Research Article

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The Napoléon Mutation 16184T is that Found in the HVS1 Sequence of the mtDNA Extracted from an Eyebrow Included in the Plaster of the Antommarchi Death Mask of Napoléon

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Abstract : We have extracted the mtDNA (mitochondrial DNA) from one eyebrow included in the plaster of the Antommarchi death-mask of Napoléon. The corresponding HVS1 (Hypervariable Sequence number 1) contains only one mutation, named 16184T, that is that of the Napoléon family (reference 2). That proves, in accordance with some previously established plaster particularities of the eyebrow part of the mask (reference 1) showing that it originates from the St. Helena island, that the Antommarchi death-mask of Napoléon is the nearest historical mask of the real death-mask of Napoléon.

Keywords : Napoléon, Antommarchi Death-mask of Napoléon, Plaster of the Mask, Eyebrows, mtDNA, HVS1 Sequence, 16184T Mutation

An eyebrow was taken from the left eyebrow of the Antommarchi death-mask of Napoléon (**Figure 1**). This mask, kept in the Archives of the Bois-Préau Castle since the 15th of December 1944, is now (since December 2017) exhibited to the public in the Museum of Malmaison (near Paris, in France). Careful examination of the plaster of this mask (1) shows that it is in fact of a compound nature (a sort of mosaic structure) ; it is made up of two sorts of plasters : that of the Paris plaster, and that of a lessquality plaster originating from the gypsum of the St-Helena island.

DNA was extracted from this eyebrow, to verify (2) that it is well an eyebrow of Napoléon.

MATERIAL AND METHODS.

Plaster of the mask at the left eyebrow represented on the photograph of figure 1 was scratched with a sterile Gillette blade and then transferred to a dedicated sticky paper (SP); it is at the occasion of this transfer that we observed an eyebrow fragment on the SP. SP and the eyebrow were first observed in optical microscopy, with a binocular stereoscopic microscope.

Eyebrows were then observed and analysed by SEM (Scanning Electron Microscopy) – EDX (Energy Dispersive X-ray). The observations were conducted by SEM, using a Philips XL30 instrument (environmental version) ; GSE (Gaseous Secondary Electrons) and BSE (Back Scattering Electrons) procedures were used , the last one to detect heavy elements.

Elemental analysis were realized by X-ray micro fluorescence, this SEM microscope being equipped with a Bruker AXS energy dispersive X-ray; the system of analysis is PGT (Spirit Model, of Princeton Gamma Technology).

Each elemental analysis is given in the forms of a spectrum, with Kiloelectrons / Volts (ke/V) on the abscissa and elemental peak heights (cps/eV) in ordinates.

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Figure 1 : The Antommarchi death-mask of Napoléon. The circle on the left eyebrow indicates the region where the sample was taken.



Table 1 gives the numbers (among the first fifty detected) of non-plaster (gypsum) particles found on the stickypaper corresponding to the eyebrow region of the death-mask.

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Table 1 : Numbers of non-plaster particles found in the eyebrow region (from 1).		
Particles	Numbers found	
St. Helena lavas	2	
Iron-titanium inclusions	1	
Iron' ores	2	
Potassium phosphate	2	
Plaster impurities :	1	
. halite	1	
	1	
Whites : harvum sulphate	2	
. lead carbonate	2	
	28	
Lead phosphate	9	
Artefacts :		
. zinc oxyde	1	
. iron chrome plated	1	

DNA extraction from the eyebrow and obtention of DNA sequence of the HVS1 (Hyper Variable Sequence 1) of mtDNA (mitochondrial DNA) was realized as in (2). Briefly : DNA extraction for the eyebrow specimen was conducted using a standard method (0.5 M EDTA, sarcosyl 20% and proteinase K 10 mg/ml) ; the genomic DNA obtained was purified using a commercial kit (NucleoSpin® Kit ; Macherey-Nagel, Duren, Germany), in accordance with the manufacturer instructions and with some modifications.

PCR procedure was performed in a sterile PCR hood in accordance with standards for ancient DNA (a-DNA) work, with regular decontamination measures and all precautions taken to avoid any risk of contamination by modern DNA molecules. The mtDNA genomic sequence interval of HVS1 from positions 15,991 to 16,390 was amplified by PCR with primers F15971 and R16410.

