ink is a “ferro-gallic” ink, iron sulphate particles added darkening again the blackness of the organic matter constituting the ink. This sort of ferro-gallic ink was commonly used in Europe, from the Middle-Age to the end of the 18th Century (and even after).

The hairs

One of the difficulties of such a study is to recognize hairs belonging to the King from the Queen’s hairs, because the lock of hair (Figure 2) contains both these hairs (as indicated : “*cheveux du Roi et de la reine*”).

So we have analysed separately blond hairs and brown hairs. Three blond hair portions (numbers 1, 2 and 3) are represented on the optical photography of Figure 11 ; in the enlarged photography of hair number 3, we can observe (by transparency) the medullar canal of the hair. All the blond hairs observed are cut at both extremities. Figure 12 shows a SEM photography of the extremity of the of the blond hair number 3 ; its elementary composition is characteristic of one hair (C : carbon, and O : oxygen represent the organic matter of the hair; S: sulphur peak corresponds to the keratin; Cl: chloride and Na: sodium correspond to sweat salt). The mean feature of blond hairs is that they are very clean: we can observe only very few deposit particles at the scale surface; so they correspond to well prepared washed and cleaned hairs. Figure 13 shows residual particles of the “black soap” potassium chloride that was used in the cleaning process.

Three brown hairs (numbers 1,2 and 3) are represented on the optical photography of Figure 14. All the brown hairs observed are also cut at both extremities.

Both extremities of the cut brown hair number 1 are shown on the SEM photographs of figure 15. A surface portion of brown hair number 1 is shown on the MEB photography of Figure 16. We can distinguish at this surface a great number of mineral deposits particles (from D1 to D17); the mean feature of brown hairs-contrary to blond hairs-is that they are “dirty”.

Numerous deposits of particles can be observed at the surface of hair number 1 (figure 17): they can correspond to modern deposits of synthetic fibres, spores and even acari egg-layings, or mineral deposits of calcium carbonate (Figure 17’ spectrum).

Among the mineral (and metallic) deposits shown on Figure 17, EDX spectrum analysis (figure 18) establishes that one particle (number10) is a clay, another one (number 9) is a cement, and a last one (number 8) is a titanium dioxide metallic micro-plaque.

The SEM photography of a surface portion of hair number 1 (Figure19) shows a dandruff. We can observe a total of five dandruff particles along the surface of hair number 1.

Table 2 gives the main features (observations based on 10 hairs of each category) of blond and brown hairs.

Table 2: Characteristic features of blond and brown hairs.

	Blond hairs	Brown hairs
Cut at both extremities	all	all
Mean thickness in the middle part	62.4 ±19.6µ	91.9 ±13.4µ
General aspect	clean	dirty
Dandruffs	five	no

We can attribute blond hairs to Marie-Antoinette, for the following reasons: 1/ We know that her hairs were of blond (or red-blond) colour. 2/ The blond hairs are finer hairs than brown hairs (mean thickness of 62.4 µ *versus* 91.9 µ, table 3); in general at comparable ages, woman hairs are finer than men's. 3/ Carefully washed and cleaned, Marie- Antoinette hairs were probably conserved previously in an envelope (or in a medallion).

obtain mitochondrial-DNA (mt-DNA) only (**11**) ; but it is possible to obtain some large amounts of genomic DNA from dandruffs joined to hairs (**12**).

The Y-STRs profile of brown hairs

And we can attribute brown hairs to Louis XVI ; they were cut during his imprisonment in the Temple (“cheveux de coiffeur”). We know that, at this time, his hairs were brown (and bleach). It is secondarily that the two sorts of hairs were mixed, in the lock of hair under study (Figure 2).

It is the same DNA profile (Table 3) that was obtained, repeatedly, for genomic DNA extracted from the three cut portions of the hair number one and from the hair number 2.

Table 3. Allele values of Louis XVI for 16 STRs, compared to those of the three living Bourbons already published (allele values of Louis XVI that differ from those of the three living Bourbons are in italic). Allele values of Naundorff that differ from those of Louis XVI are in italic.

It is possible to extract genomic DNA from hairs with bulbs (**5, 10**). On cut hairs, it is relatively easy to

Numbers	Y-STRs	Y-STRs profile of Louis XVI	Y-STRs profiles of the three Bourbons	Y-STRs profile of Naundorff
1	DYS19	14	14	14
2	[DYS385.a DYS385.b	11	11	11
3		14	14	14
4	[DYS389.I DYS389.b	14	13-14	13
5		16	16	16
6	DYS390	23	23	24
7	DYS391	11	10	12
8	DYS392	13	13	13
9	DYS393	13	13	13
10	DYS438	12	12	12
11	Y-GATA-A4	13	12	12
12	DYS448	19	19	18?
13	DYS456	16	17	15
14	DYS458	18	18	18
15	Y-GATA-C4	?	23	23
16	Y-GATA-H4	12	12	10

Results about amplification of the amelogenin gene show that the man with these hairs is XY. For a total of 15 sites compared (DYS19, DYS385.a and .b, DYS389. I and .b, DYS390, DYS391, DYS392, DYS393, DYS438, Y-GATA-A4, DYS448, DYS456, DYS458 and Y-GATA-H4), the Y-STRs profile of the man bearing brown hairs is greatly similar to those of the TLB : identical allele values are found for 12 of these (a percentage homology of 12/15 = 80%). This profile differs from the Y-STRs profiles of the TLB for three Y-STR allele values only : allele values at DYS391 of 11 *versus* 10, at Y-GATA-A4 of 13 *versus* 12, and at DYS456 of 16 *versus* 17 : moreover these three are one-step mutation only, in the *plus* sense for DYS391 and for Y-GATA-A4, and in the *minus* sense for DYS456.

So we conclude that the Louis XVI Y-STRs profile is perfectly compatible to that of the common Bourbon Y-STRs profile previously established (2) ; allele Y-

STR values that differ between the two are easily explained by accumulation during time of single-step mutations since the common ancestor Louis XIII (see Figure 1) between the two lineages. The Louis XVI Y-haplotype, submitted to the Wit Athey's Haplogroup Predictor test (9), had more than 95% of chance to be of the sub-haplogroup R1b.

Dating

Because there is a time gap between Louis XVI and the three living relatives, we calculated the ages based on the genetic datas of the three living Bourbons and they plus Louis XVI, respectively (Table 4) ; the TLV and Louis XVI trace their common ancestor to Louis XIII (1601-1643).

Table 4. Estimations of the TMRCA (Time of the Most Recent Common Ancestor), in years (ya), based on the two approaches.

Clusters	TMRCA (ya)	95% CI
The three living Bourbons (TLB)	220	426
TLB plus Louis XVI	405	441

The former can trace common ancestor to 220 ya by using genealogical mutation rates, and the latter accordingly gives 405 ya. So, the two approaches give similar TMRCA times, the second being relatively more conform to the real time estimate (Figure 1).

DISCUSSION

We have established in the present study, based on his hairs, the Y-STRs profile of Louis XVI. The first problem to take in consideration is that of the authenticity (and the traceability) of the hairs examined.

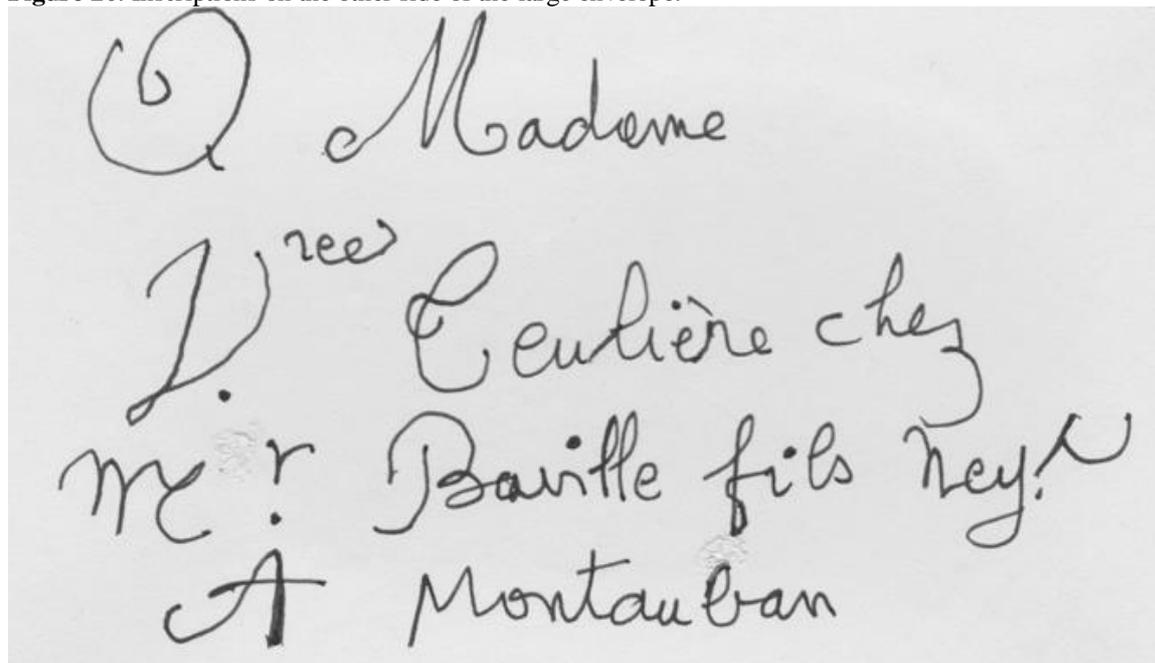
The small envelope (object 3, Figure 2) contained hairs. We showed here that the paper constituting the envelope is a "papier-chiffon" (a special sort of paper used in the past for the official documents) and that the ink used for the writing is a "ferro-gallic" ink (commonly used at that time). The large envelope (object 5, Figure 2) contained both the tie fragment (object 7, Figure 2) and the small envelope.

