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REPORT

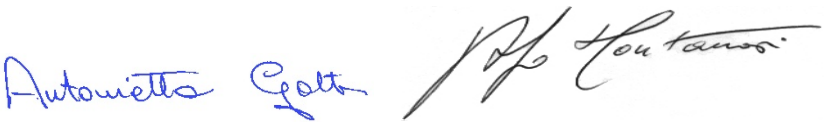
N° 34/2016

EVALUATION OF A VACCINE (INFANRIX) SAMPLE THROUGH AN ENVIRONMENTAL SCANNING ELECTRON MICROSCOPY INVESTIGATION AND AN X-RAY MICRO-ANALYSIS

Client : UNACS

Sample checked : Vaccine Infanrix

Analyses and conclusions by: Dr. Antonietta Gatti, Dr. Stefano Montanari

Signatures: 

Date: San Vito, 22nd August 2016

This report is made up of 41 (forty-one) numbered pages including the cover.

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1. INTRODUCTION

Nanodiagnostics srl is a consulting firm in the fields of medicine, biology, industry, environment and ecology. Its main activity is detecting inorganic micro- and nano-particles in any medium (biological tissues, food, drugs, cosmetics, environmental samples, garments, etc.), through an innovative system of environmental scanning electron microscopy. The investigation is carried out mainly by means of an ESEM or a FEG - ESEM (Field Emission Gun - Environmental Scanning Electron Microscope, QUANTA 200, FEI Company, Holland) with suitable protocols according to the type of substrate.

This analysis offers the possibility to observe biological samples in wet-mode, i.e. in the condition of their normal hydration, at environmental pressure, without the need to dehydrate them or make them electro-conductive through a coating of Carbon, or metals like Gold and Palladium, a procedure that is necessary with the traditional scanning electron microscopes.

By applying appropriate protocols to be adapted to each kind of observation, this feature allows to check biological samples, including living cells, without impairing their integrity, and to repeat the test every time one wishes. The main objective of the study is the detection of inorganic micro- and nano-particulate matter, in case there should be any in the sample, and this is achieved without the need of any process of the specimen.

An x-ray microprobe of an EDS (Energy Dispersive System, EDAX, USA) supplements the investigation, as the spectrum thus obtained shows the chemical elemental composition of the particulate matter. In fact, it measures the energy characteristic of each element making up the particles in the specimen, and returned as X-rays after the sample has been hit by the electron beam delivered by the FEG - ESEM.

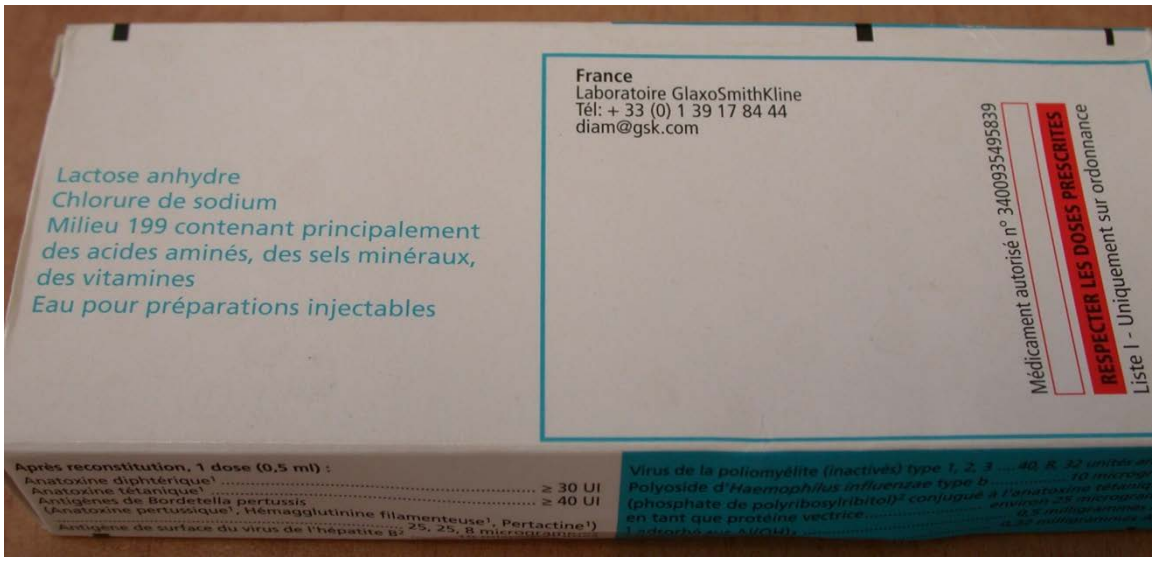
So, through such an integrated analysis, the micro- and nano-sized inorganic particles are photographed, measured and chemically characterized in a non-invasive, non-destructive, repeatable way, the only exception being fluids, which are often hard to recover in their liquid form after some time. (1,2,3)