For each PCR, the DNA extract for eyebrow specimen was amplified in a 12.5μ l reaction mixture (2mM MgCl₂, 50 mM KCl, 10 mM Tris/ HCl pH=9,

0.1% Triton X-100, 0.2 mM of each dNTPs, 0.1 μ M each primers) and 2.5 U of DNA polymerase (Ampli Tap Gold; Applied Biosystems, Foster City, CA, USA). The amplification was carried out with an initial denaturation step at 95°C for 6 min, followed by 35 cycles at 95°C for 1 min, and 72°C for 1 min.

PCR product was purified from agarose gel (QIAQuick PCR purification Kit, Valencia, CA, USA). Both strands of all the amplified mtDNA fragments eluted from the agarose gel slices were directly sequenced (Big Dye Terminator Cycle Sequencing Kit, Applied Biosystems) and separated (ABI PRISM3130x1 Genetic Analyser, Applied Biosystems).

The Sequences obtained were aligned against the revised Cambridge Reference Sequence, to identify the presence of polymorphic sites. SeqScape software (Applied Biosystems) and Clustal analysis were used for pairwise alignments.

G.L. is the only DNA experimentator. He had only one mutation in his mtDNA HVS1 sequence:

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16298C.

RESULTS.

1. Another eyebrow on the sticky-paper.

Examination by optical microscopy of the SP (**Figure2**) shows another eyebrow on it. The nomenclature given for the two eyebrows is eyebrow number 2 (EB2) for the first eyebrow observed (located at the left part of SP), and eyebrow number 1 (EB1) for the second eyebrow observed (located on the SP middle part).

Only the adhering part of EB2 is visible on the SP. The two eyebrows seem to differ for their thickness, EB2 being lightly lesser thick than EB1. They differ also for their apparent colours: EB1 is brown in colour, while EB2 is of a strange dark-blue colour.

Figure 3 shows the both eyebrows in SEM. EB2 is longer than EB1. Disposition of the points of the transversal scale rows and progressive slimming of the eyebrow diameter permit the eyebrow orientations, from the basis to the extremity.

Both eyebrow basis are broken. Both extremities correspond to natural eyebrow ends. While the EB1 surface is smooth, that of EB2 is covered by numerous deposits.

2. Study of the eyebrow number 1.

The SEM photograph of **Figure 4** shows a portion of EB1 located at its medial part; points of scale rows, orientated towards the eyebrow extremity, are well visible. The elemental analysis of this portion establishes that it is mainly constituted of organic matter (carbon, nitrogen and oxygen), with a well marked sulphur peak that is characteristic of the keratine of hairs.

The photograph of **Figure 5** shows clearly the main peculiarity of the EB1 basis: that of a "splice-end" (**3**), a longitudinal fracture indicating an intense traction in that orientation. This longitudinal breakage corresponds to the traction exerted on the eyebrow 1 basis during its transfer from the plaster of the hollow to that of the mask.

This photograph, and the corresponding spectras, show also that the EB1 basis was coated in mineral particles of gypsum (calcium sulphate of the plaster of the mask). It shows also that the hollow was initially covered, to favour the transfer, with a cotton tissue; we can observe on the photograph residual cotton textile fibers (large and flat fibers, with twisted segments, and some potassium component that is characteristic of vegetal fibers).

The photograph of **Figure 6** shows an enlargement (1265x) of the eyebrow basis ; we can see fibrillation (which indicates a trauma) of the EB1 basis.

The photograph of **Figure 7** shows the EB1 extremity, which is a natural end. Spectrum in the corpus corresponds to that shown on figure 4 (with calcite deposits); spectrum at the end shows some terminal silice deposits.

3. Study of the eyebrow number 2.

The photograph of **Figure 8** is an enlarged view of EB2 in its initial position.

The photograph of **Figure 9** shows an EB2 portion in the eyebrow region1. The corresponding spectrum, compared to that shown on figure 4, shows three (those of sodium, of aluminium and of chromium) supplementary peaks. The sodium, combined with chloride, represents probably the salt (ClNa) of the sweat.

The chromium combined with potassium represents probably the potassium dichromate $(Cr_2K_2O_7)$, a chemical product used since the XVIII th century for its oxidative properties. In the present case, it was used to bleach the eyebrow. Presence of aluminium is more difficult to explain; possibly it is that of a biting, used for some form of eyebrow colouring.