On the apparent side of the large envelope (of the same paper as the small envelope) is indicated (same

writing as the small envelope, and same ink) both origins of the tie and of the hairs. Toulan is explicitly designated as the second donator ("doné à toulan...") of both the tie and the hairs. We know (13) that the bookseller François-Adrien Toulan (1761-1794), Conventional but supporter of the royal family members (Madame Elisabeth, the King's sister, called him "Fidèle"), broke the seals appended to the last Louis XVI objects (a ring, his hairs and the seal that he intended to give to his son), retrieved these rests and handed them to Marie-Antoinette. The Queen will be further successful in getting these precious relics out of the Temple, and gave them to the Count of Provence and to the Count d'Artois (both the King 's brothers).

We depict on Figure 20 the writing located at the other side of the large envelope paper. It is in fact a letter-paper, where the name and the address of the last addresser ("Madame Teulière, à Montauban") were written. After the lock of hair was left out to the small envelope and was sewn to the cardboard located in the glass-frame, to constitute the object 2 (Figure 2).

Figure 20. Inscriptions on the other side of the large envelope.



The second difficulty surmounted concerns the parting between Louis XVI and Marie-Antoinette 's hair. Probably the mixture between the two sorts of hairs was realized by Toulan himself : he had Louis XVI's hair, and he probably obtained Marie-Antoinette's hair by another way. Hair mixture in a lock between hair coming from two affectively linked persons is not an uncommon procedure used to make these sorts of relics ; for instance we had recently access (Lucotte, unpublished) to a peculiar relic where hair from the Dauphin and the one from his sister Madame Royale were mixed together.

We have separated Louis XVI's hair from the one of Marie-Antoinette on the basis (Table 2) of colour, thickness and general aspect. Contrary to Louis XVI's hair, Marie-Antoinette's blond hair was carefully washed, cleaned and prepared; it was kept separately from Louis XVI 's hair , and it is in a second time that both sorts of hair was mixed.

Both sorts of hairs are cut-hairs, that excludes to obtain genomic DNA from them. Fortunately, because Louis XVI hair are "unprepared hair" ; their surfaces are literally covered by numerous deposit particles. It is the dandruffs that adhere to these hairs that allows us to obtain Louis XVI 's Y-STR profile.

Louis XVI 's Y-STR profile obtained here (Table 3) is greatly similar to those of the TLV previously studied (2) : it differs from them by three allele values only, at the Y-STRs DYS391, Y-GATA-A4 and DYS456 (alleles 11 instead of 10, 13 instead of 12, and 16 instead of 17, respectively) ; moreover, these three variant values of Louis XVI's Y-STRs profile differ each from those of the TLV by one-step mutation only. This allows us to estimate securely the

divergence time (Table 4) between the two main branches (the one that goes to Louis XVI , and the one that goes to TLV), time (in ya) that corresponds to the observed genealogical time (Figure 1).

We ignore for the moment what is the Y-STRs profile of the common ancestor of these two branches, Louis XIII. But, in a similar manner to the one we have used for Napoléon's Y-STRs reconstruction profile (14) from those of his living direct descendant and of descendants of two of his brothers, we can predict-by application of the parsimony principle- that Louis XVI's Y-STRs profile includes the non-variable allele values of 14 for DYS19, 11 and 14 for DYS385.a and .b, 16 for DYS389.b, 23 for DYS390, 13 for DYS392 and for DYS393, 12 for DYS438, 19 for DYS448, 18 for DYS458, and 12 for Y-GATA-H4. But we cannot predict with certainty what is the ancestral form for variable Y-STRs DYS391, Y-GATA-A4 and DYS456, nor for DYS389.I (which had an allele value of 14 – identical to that of Louis XVI – for Jao d'Orléans –Bragance, instead of 13 for the two other living Bourbons).

For a total number of fifteen Y-STRs allele values compared (Table 3), Karl Wilhelm Naundorff 's Y-STRs profile (4) differs from the one of Louis XVI by seven of them : allele values of 13 (instead of 14) for DYS389.I, of 24 (instead of 23) for DYS390, of 12 (instead of 11) for DYS391, of 18 (instead of 19) for DYS448, of 15 (instead of 16) for DYS456, and for 10 (instead of 12) for Y-GATA-H4. So Naundorff is certainly not a Louis XVI's natural son.

To conclude, based on the study of his authentic

hairs, we have established the Y-STRs profile of the French King Louis XVI. This profile is similar to those of three living Bourbons previously published. Estimation of the divergence time between Louis XVI and these three other Bourbons, starting at their common ancestor Louis XIII, is in accordance to the genealogical time observed. We move now to the detection of the Dauphin's Y-STRs profile.

List of abbreviations

a-DNA : ancient DNA ; ASD : Average Squared Distance ; PCR : Polymerase Chain Reaction ; SEM-EDX : Scanning Electronic Microscopy-Energy Dispersive X-rays ; STRs : Short Tandem Repeats ; TLB : the Three Living Bourbons ; TMRCA : Time of the Most Recent Common Ancestor ; Y : Y-chromosome.

Acknowledgments

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Figure 2. A photography showing the lock of hair position (indicated by number 2) in the glass-frame.



Figure 3. Optical photographs of a cut up portion of the tie. Above (x10) : disposition of the threads in the cloth. Below (x100) : details of fibres (F) composing threads (T).

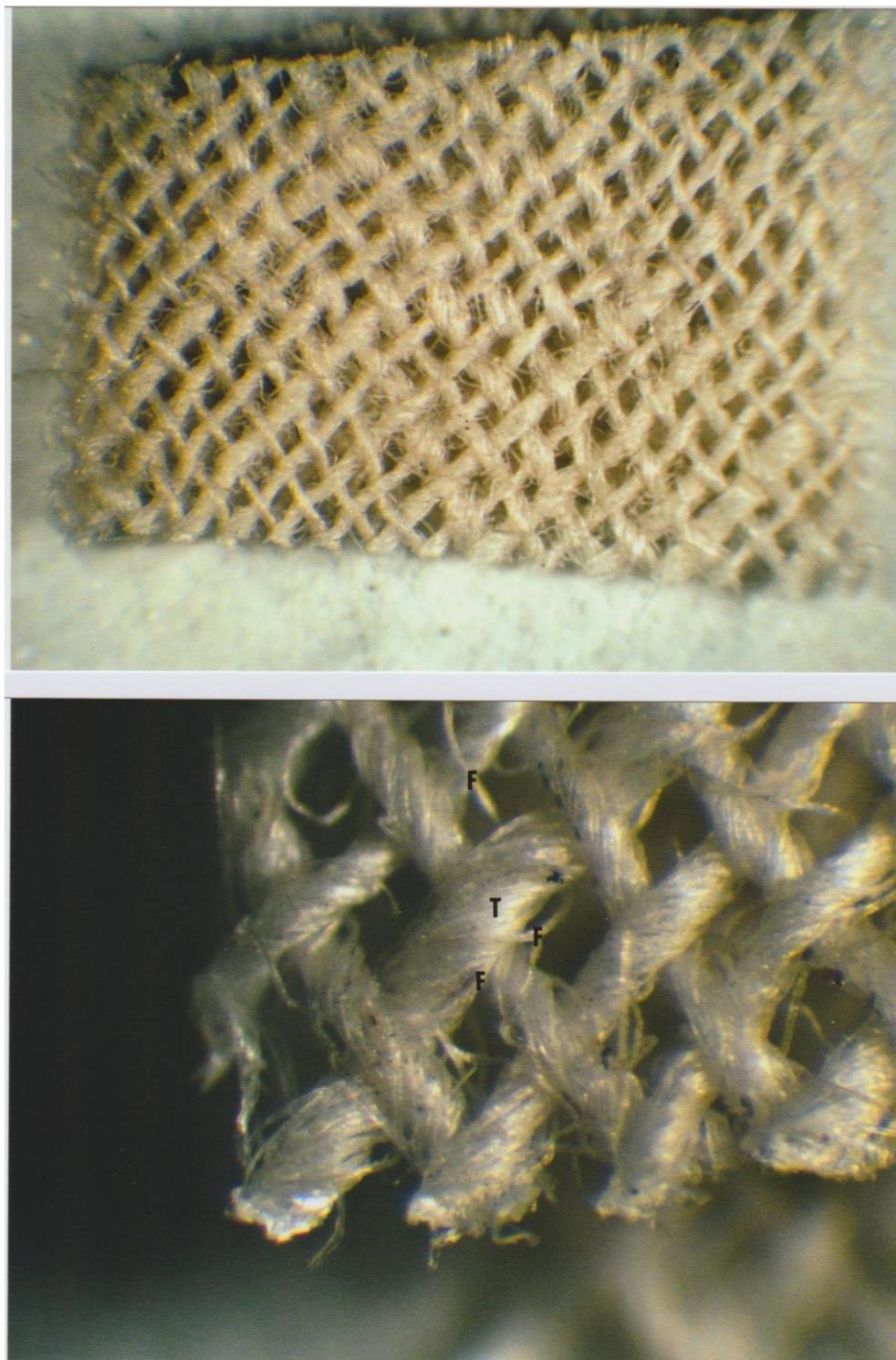


Figure 4 : SEM photographs of a portion of the tie. Above (x50) : dispositions of the linen threads (comprising 30-50 linen fibres) in the cloth ; some fibres (indicated by arrow points) are disposed at the exterior of linen threads. Below (x200) : details of an exterior fibre, showing twists (T).