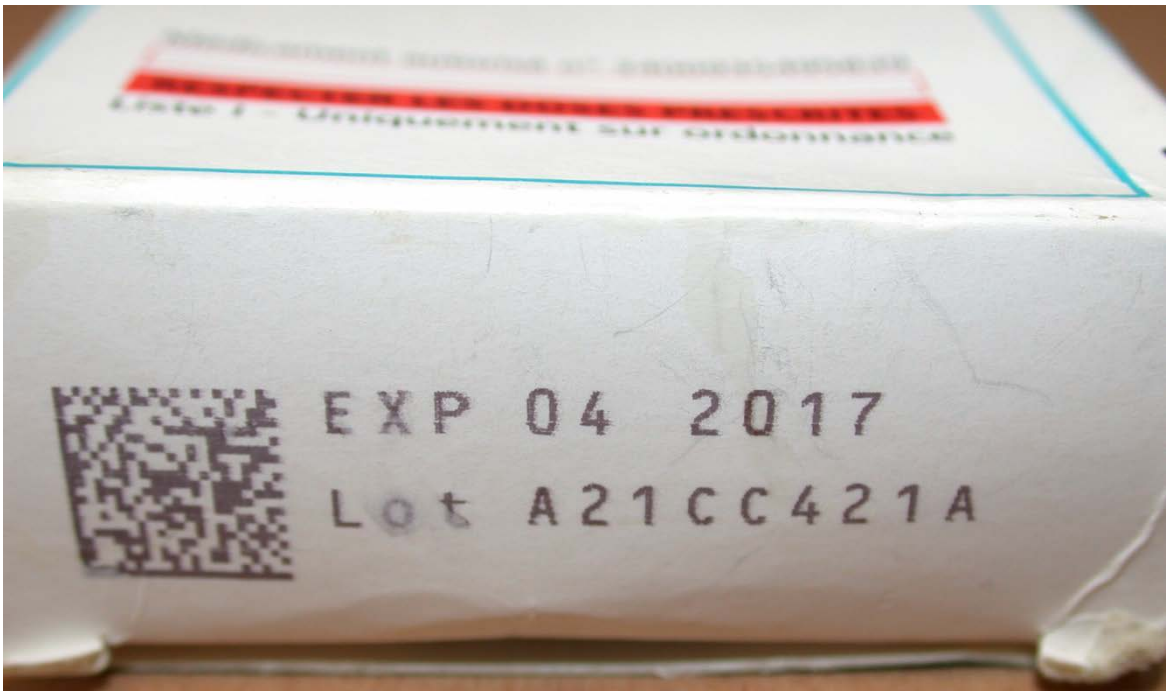
The investigation can be carried out on specimens of biological origin like biopsies, autopsies and organic fluids, but is equally feasible on many other kinds of materials like, for example, environmental samples, food, drugs or cosmetics. As the principal object of the investigation is inorganic and non biodegradable, there is no particular difficulty in detecting such particulate matter either in fresh or in archived samples.

2. MATERIAL

A whole package of vaccine INFANRIX by GlaxoSmithKline Biologicals s.a, distributed in France, was sent to the Laboratory (lot A21CC421A - expiry date 04/2017) for the ultrastructural analyses. The package contained a syringe pre-filled with injectable water, a vial containing the vaccine as powder and two sterile needles. The vaccine powder consists of 10 microg “Neisseria meningitidis” oligosaccharides. That was conjugated with 15 microg of CRM197 protein adsorbed on Aluminum phosphate (0.125 mg Al^{3+}) in presence of saline.

The specimen was sent by UNACS and was encoder at the Laboratory as STD 1225.





3. TEST CONDITIONS

The sealed sample was preserved in a +4 °C refrigerator at the Laboratory until preparation. After verifying the integrity of the package, the package was opened inside a laminar-flow hood. The needle was connected to the syringe wearing talc-free gloves and the syringe was shook horizontally in order to make the solution homogeneous and simulate the procedure as suggested by the instructions for the use before administration.

Then, a drop of about 20 microliters of liquid was deposited on a nitrocellulose filter (Millipore, type HAWG, 0.45 micron) mounted on a Carbon disc, adherent to the Aluminum

stub for the electron-microscopy observation. Thus prepared, the sample was inserted in a specific, clean container, sealed and left to dry at room temperature inside the laminar-flow hood where the whole operations had been carried out.

After about 24 hours the sample was entered in the electron-microscope chamber for the analysis.

All procedures were carried out in an environment protected from environmental pollution and with clean instruments.

4. TIPES OF ANALYSES CARRIED OUT

The electron-microscopy study was meant to ascertain the presence of the declared inorganic components in the vaccine solution and to investigate on the presence of possible foreign bodies. The technique used was developed within the European project called Nanopathology (QLRT-2002-05-147) employing an environmental scanning electron microscope FEG – ESEM Quanta 200, by FEI (The Netherlands), that allows to observe solid micro- and nanoparticles in the sample investigated. The energy-dispersive X-ray spectroscopy carried out through an EDAX (USA) instrument allows to evaluate the elemental composition of the particles detected (1,2).

5. RESULTS

The electron-microscopy analyses of the sample showed the presence of particulate matter with different chemical compositions (see Table).
The chemical and morphological results are shown in the pictures that follow.
The spectrum peaks without an indication of the chemical element to which they refer are the secondary peaks of an already reported element at its primary peak.

Note: The list of the elements reported in the table is done according to their degree of representativeness, starting from the highest in the EDS spectrum.

INFANRIX – vaccine distributed in France (Lot A21CC421A – expiry date 04/2017)

The table contains all the most meaningful analyses carried out on the sample. The product contains Aluminum salts that create precipitates. Identification of further foreign bodies in the Al-precipitates was particularly hard.