The photograph of **Figure10** shows the EB2 portion at the basis; the basis of this eyebrow is also splitended. The corresponding spectrum of this splitended eyebrow shows that it is partly calcified.

The detailed photograph of **Figure 11** shows the terminal part of the portion of the EB2 basis. Under the action of the X-rays of the SEM beams, the covering matter melts under the form of bubbles that look like to glue bubbles. The corresponding spectrum of the matter contains phosphorous, so the glue is an osseous glue (of PCa).

That establishes that the eyebrow basis was fixated, by the mean of an osseous glue, on the plaster of the mask.

The photograph of **Figure 12** shows that the eyebrow part, partially fixated on the sticky-paper, is located further than the distal part of the eyebrow basis; it is constituted of a sleeve, of a thickness greater than that of the eyebrow basis; the corresponding spectrum shows that the sleeve is more calcified.

The photograph of **Figure 13** shows the breakage point between the distal part (more stick) of the sleeve and the free part of EB2. Certainly, the breakage was caused by the load of the eyebrow on the sticky-paper.

The photograph of **Figure 14** shows the EB2 extremity, which is also a natural end. The spectrum of the detached scale layer is practically the same (aluminium peak excepted) than that of the eyebrow corpus.

Contrarily to EB1, there are some numerous and relatively large calcium deposit particles on the eyebrow2 surface. Photograph of **Figure 15** shows three of such deposits; they can to be calcium carbonate (CaO) and calcite (CaCO₃) particles but, because mainly of them has a magnesium peak in the spectrum, we think that they are carbonate-doubles of calcium and magnesium (tartar).

There are also lava particles on the eyebrow2 surface. Photograph of **Figure 16** shows an example of such a lava particle, with a relatively elevated peak of magnesium. Such a lava is a "mineral marker" of the St- Helena island (**4**). A total number of four of these particles were found on the EB2 surface.

4. Skin-debris and dandruffs on the EB2 surface.

Skin-debris (SD) debris are human skin residues that were well studied by SEM-EDX analyses in some balistic investigations (5, 6). Several SD, located on or at the vicinity of the eyebrow2 surface (figure 8), are detected in the present study. The three largest of them (SD1, SD2 and SD3) are associated to microblades of silice or to other contendent materials.

The photograph of **Figure 17** shows SD1, located on a portion of EB2 under the tartar particles number 3. We can see clearly on this photograph the skin shred that extrudes from the eyebrow corpus, outside of the silice (rich in silicium) micro-blade; this micro-blade cuts practically all the eyebrow thickness.

The photograph of **Figure 18** shows that SD2 is a relatively great skin-debris, that is located under the eyebrow corpus. Its size diameter is about 50 μ m; as

the thickness of the eyebrow at that level is less than 20 μ m, the two parts of it (P1 and P2) are visible at each sides. The silice micro-blade is located inside of the skin-debris.

The photograph of **Figure 19** shows that SD3 is located on the EB2 corpus. It is divides into three distinct parts (1, 2 and 3), the second one being partitioned from the third by a contendent segment. Elementary composition of SD3 is the same than that of the eyebrow, but the sulphur peak is less elevated.

Contrary to squamed epidermis cells (that are devoided of DNA), dandruffs are relatively great bulging multinuclear formations (7). We previously used hair' dandruffs to establish the autosomal and the Y-chromosome STRs profiles of Napoléon (8). Several dandruffs (D) are detected on the eyebrow2 surface.

The largest of them (D1) is illustrated on the photograph of **Figure 20**. It is a relatively angular bulging formation of about 13 μ m of length. Her elemental composition is similar to that of the EB2 surface. Another dandruff of a more little size, located under P1, is represented on the photograph of **Figure 21** and three other ones on the photograph of

Figure 22

Table 2 summarizes the comparison between the twoeyebrows, based on twelve different criterias.

Table 2 : Comparison between eyebrows 1 and 2.