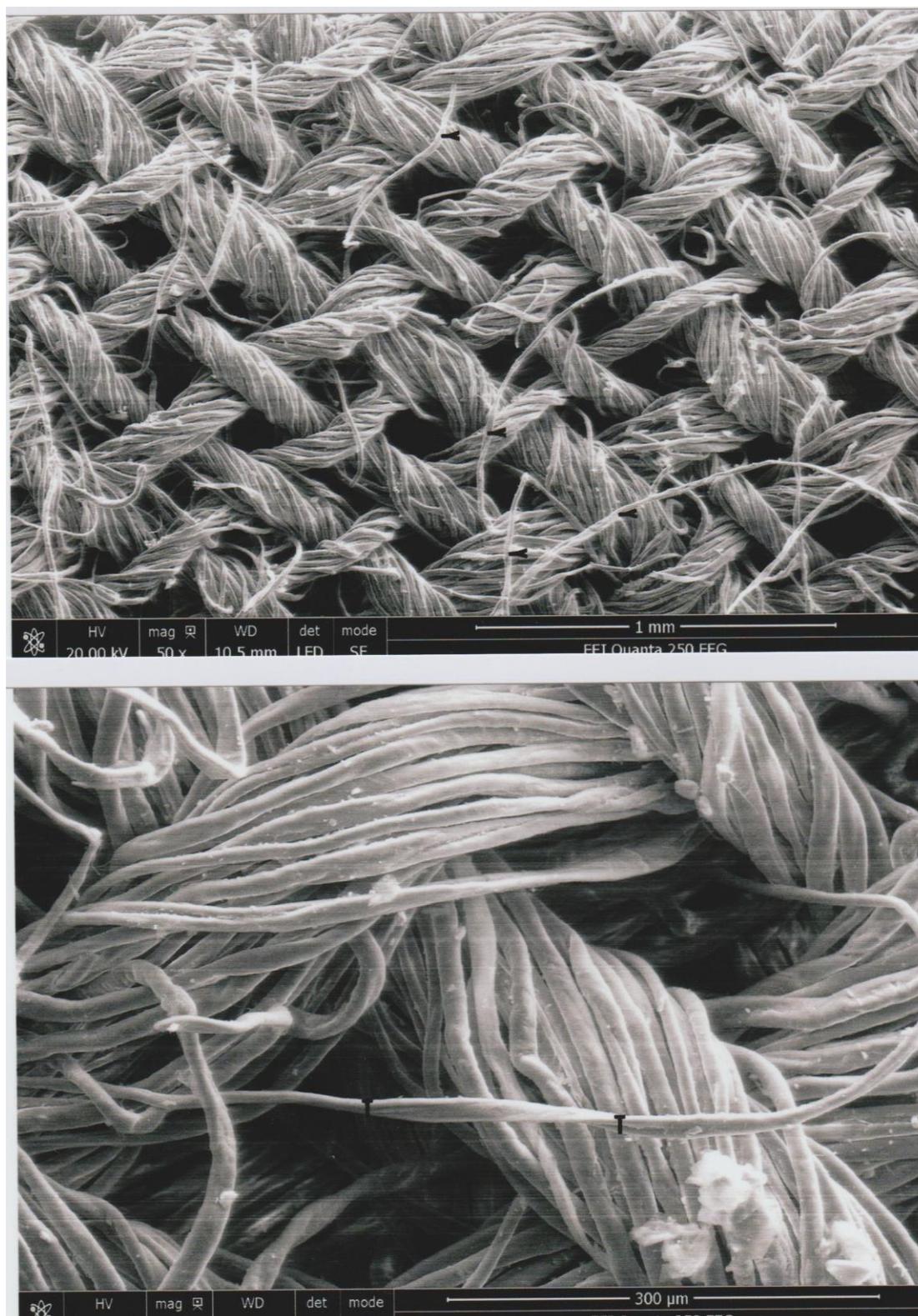


Figure 5 : SEM photographs showing the distinction between linen fibres of the threads and cotton fibres exterior to the threads. Above (x400) : details of a linen fibre (indicated by an arrow point) showing nodes (N). Below (x200) : details of cotton fibres (numbers 1, 2 and 3) showing twists (T).

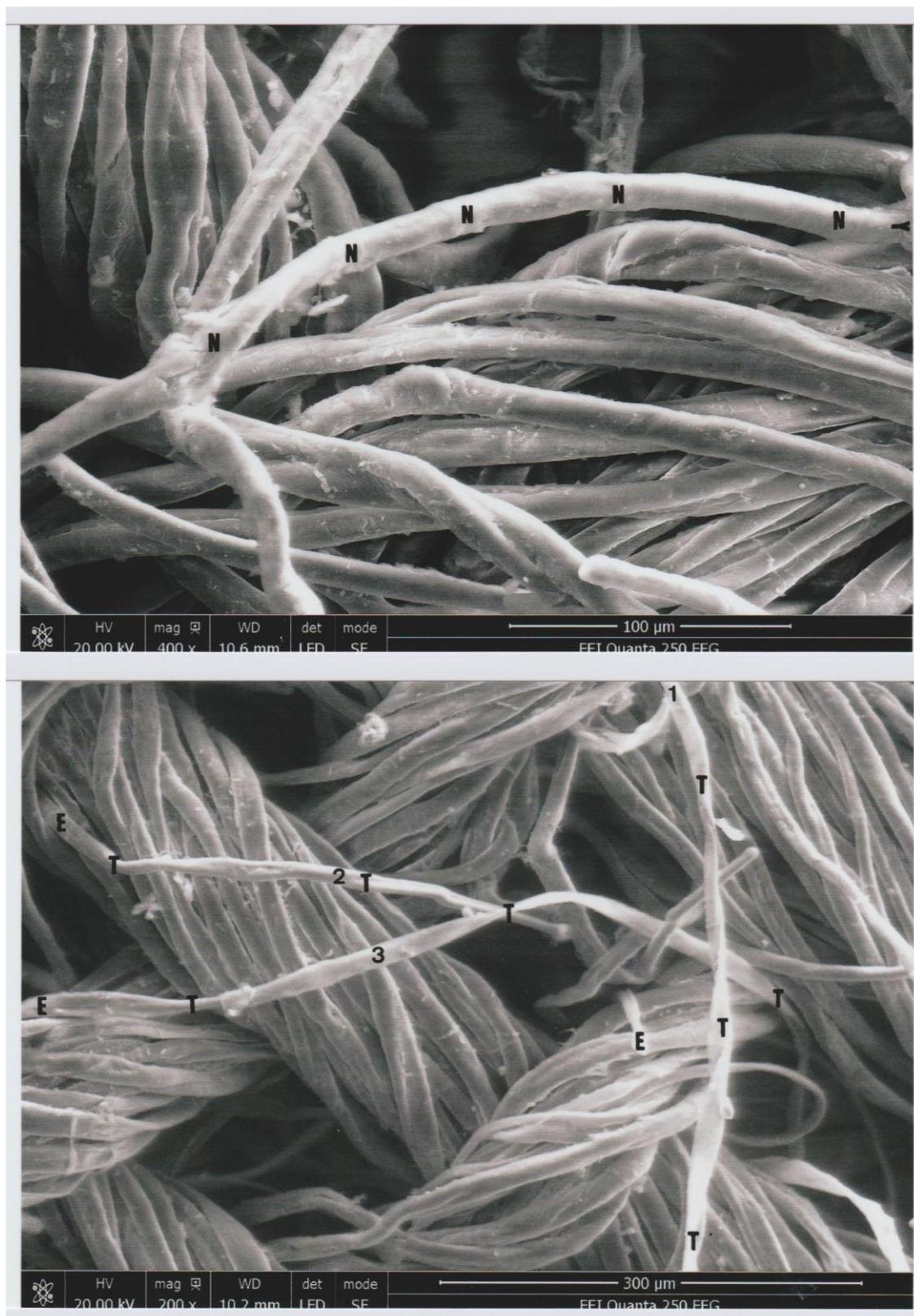


Figure 6 : Photographs of the upper left corner of the small envelope paper. Above (x200) : optical photography. Below (x200) : SEM photography showing fibres.