N. analysis	Description (magnification, morphology)	Elements detected
1	Vaccine on filter – 60x	Morphology image
2	400x	
3	1486x	AlOClPNaCS
3BIS		OAlClPNaC
3TER		Subtraction 3-3bis=AlCl
4	19594x (54 spherules 0.5m)	OWAlPNaClCaCSFe
5		38236x
6	Debris 5 µm, 6971x	OAlWPClNaCaSFe
7	10582x	OAlWPClNaCaSFe
8	6 µm; 10103x	WAlCa
9	2-1-0.5 µm, 40000x	OWAlCaClNaPCFe
10	5287x	
11	2007x	AlOClCNaP/OAlPClCNa
		Subtraction 11-11bis, AlClOCNaP
12	2252x	
13	dx5 µm + 0.8 µm6988x	FeOAlCrNiClPNaSiMn/ OWAlCaClNaPCFe
14	3808x	OWAlCaClNaPCFe
15	1.5 µm,4998x	OWAlCaClNaPCFe
16	5 µm, 10235x	TiAlOPNaCl
17	8807x	OWAlCaClNaPCFe
18	4597x	OWAlCaClNaPCFe
19	5185x	OWAlCaClNaPCFe
20	2986x	OWAlCaClNaPCFe
21	White block 50 µm, 5185x	OAlPClNaC + OWAlCaClNaPCFe
22	22484x	OWAlCaClNaPCFe
23	protein corona,40000x	OWAlCaClNaPCFe
24	13490x	OWAlCaClNaPCFe
25	spherules W, 32180x	OWAlCaClNaPCFe
26	cubes W, 64479x	OWAlCaClNaPCFe
27	62x	OWAlCaClNaPCFe
N. analysis	**Description(magnification,**	**Elements detected**

	morphology)	
28	**1599x**	**OWAlCaClNaPCFe**
29	**2895x**	**OWAlCaClNaPCFe**
30	**12477x**	**OWAlCaClNaPCFe**
31	**2404x**	**W**

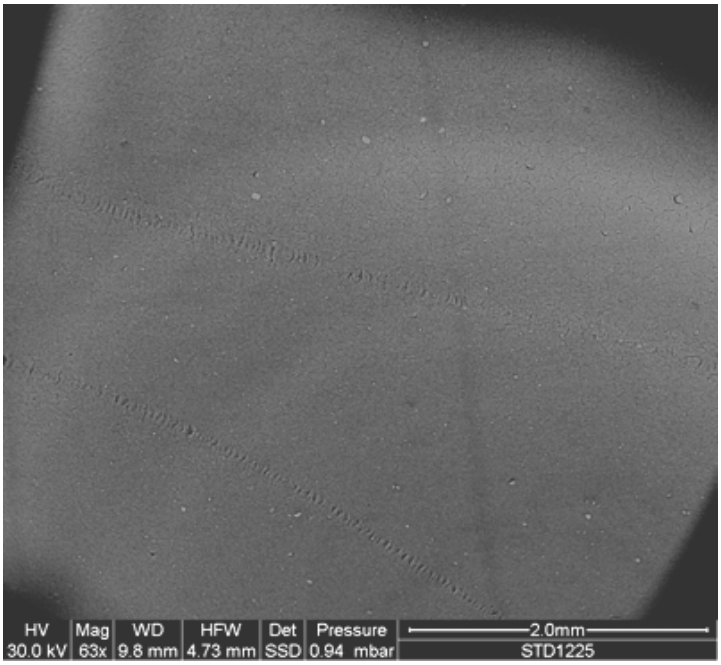


Image 1. The low-magnification (60x) image shows the surface of the nitrocellulose filter on which the droplet of vaccine was deposited.

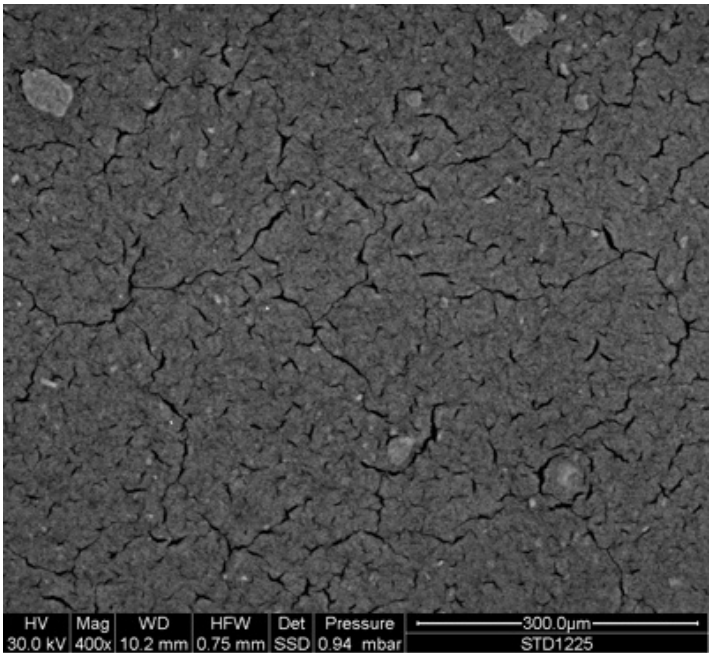


Image 2. The medium-magnification (400x) picture shows a section of the sample where crystals and precipitates are visible.