	Criterias	EB1	EB2	
1.	Approximate length	1.2 cm	1.8 cm	
2.	Thickness in the median	27µm	16µm	
	part			
3.	Broken basis	+	+	
4.	Split-ended basis	+	+	
5.	Natural end at the	+	+	
	extremity			
6.	Typical composition of an	+	+	
	hair			
7.	Chromium	-	+	
8.	Surface	smooth	rough	
9.	Calcium carbonate	very few, of little	numerous, of relatively	
	deposits	sizes	great sizes	
10.	Lavas detected	no	3	
11.	Skin-debris detected	no	3	
12.	Dandruffs detected	no	5	

5. The mutation of the HVS1 mtDNA sequence of eyebrow 2.

Only the free part (that located after the breakage point) of EB2 was used for DNA extraction. We found only one mutation (**Figure 23**) in the HVS1 corresponding sequence (sequencing repeated twice) : in position 16, 184, the cytosine (C) is replaced by a thymine (T) ; this transversion 16,184C > T, named

16184T, is the HVS1 mtDNA mutation characteristic of Napoléon (2).

Figure 23 : HVS1 DNA sequence (from positions 16149 to 16189) of the EB2 mtDNA. A (in green) : adenine ; C (in blue) : cytosine ; G (in yellow) : guanine , T (in red) : thymine. The little black point and the column indicate the mutation site.



DISCUSSION

We have previously shown that the plaster of the Antommarchi death-mask of Napoléon is partly composed of a bad-quality plaster, whose gypsum originates from the St-Helena island (1); on the surface of that plaster are included numerous particles of a special sort of aluminosilicate iron-rich (lavas), which also constitute a mineral signature on this island. We found in the present study three particles of such a lava on an eyebrow of the left eyebrow of the mask, that establishes its geographic origin.

The detection of the 16184T mutation in the HVS1sequence of the mtDNA of one eyebrow adhering to the death-mask is the genetic proof that this eyebrow is well an hair of Napoléon. Secondarily, it demonstrates that the corresponding part of this death-mask is directly derived to some portion of the initial death-mask that was applied in St. Helena to the Napoleon face at the time of his death.

At the time of her discovery (2) the 16184T mutation of the HVS1 mtDNA sequence was attributed to hairs of Napoléon, of his mother Letizia and of his sister Caroline. Recent information on mtDNA mutation frequencies obtained in a public data-base (EMPOP) establish that, at the present time, 14 subjects only (on more than a thousand of European subjects tested) bear the 16184T mutation in their HVS1 sequences. With a so very low incidence, this rare mutation constitutes a highly resolutive genetic marker of Napoléon and his family (It is a private, quasi-familial DNA marker).

From what part of the eyebrow the mtDNA come from? A first possibility is that it originates from the eyebrow cuticle. Hair cuticle, which contains DNA, can effectively be seen (for example on the photograph of figure 4) at the limits and between individual scales; the fact that the scales are not completely flattened on the eyebrow matrix is also in favour to the existence of some cuticle part). Probably DNA originates also from the skin-debris, at least from those (scraped) shown on the photographs of figure 18 and 19 from which thickness indicates that there is some dermis part in the skin layers.

Figure 24 explains how eyebrows (and skin-debris detached from, and also dandruffs adhering to) of the copse face were transferred to the mask. In a first time the mould (constituted of the bad quality plaster of St. Helena) was applied to the face ; the face was

probably coated by oil, and locally covered by some textile piece of cotton' tissues (figure 5). The pulling up of the mould from the face drived the *in situ* transfer of numerous of some residual eyebrows to the mould.

Figure 24 : The different steps (1,2 and 3) in the transfer of the eyebrows from the face to the mould (in yellow) and then to the initial mask (in red); levels and abundances of eyebrows : e (on the face), e1(on the mould), e2 (on the mask), are indicated.



Then the initial mask (also constituted of the badquality plaster) was obtained in casting this liquid plaster into the mould hollow; this initial mask was finally obtained in separating it from the hollow. A second *in situ* transfer, from the hollow to the initial mask, of some eyebrows was in this way carried out.

We have formal historical proofs (1) that the mould was destroyed. The splitted-end aspects of the broken basis of the two eyebrows examined (figures 5 and 10) occurred very probably during the first transfer, when eyebrows were pulled up from the skin' face. We have also evidence (figure11) that osseous glue was applied to the basis of one of the eyebrow, to maintain it on the death-mask.