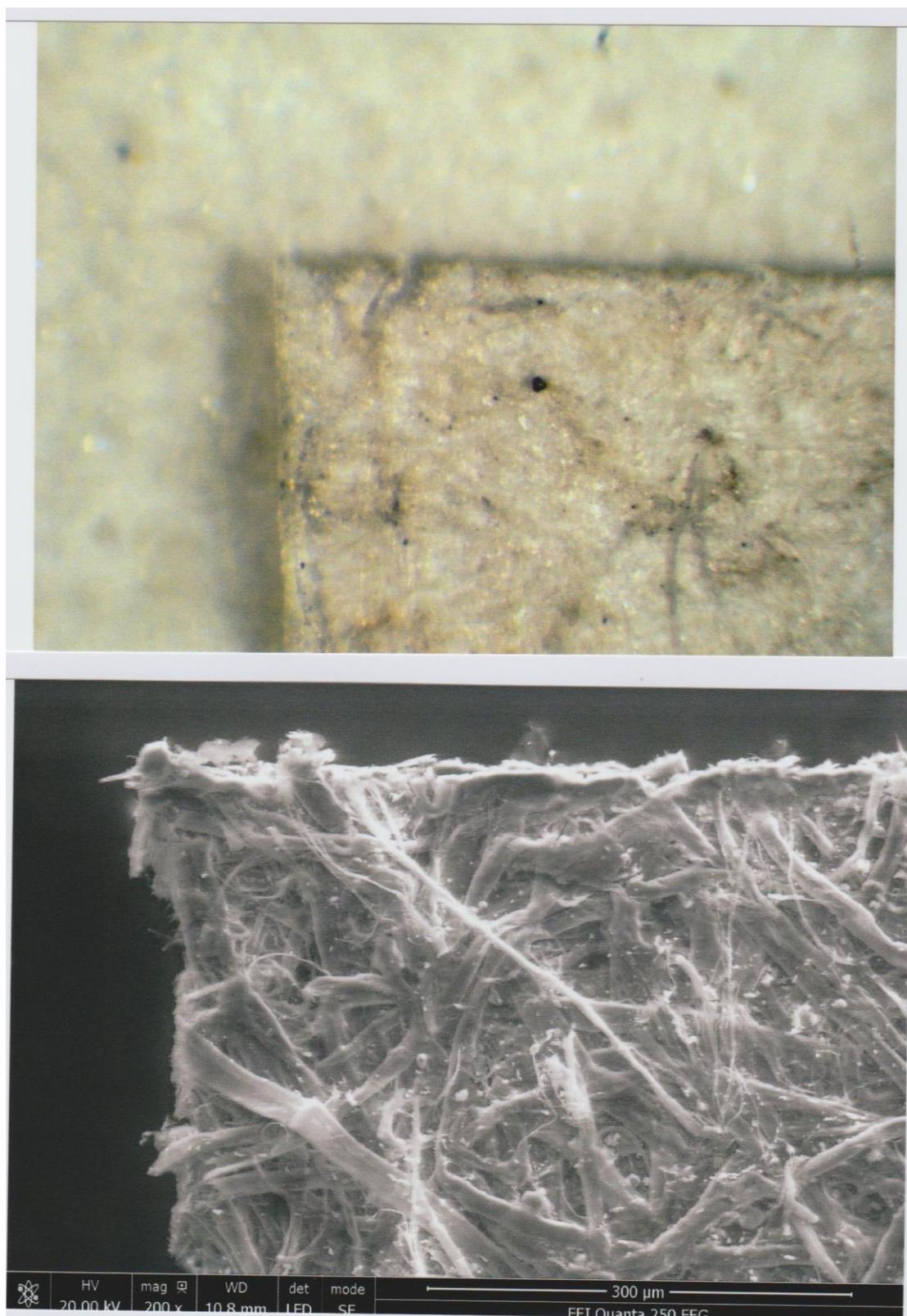


Figure 7 : SEM photographs showing the details of some paper fibres. Above (x800) : a portion of a hemp fibre (thickness in μ). Below (x1500) : a portion of a linen fibre (thickness and distance between two nodes in μ).

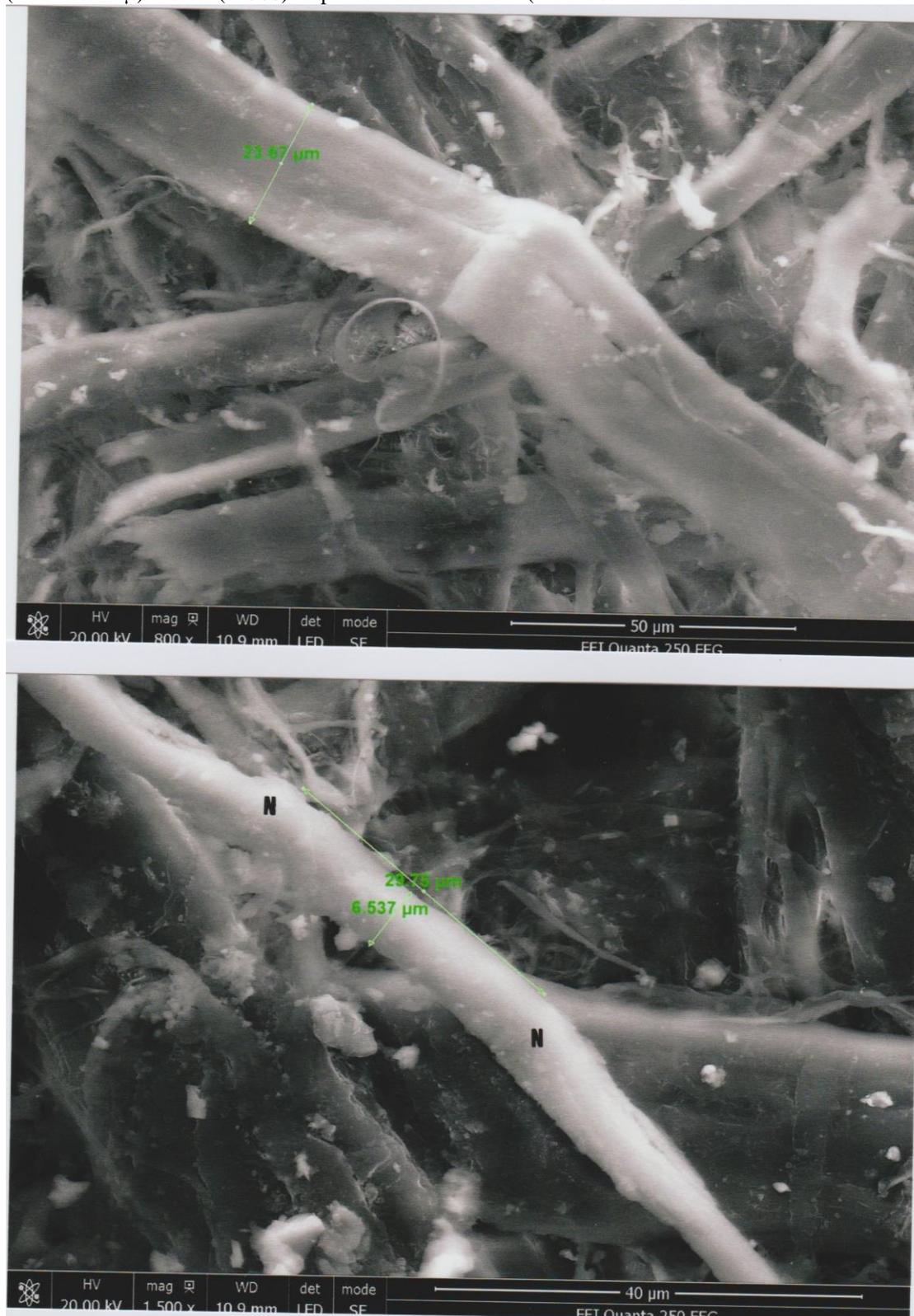


Figure 8 : Optical photographs showing two ink blots (1 and 2) on the surface of the paper (P). Above (x5). Below (x20) ; PM1 and PM2 are two triangular plastic marks.

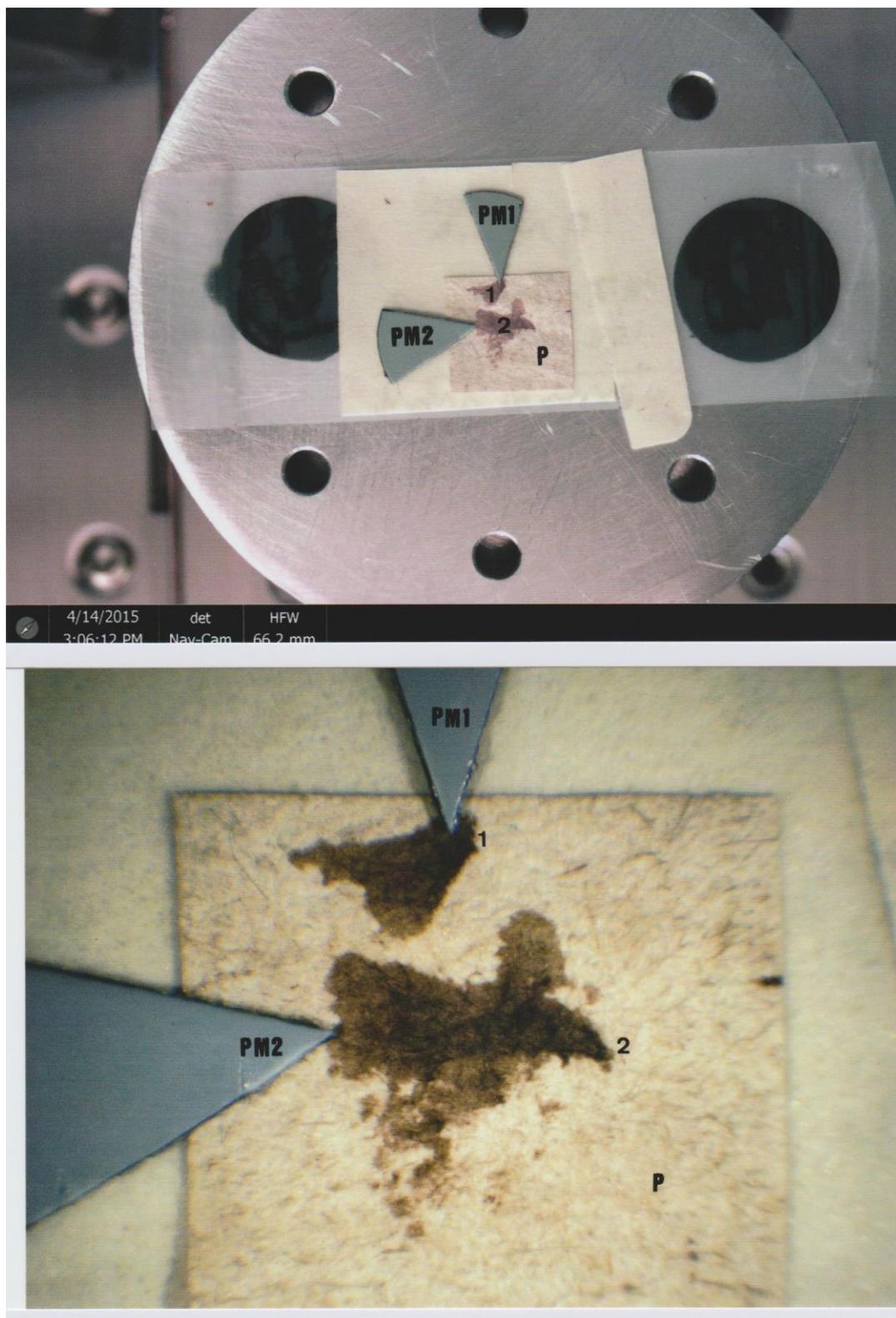


Figure 9. SEM photographs (in retrodiffusion) showing the ink blot 1. Above (x25) : ink blot 1 under the plastic mark PM1. Below (x200) : details in the ink blot ; the little black spot indicates the particle enlarged in the following figure.