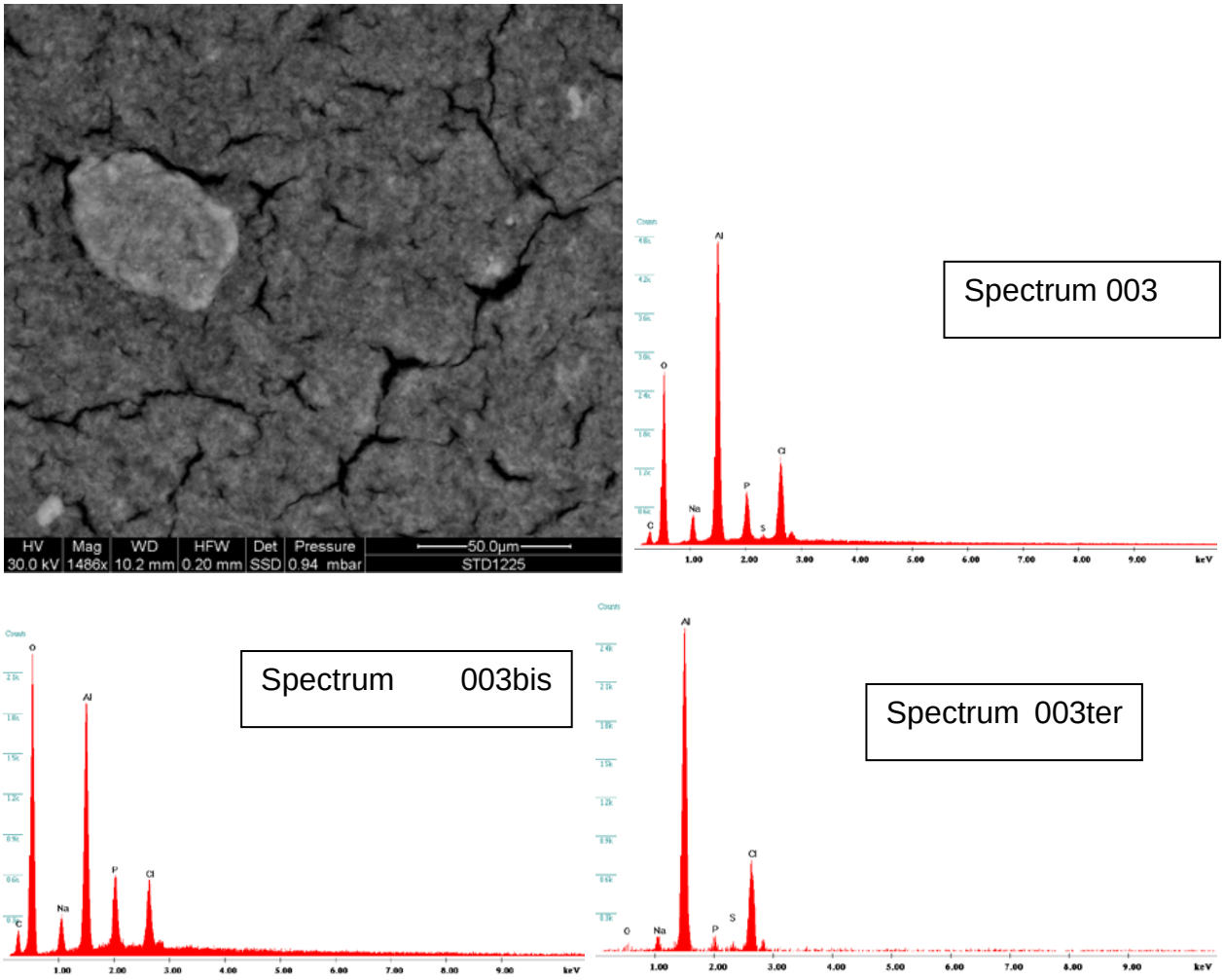
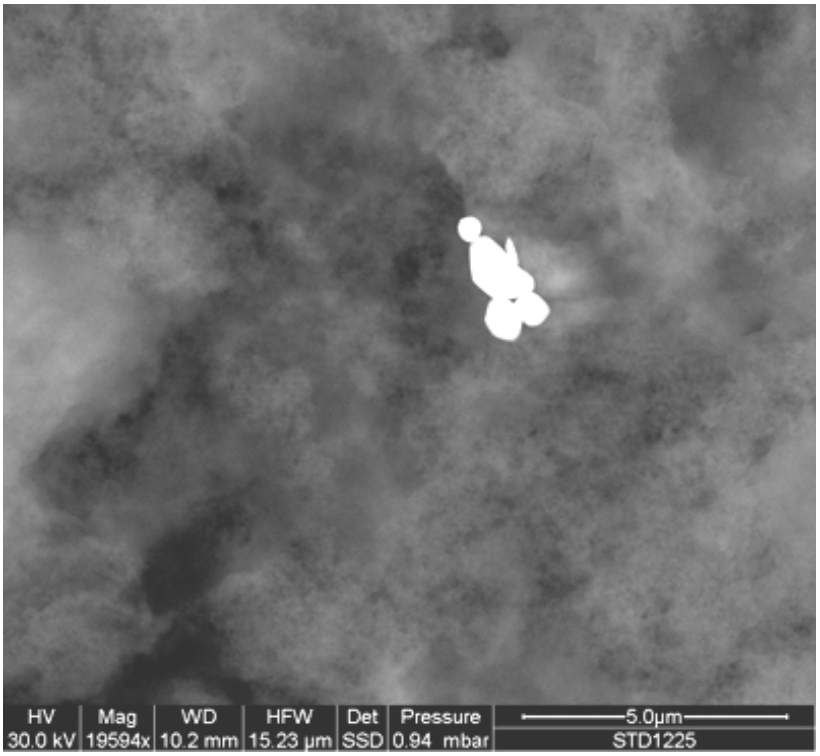


Image 3. A section of the sample is shown at high magnification (1486x). A 50-micron detritus is visible. The EDS analysis shows its composition: Aluminum, Oxygen, Chlorine, Sodium, Carbon, Sulfur. The filter is completely covered by Aluminum-phosphate salts including Sodium chloride.