Our previous study on the death-mask (1) had shown that there are numerous particles of white on the mask surface (table1) ; these whites are of three different sorts (**Table 3**): ceruse (a lead carbonate), barytime (the baryum sulphate) and occasionally white of zinc (a zinc oxide). The concentration in white particles of ceruse and barytine is especially intense in the eyebrow region of the mask, where they represent more than 62% of the total number of non - plaster particles detected.

Table 5. Whites on the eyebrow sticky-paper (see table 1	Table 3:	Whites on	the eyebrow	sticky-paper	(see table 1
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Whites	Chemical formulaes	First dates of their use
Ceruse	$2PbCO_3$. $Pb(OH)_2$	since the Antiquity
Barytine	$BaSO_4$	since 1820
White of zinc	Zn0	since 1779

There is no doubt that white particles of ceruse and barytine are micro-scales of the painting used to paint the mask in white. In the eyebrow region the white painting was certainly applied to hide the darkness of the eyebrows.

Nine (of 50 detected) of lead phosphate particles

(Figure 25 shows of the spectrum of another one) were found on the eyebrow sticky-paper (see table 1). They probably correspond to the lead phosphate $(Pb_3(PO_4)_2)$, a chemical product that was widely used since the 18 th century for her stripping properties.

We have previously presented here some evidence (for example figure 9) that the eyebrow 2' surface is covered by potassium bichromate, its indicates an early attempt to bleach it.

In his predictive text book (9) Gérard Azémar indicated two sorts of experimentations to do about the Antommarchi death-mask of Napoléon, to valid it : i. The study of the plaster of the mask; ii. The study of eyebrows included in the plaster. We have previously published (1) this first study; the present paper concerns this second study. Both confirm that the Antommarchi death-mask of Napoléon is, among the historical masks, the one that is the nearest from the real death-mask of Napoléon.

At least two other historical death-masks of Napoléon have Napoléon's hairs at their surfaces. Among them is one wax mask of Napoléon, said "the Noverraz mask" (10).

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Figure 2: Optical microscopy of the SP.1 : micro-photograph (2x) of the SP; e2: EB2; e1:EB1.2: micro-photograph (80x) of EB1.3: micro-photograph ($80x^{\circ}$ of EB2.



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Figure 3: Electron microscopy (in GSE, 100x) of EB1 and EB2.1: SEM photograph of EB1.2: SEM photograph of the entire part of EB2. B: basis; E : extremity.



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Figure 4: Some portion of EB1 and its elemental composition. Above: SEM photograph (in GSE, 633x) of that portion. The black dot indicates the region where EDX elemental analysis is realized. Below: spectrum at the black dot. C:carbon; N: nitrogen; O: oxygen; S: sulphur; Ca (two peaks): calcium.



Figure 5: Basis of EB1. Above: SEM photograph (in GSE, 633x) of the EB1 basis. Arrow points indicate the splitend limits (F: cotton textile fibers; T: a twisted portion of a cotton fiber). The little black dot indicates EB1, the middle black dot the mineral material where the EB1 basis is located, and the large black dot a portion of cotton fiber. Below: The corresponding spectras of the fiber (below) and (above) of the mineral ; in the below spectrum, CI: chlorine and K: potassium.





Figure 6: SEM photograph (in BSE, 1265x) of the EB1 basis (1 and 2 indicate the two terminal parts of the eyebrow basis at each sides of the split end).





Figure 7: the Eyebrow 1 extremity. Above: SEM photograph (in GSE, 1600x) of the EB1 extremity (measurement in μ m). The black dot indicates the location (in the eyebrow corpus) where elemental analysis is realized; the little black dot is that of the end. Below: spectras at the (below) black dot, and at the little (above) black dot (Si: silicium).



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Figure 8 : The different parts of eyebrow 2 (SEM photograph, in GSE, 100x). F: a portion of a synthetic fiber. LP: left border of the sticky-paper. B: EB2 extremity. 1, 2, 3 and 4 indicate various parts of EB2 since the extremity, 5 indicating the eyebrow portion loaded on SP. G : EB2 basis. S2: the limit between calcified and sleeved parts. C: the break point. S: the largest silica blade (see figure 18). L: the first lava discovered (see figure 16). P: position of a skin-debris, PX indicating that illustrated on figure 19. P1 is the dandruff D1 (see figure 20), and P' and P'' other dandruffs (see figure 22). A: zone of desquamation of eyebrow scales (see figure14) in the region of the extremity.