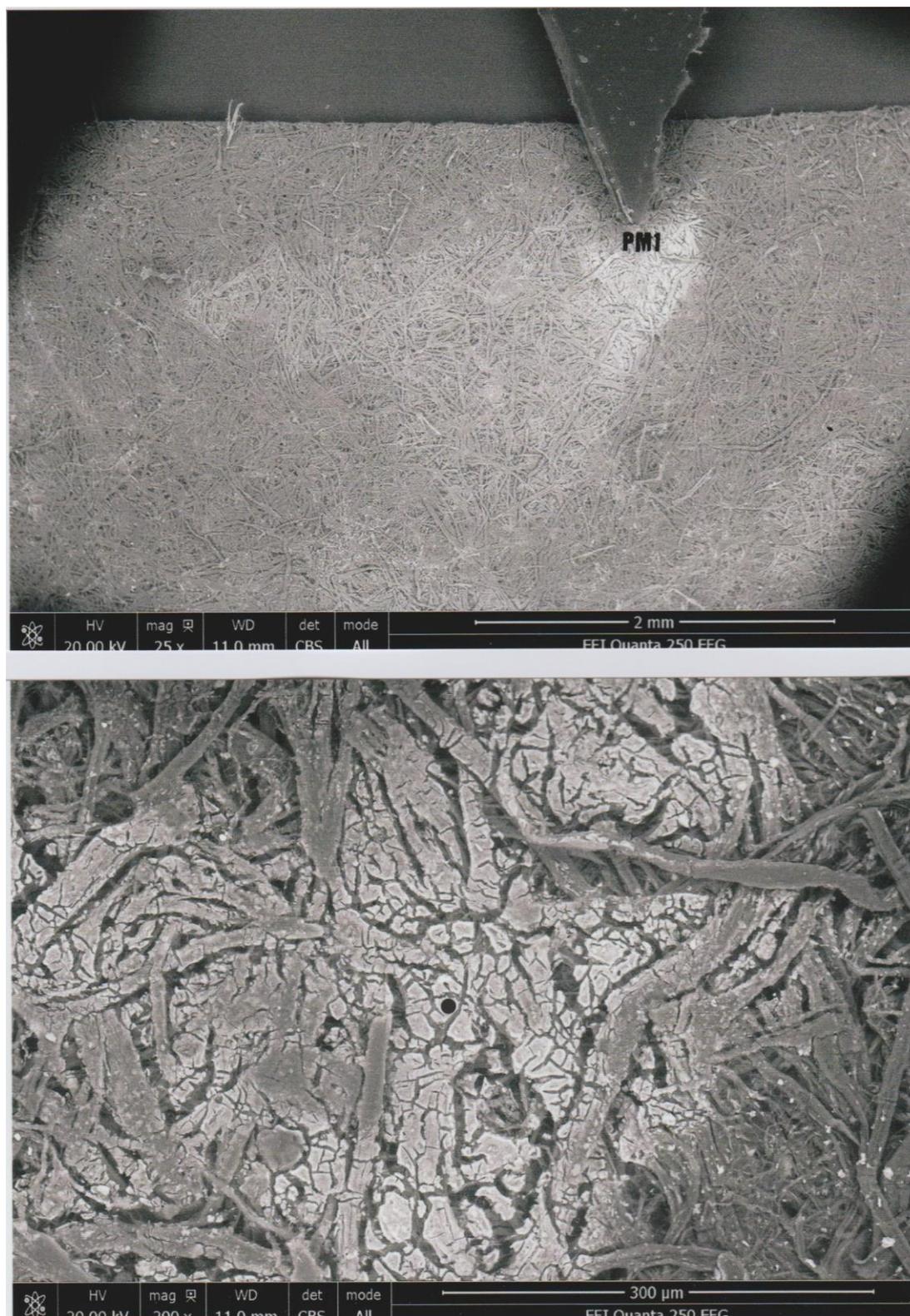


Figure 10. Above : a SEM photograph (x3 000) showing the particle indicated in the previous photography. Below : elementary analysis, by EDX, of the particle at the black spot indicated. On the spectrum, both sulphur(S) and iron (Fe) represent the two predominant peaks ; together, they allied to form iron sulphate.

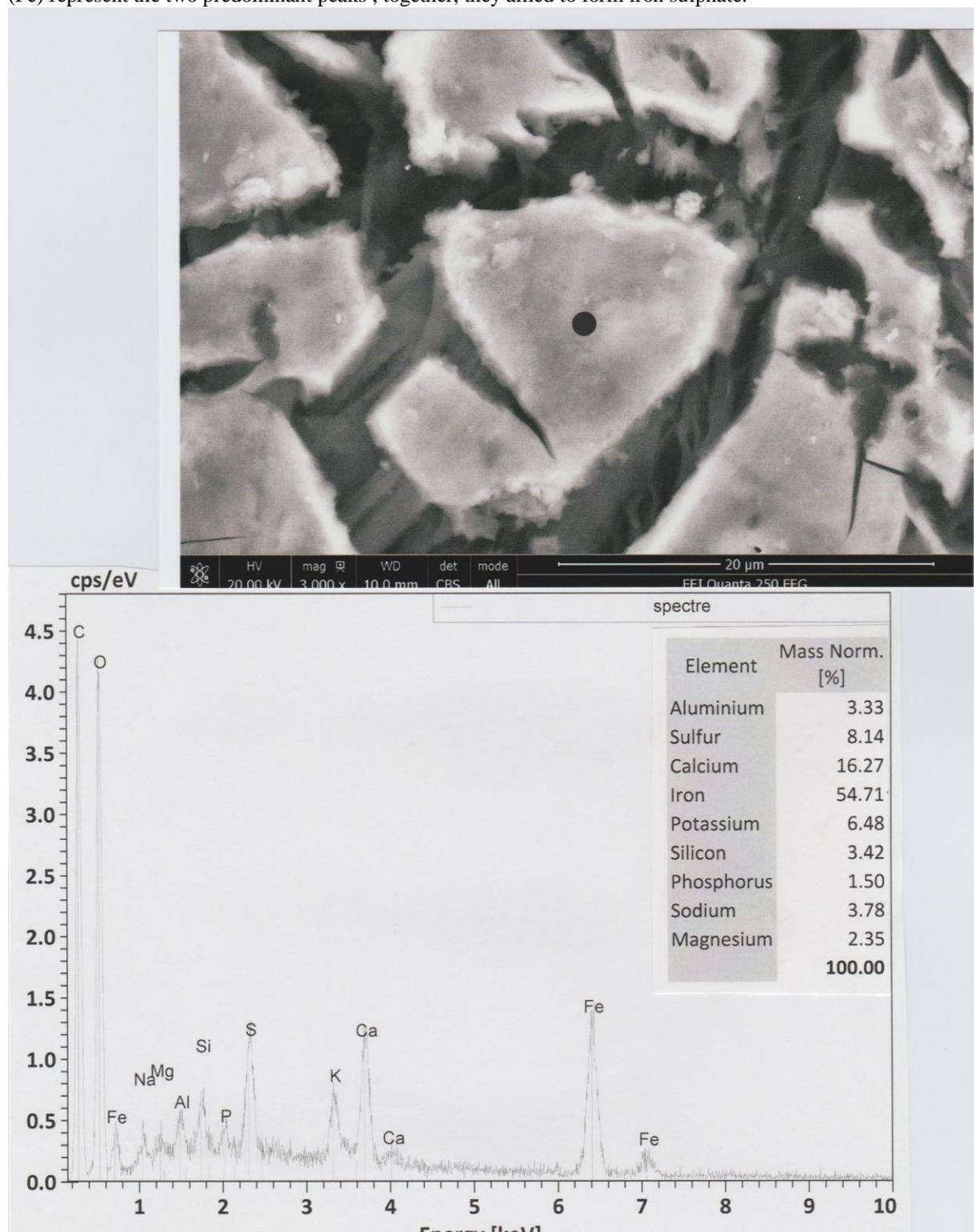


Figure 11. Optical photographs of the blond hairs. Above (x10) : three portions of blond hairs numbers 1, 2 and 3. Below (x50) : an enlarged portion of bond hair number 3 (the two dashes at the extremities indicate the medullar canal).