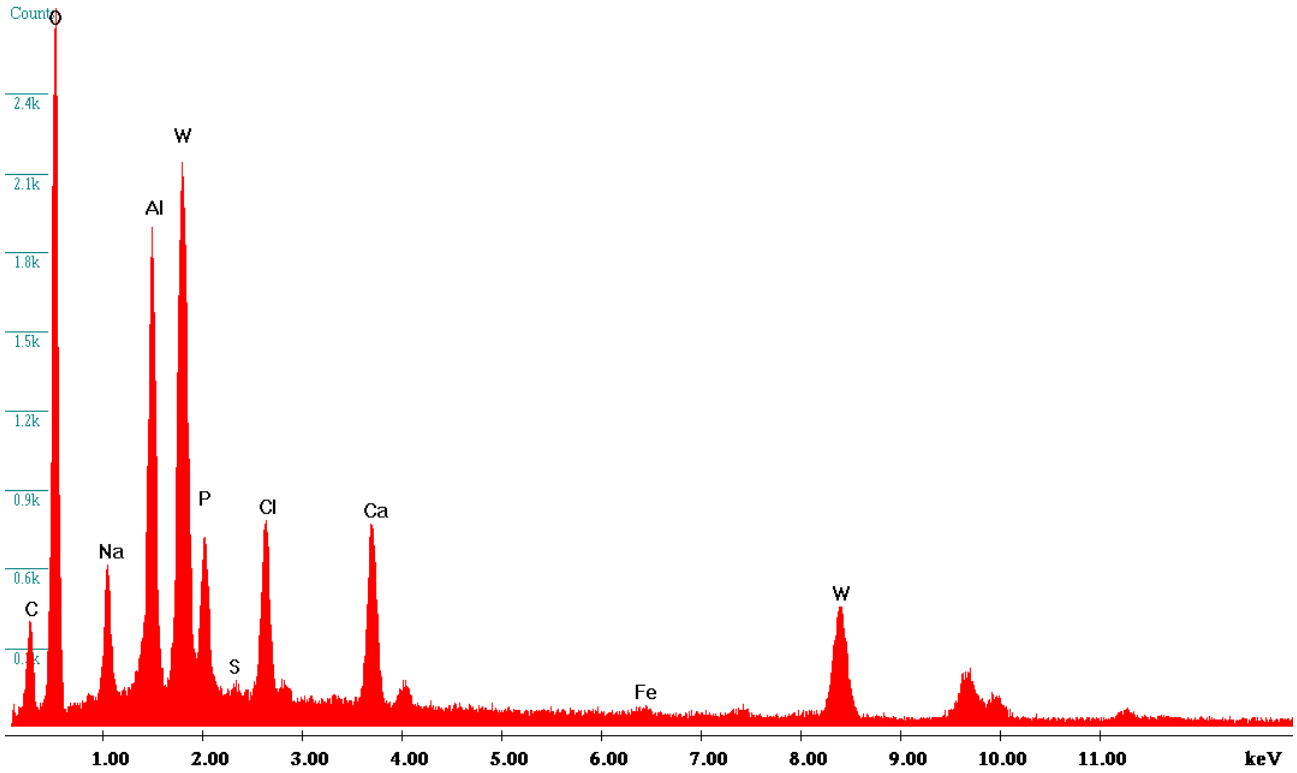
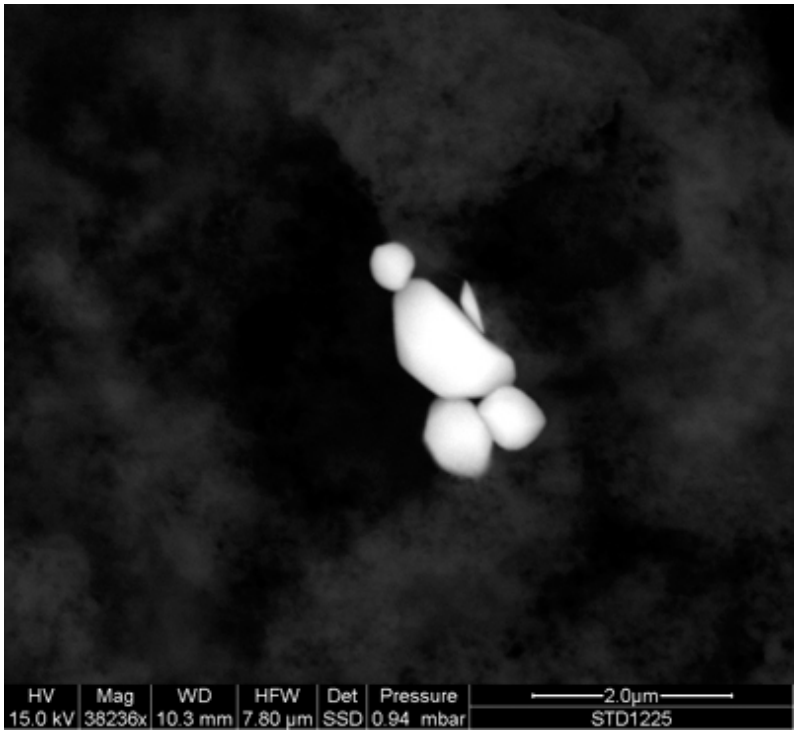


Image 4. A high-magnification (19594x) section of the sample is shown where clustered particles of Oxygen, Tungsten, Aluminum, Phosphorous, Sodium, Chlorine, Calcium, Carbon, Sulfur and Iron are visible. The size of the cluster is about 3 microns. This is a foreign body composed of Tungsten-Calcium-Iron not belonging to the vaccine's, or to any drugs', composition.