Figure 9: Eyebrow portion in the region 1. Above: SEM photograph (in GSE, 5000x) of the eyebrow portion. The black dot indicates the location where elemental analysis is realized. Below: spectrum at the black dot. Na: sodium; Al: alumium; Cr (two peaks): chromium.





Figure 10: Eyebrow 2 at the basis. Above: SEM photograph (in GSE, 1000x) showing the EB2 basis. F : crack; 1 and 2 indicate the two splitted parts of the EB2 basis. The area inside the circle is enlarged on the following photograph. The black dot indicates the location of 2 where elemental analysis is realized. Below: spectrum at the black dot.





Figure 11: An enlarged view of the terminal portion of the 1 eyebrow part. Above: SEM photograph (in BSE, 4000x) of this part 1; b is a bubble of glue. 1 and 2 indicate locations where elemental analyses are realized. Below: spectras at 2 (above) and at 1 (below); P: phosphorous.





Figure 12 : The eyebrow part further than the basis. Above: SEM Photograph (in GSE, 1000x) of the EB2 part located further than the basis. B: The sticky-paper border. Part 2 is the distal part of the basis (already studied) and part 1 is the portion of the eyebrow coated by the sleeve (L: right limits of the sleeve). Below: spectras at 1 (below) and 2 (above).





Figure 13 : The eyebrow at the breakage point. Above: SEM photograph (in GSE, 1250x) of the angular EB2 region located between the distal part of the sleeve and the broken part of the eyebrow. B: left border of the sticky-paper; M: distal part of the sleeve; C: the broken point; S: part of the eyebrow relatively sulphur-rich. The black dot indicates the location where elemental analysis is realized. Below: spectrum at the black dot.





Figure 14 : The eyebrow 2 extremity. Above: SEM photograph (in GSE, 4000x) of the EB2 extremity; 2 indicates the eyebrow top, and 1 and 3 detached scale layers near the extremity. Below : spectras of 1 (below) and 2 (above).



Figure 15 : Calcium carbonate deposits on the eyebrow 2 surface. Above: SEM photograph (in GSE, 4000x) of three (1,2 and 3) deposits on the EB2 surface. The black dot on the deposit number 2 indicates the location where elemental analysis is realized. Below: spectrum at the black dot (Mg: magnesium).





Figure 16 : An example of one particle of lava on the eyebrow 2 surface. Above: SEM photograph (in GSE, 2000x) of a EB2 portion showing a lava (L) on it surface. The black dot indicates the location on L where elemental analysis is realized. Below: spectrum at the black dot. Fe (three peaks): iron.





Figure 17 : the skin-debris number 1. Above: SEM photograph (in GSE, 4000x) showing SD1; s : micro-blade; p: skin ; 3: tartar3. The black dot indicates location where elemental analysis is realized. Below: spectrum at the black dot.





Figure 18 : the skin-debris number 2. Above: SEM photograph (in GSE, 2000x) showing SD2. P1 and P2 indicates the two parts of the skin; s: the EB2 corpus. The black dot indicates the location where elemental analysis is realized. Below: spectrum at the black dot.





Figure 19 : The skin-debris number 3. Above: SEM photograph (in GSE, 4000x) showing SD3. A: detached scales from the EB2 corpus; 1, 2, and 3 indicate the three SD3 parts; p: skin; c : contendent fiber. The black dot indicates the location (in the skin debris) where elemental analysis is realized. Below: spectrum at the black dot.





Figure 20 : The dandruff P1; Above: SEM photograph (in GSE, 4000x) of the P1 (pe) dandruff. Black dot indicates the location on the dandruff where elemental analysis is realized. Below : spectrum at the black point.





Figure 21 : A more little dandruff. Above: SEM photograph (in GSE, 2000x) of a more little dandruff (pe). D1: the dandruff where elemental analysis is realized. Below: spectrum at the black point.





Figure 22 : Three other dandruffs. Above: SEM photograph of three other dandruffs (pe). The black dot indicates the location (in the calcium carbonate pile) where elemental analysis is realized. Below: spectrum at the black dot.





Figure 25 : Spectrum of a particle of lead phosphate. Sulphur and calcium peaks indicate the gypsum; the phosphorous peak and the two lead peaks indicate the lead phosphate.