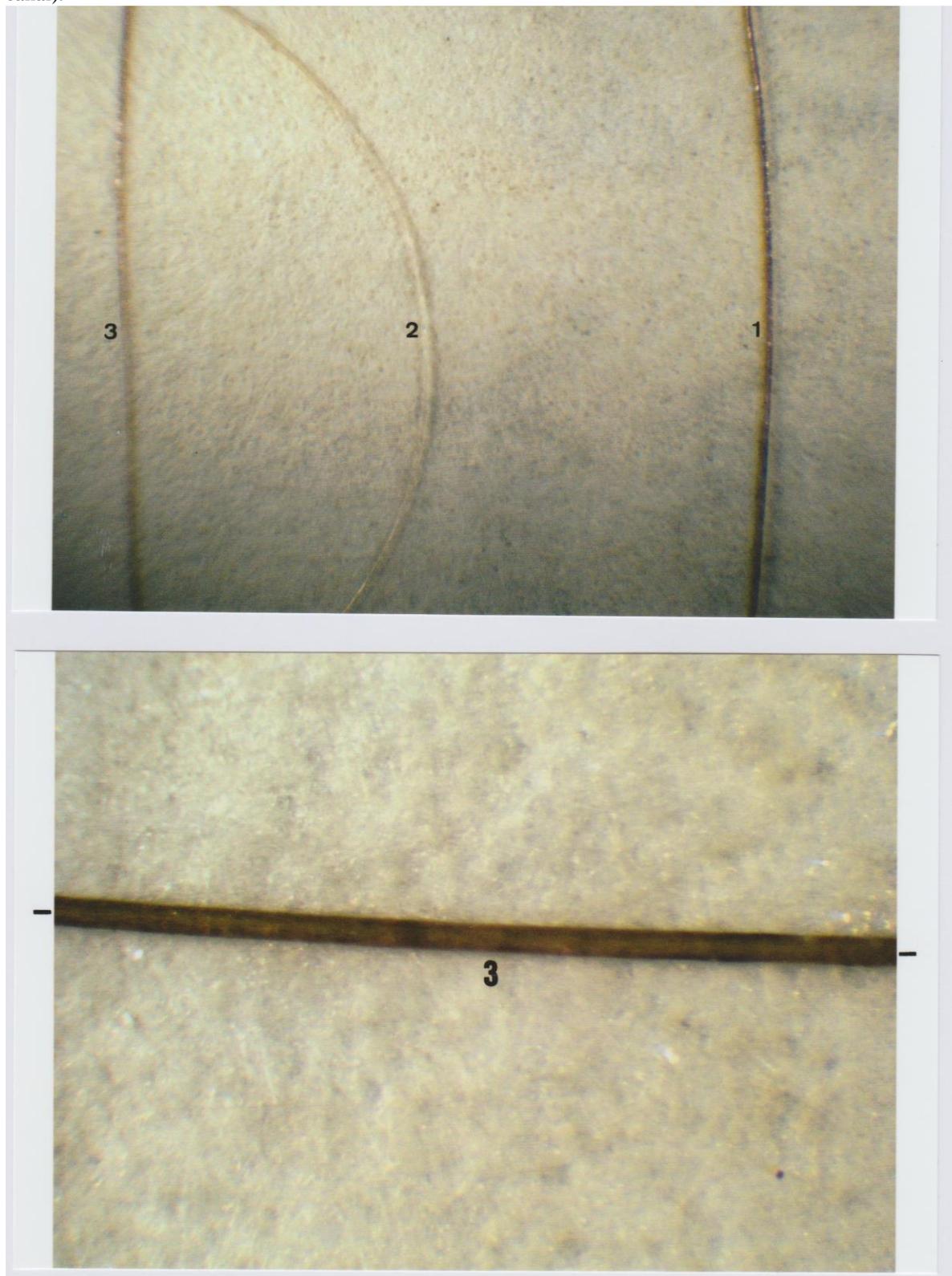


Figure 12. The blond hair number 3 extremity. Above : a SEM (x1200) photograph of the extremity (thickness in μ). Below : EDX spectrum at the black dot.

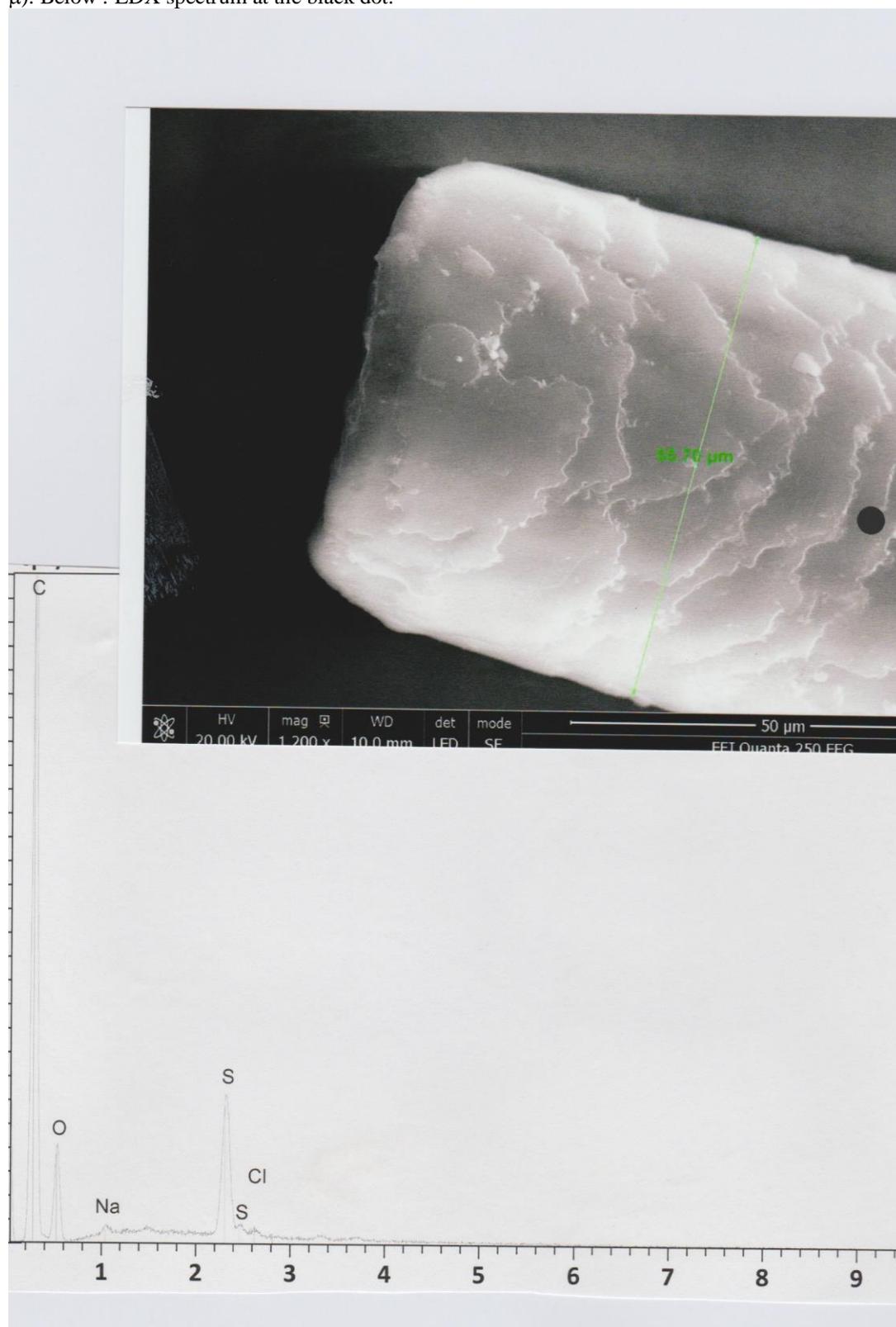


Figure 13. Examples of black soap particles on the blond hair number 3 surface. Above : a SEM (x2000) photography, showing particles 1-5 ; S is a detached scale. Below : spectrum of particles 1-5. Chloride (Cl) and potassium (K) are among the two predominant peaks of the spectrum ; together they form potassium chlorure.

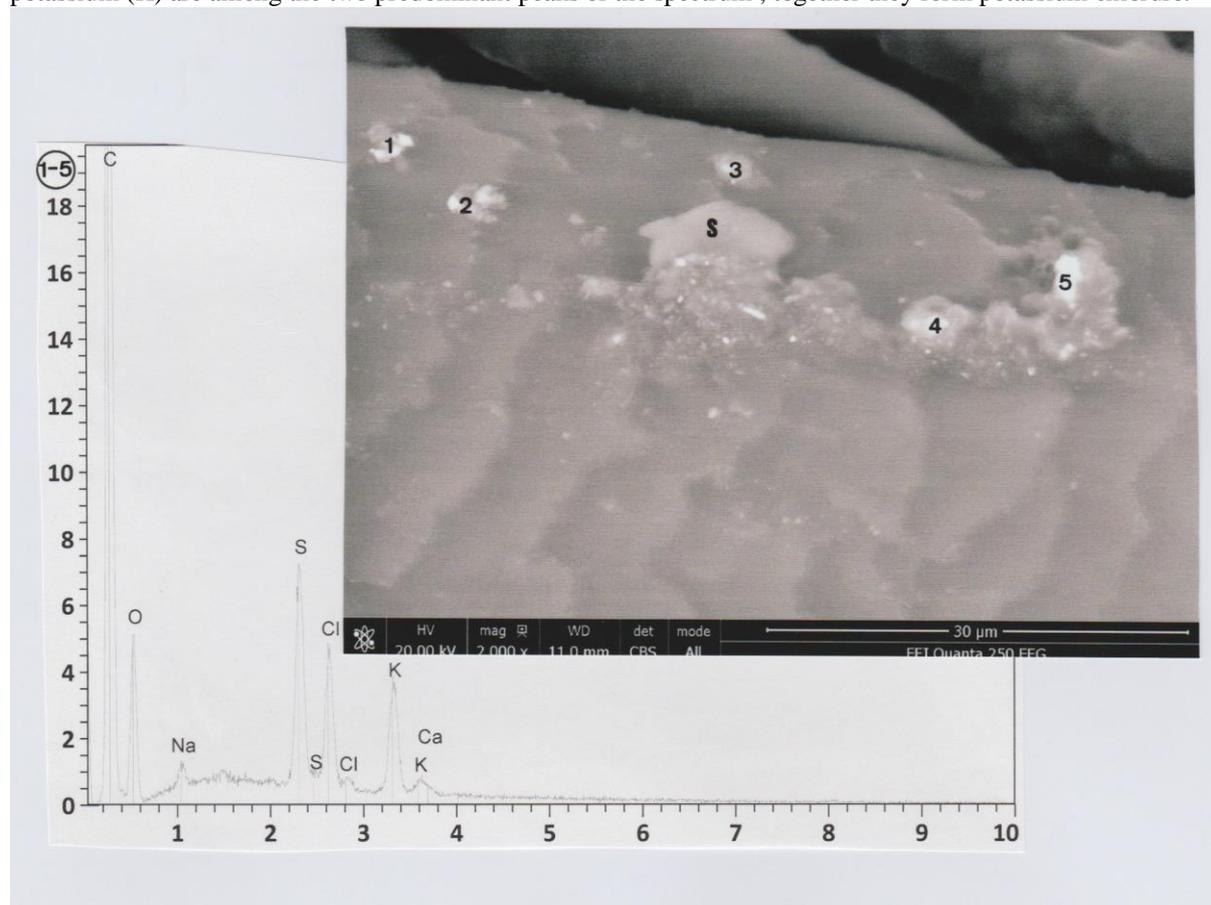


Figure 14. An optical view (x5) of three brown hairs numbers 1-3.