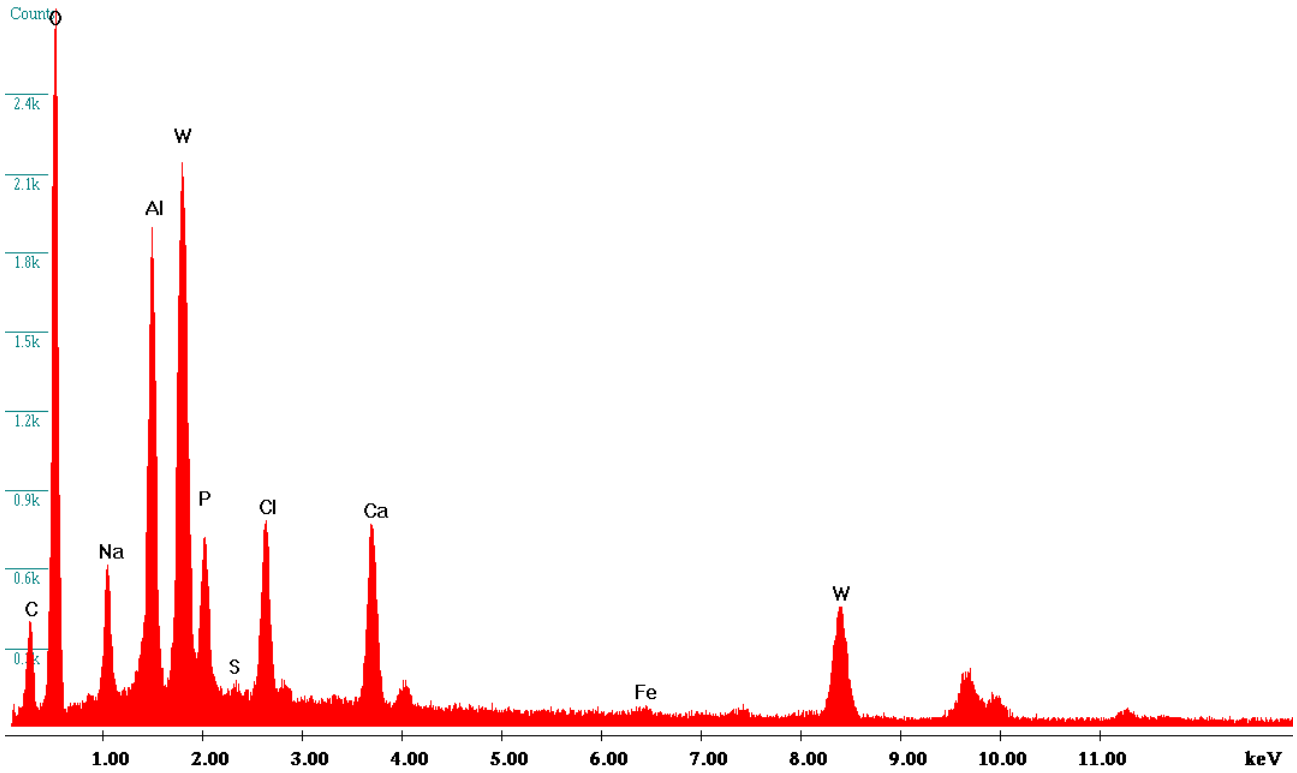
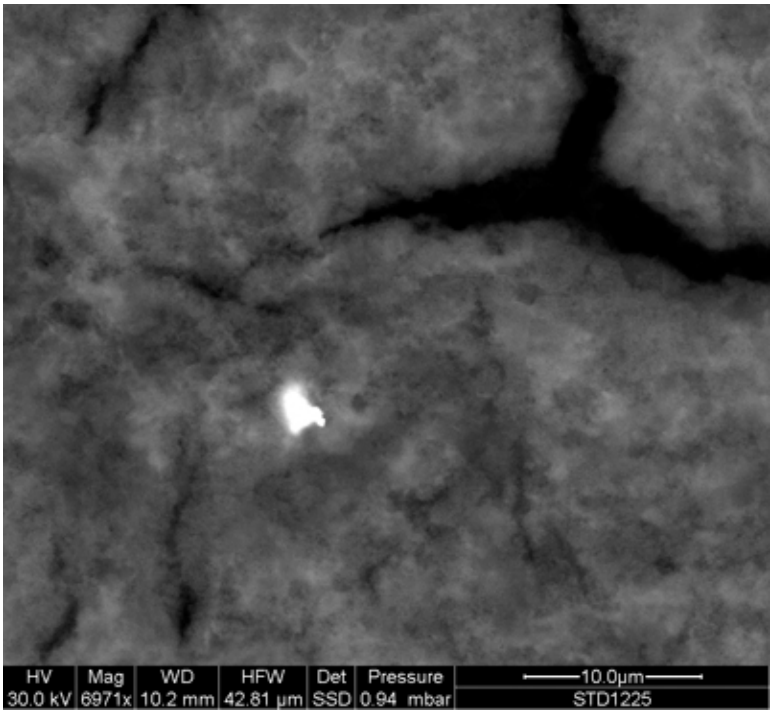


Image 5. The image shows the previous image at higher magnification (38236x) where 5 particles (0.3 – 1.5 microns) are visible. The debris are composed of Oxygen, Tungsten, Aluminum, Phosphorous, Sodium, Chlorine, Calcium, Carbon, Sulfur, Iron.