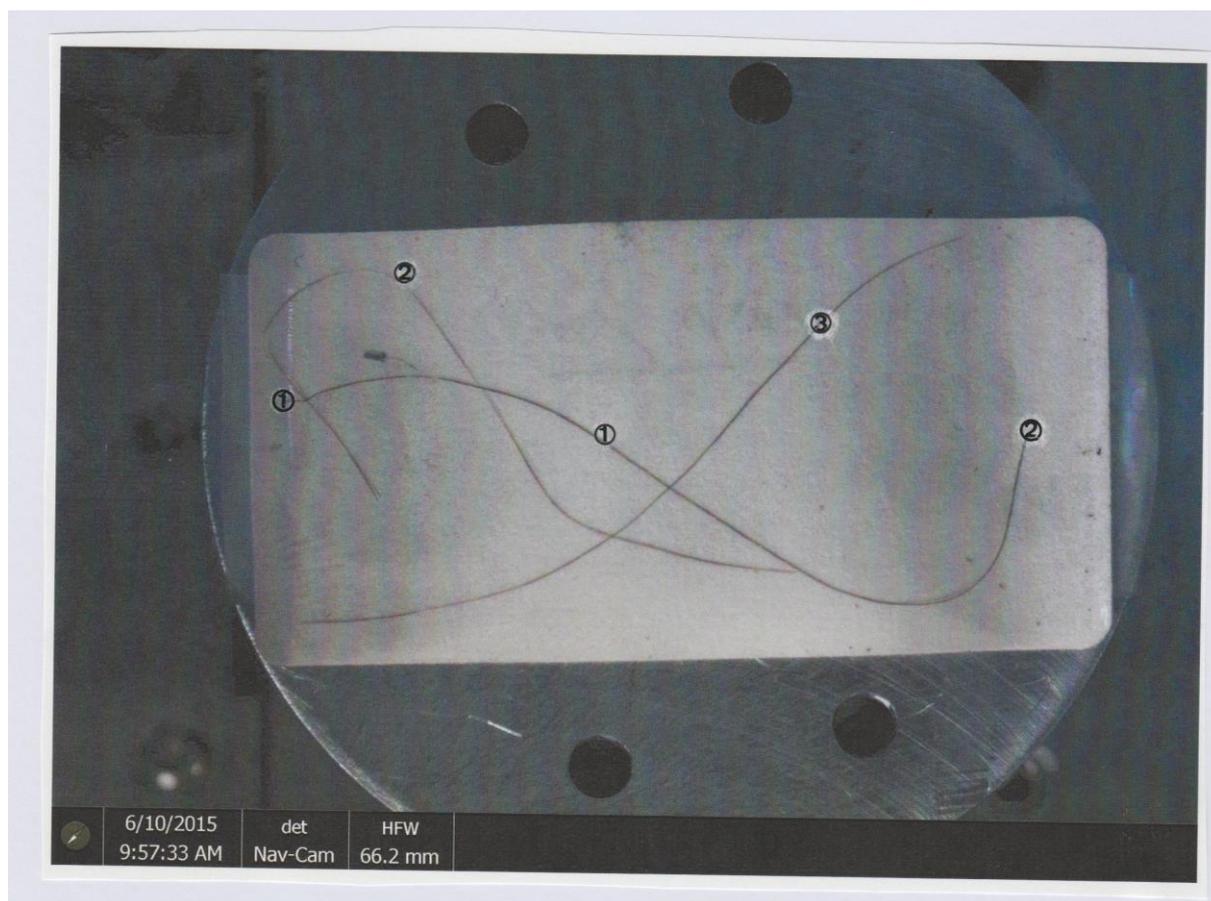


Figure 15. SEM photographs of cut number 1 extremities. Above : (x600) : the right extremity. Below (x1000) : the left extremity (thickness in μ).

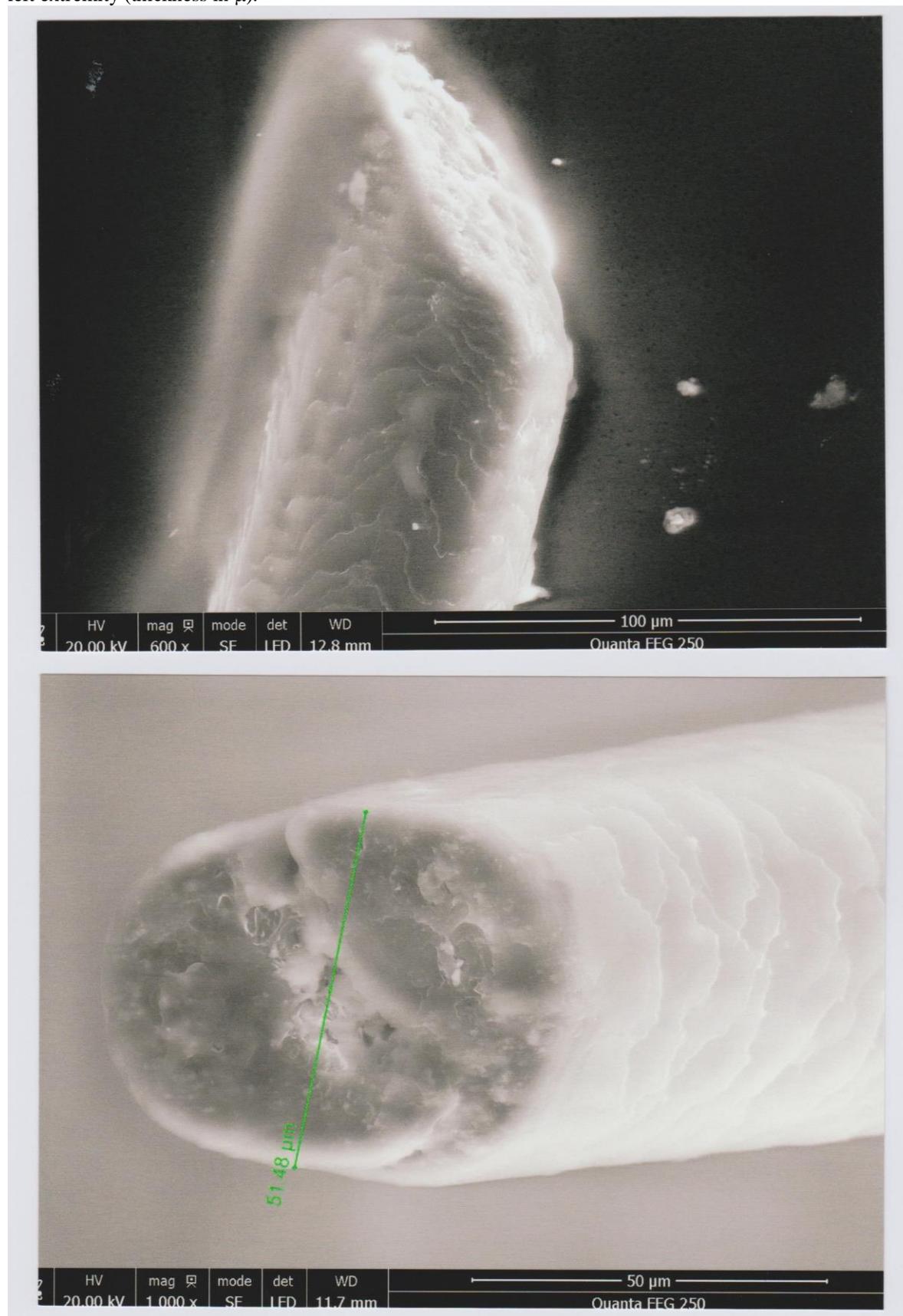


Figure 16. A portion of the hair number 1 surface. Above : SEM (x600) photography of the portion of the hair surface (thickness in μ) ; D1 to D17 indicate deposits at the hair surface. Below : EDX spectrum of hair number 1, taken at the black point (a surface of the scales without deposit particles) indicated.