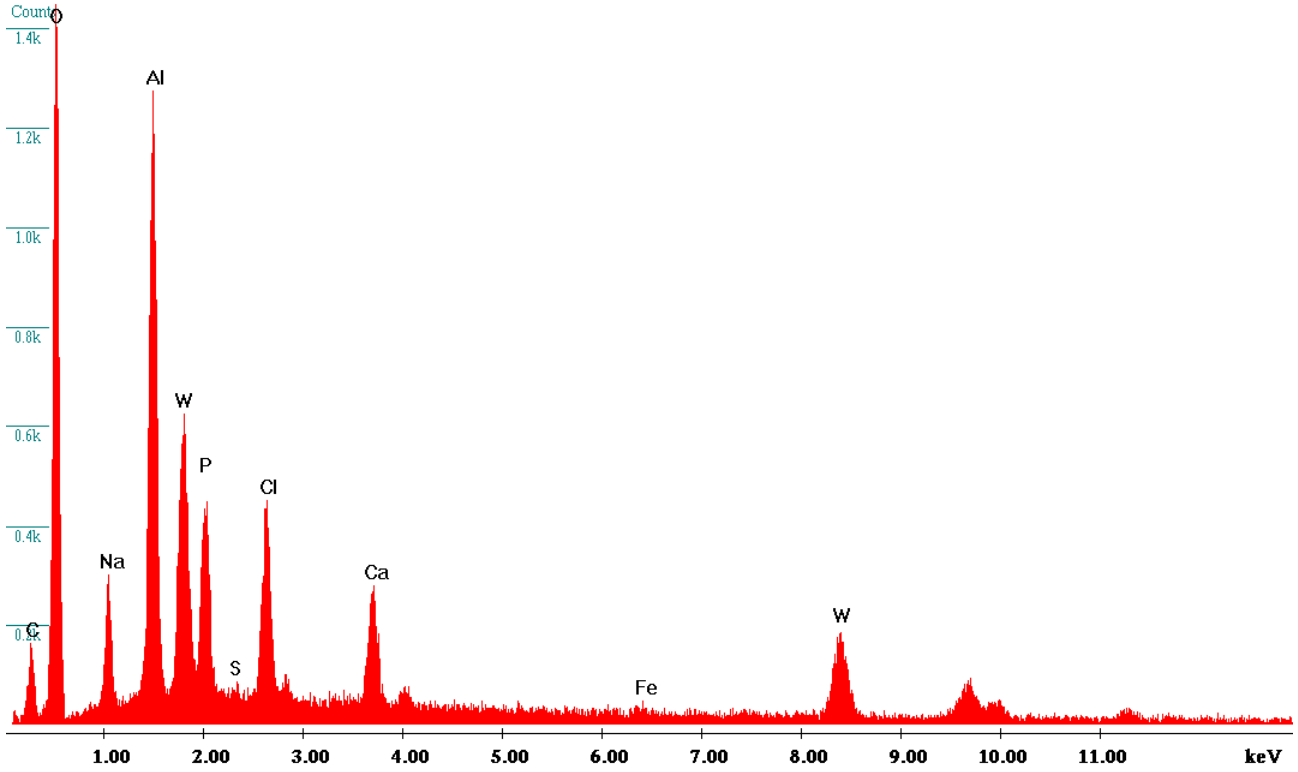
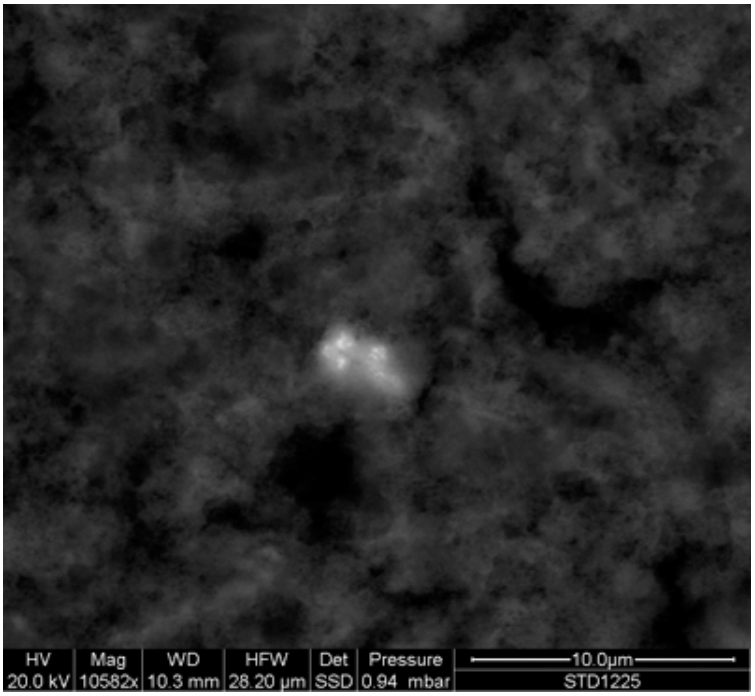


Image 6. The image (6971x) shows a 4-micron cluster of particles. The composition is Oxygen, Aluminum, Tungsten, Phosphorous, Chlorine, Sodium, Calcium, Sulfur, Iron. Qualitatively the same as the one of Analysis 5.