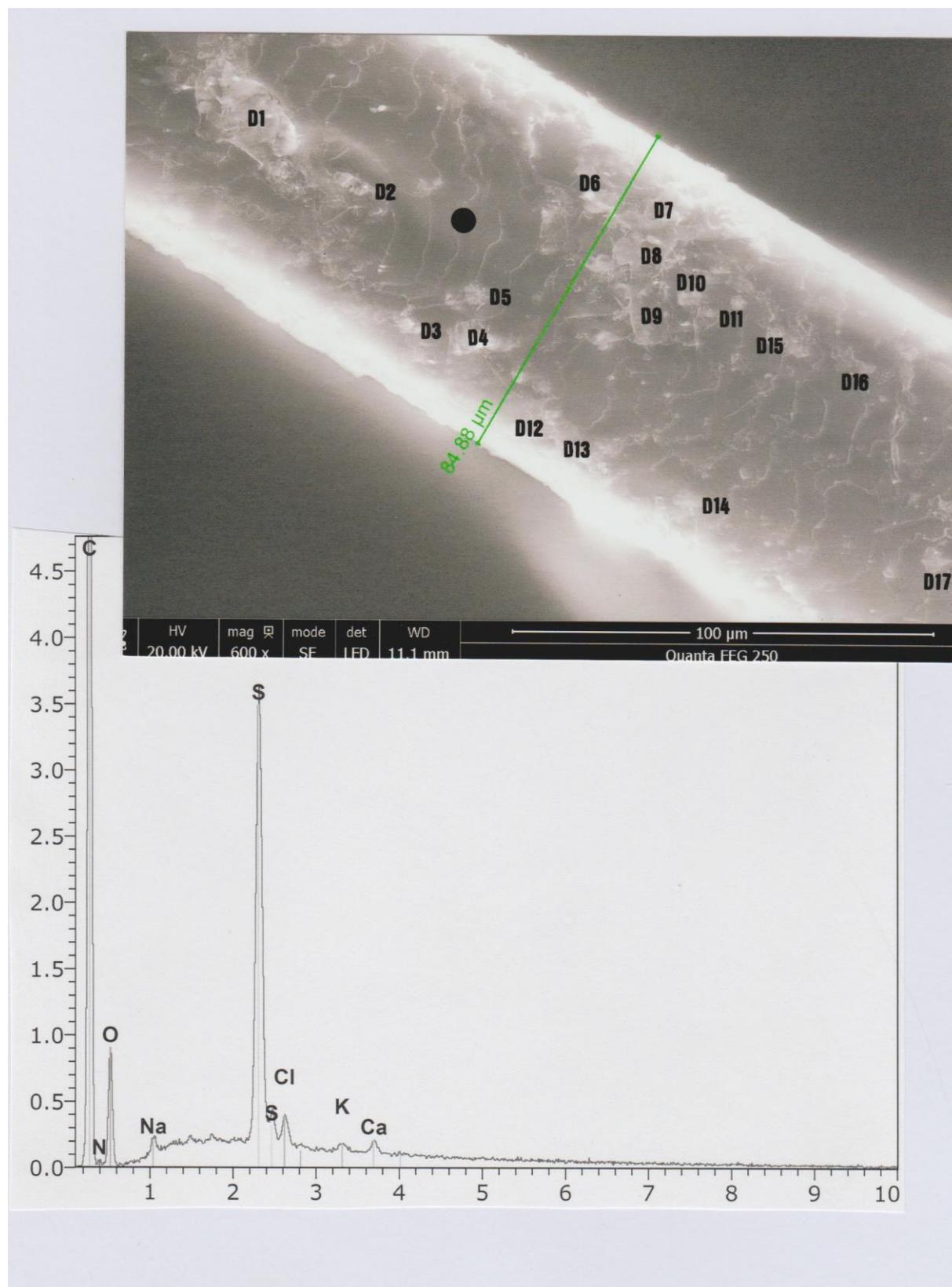


Figure 17. A portion of the hair number 1 surface, enlarged. Below : SEM (x 2400) photography of this portion (p : an acari egg-laying ; F : a synthetic fibre ; s : a modern spore) ; particles numbers 1 to 10 are indicated. Above : spectrum of particle number 3.

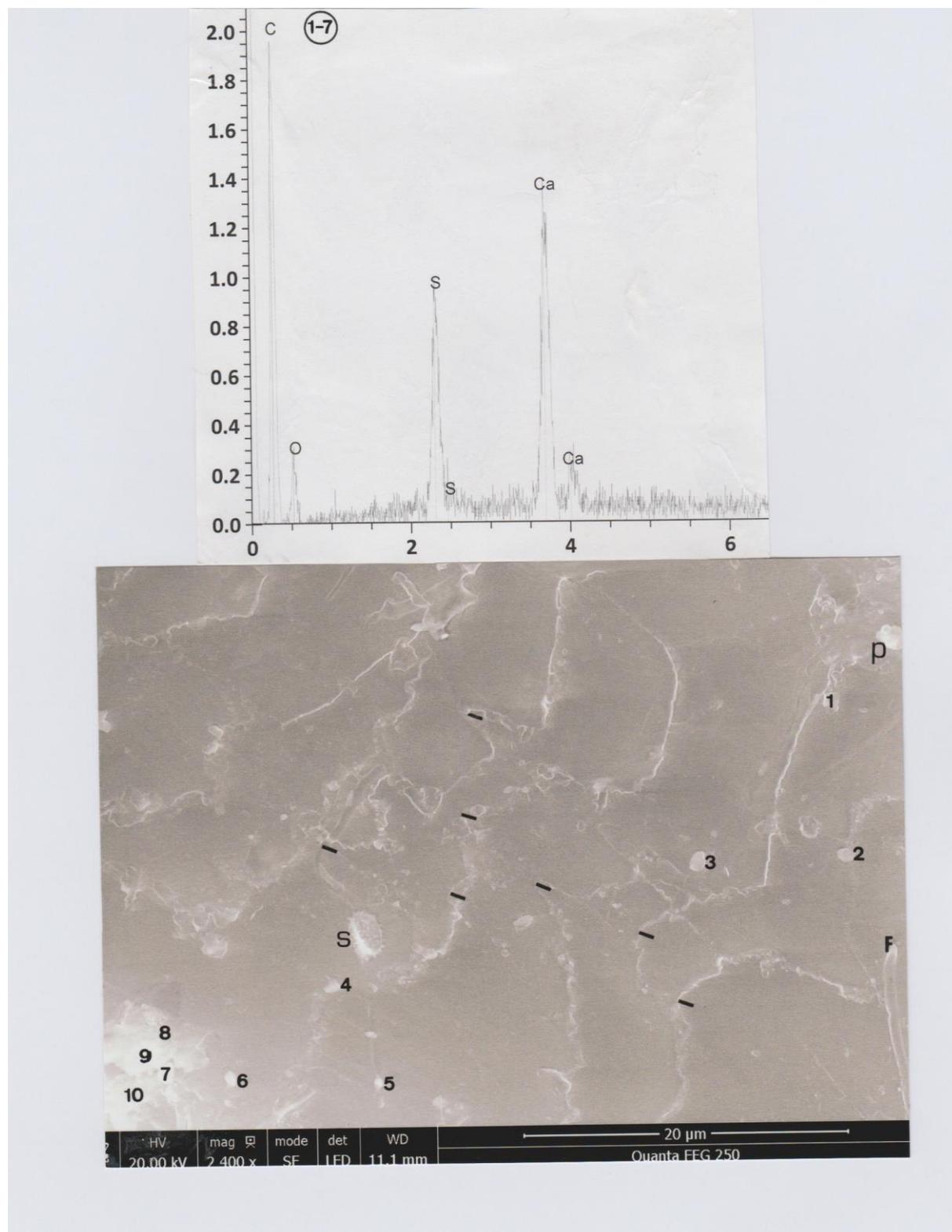


Figure 18. EDX spectrums of particles numbers 8, 9 and 10.

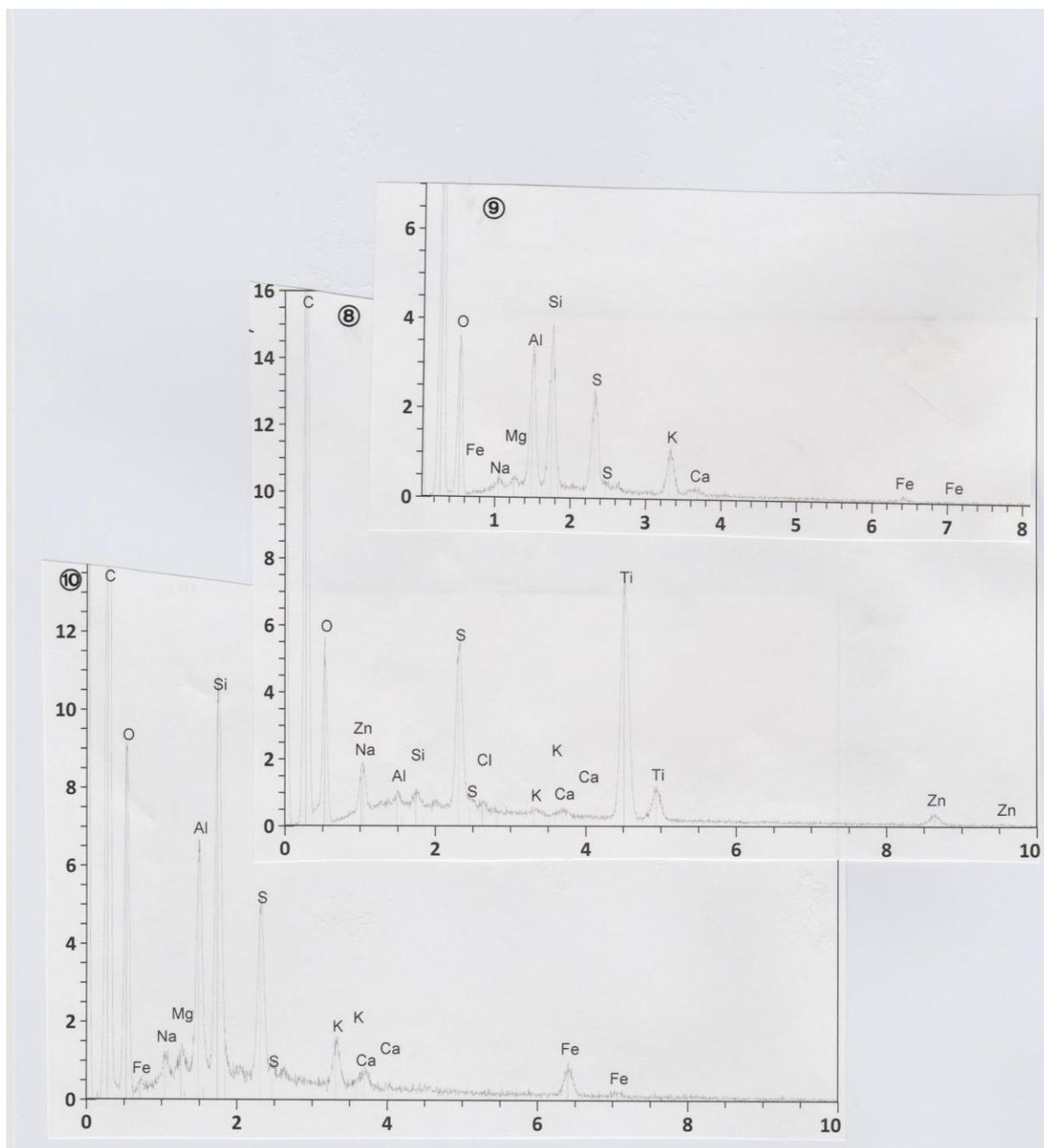


Figure 19. An example of a dandruff on the hair number 1 surface. Above : SEM (x1200) photography of the dandruff. Below : dandruff spectrum at the black point indicated. Compared to that of scale, the dandruff spectrum is relatively poor in S (sulphur) and relatively rich in organic material (C : carbon, N : nitrogen and O : oxygen).