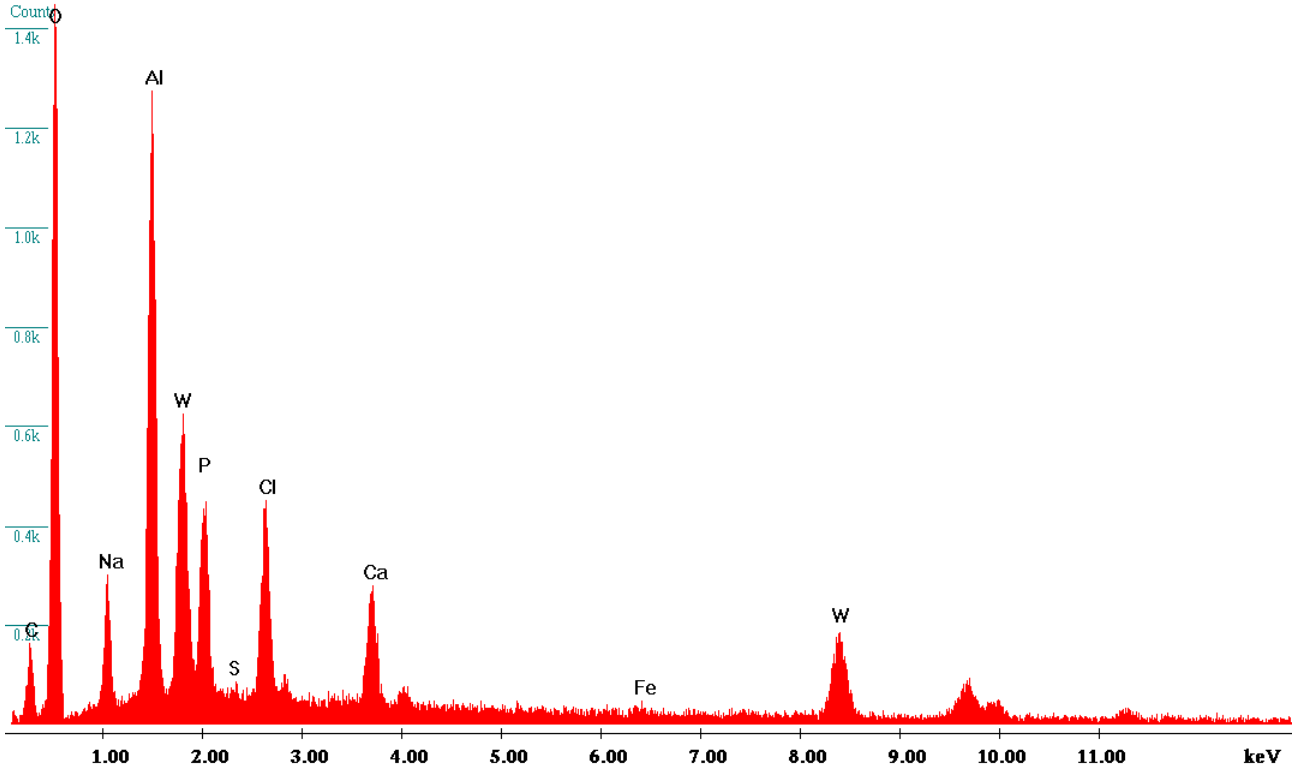
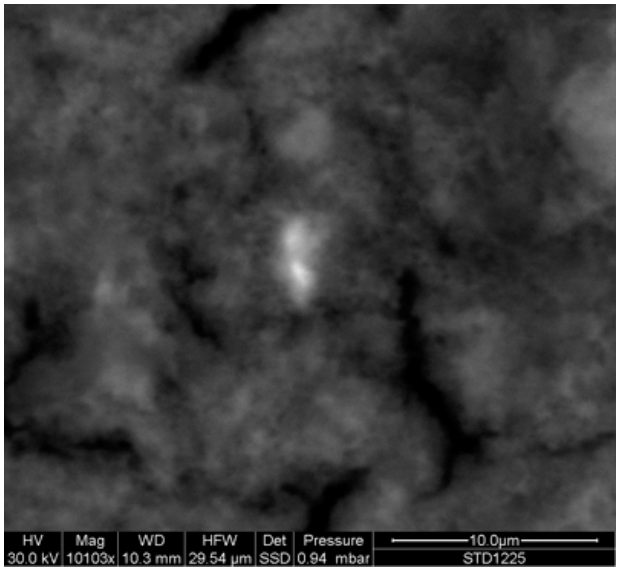
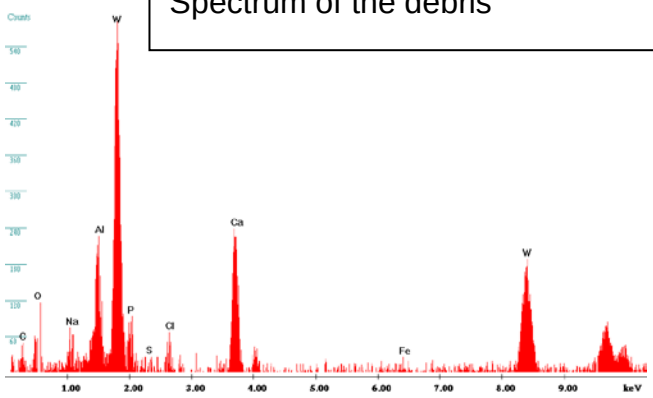
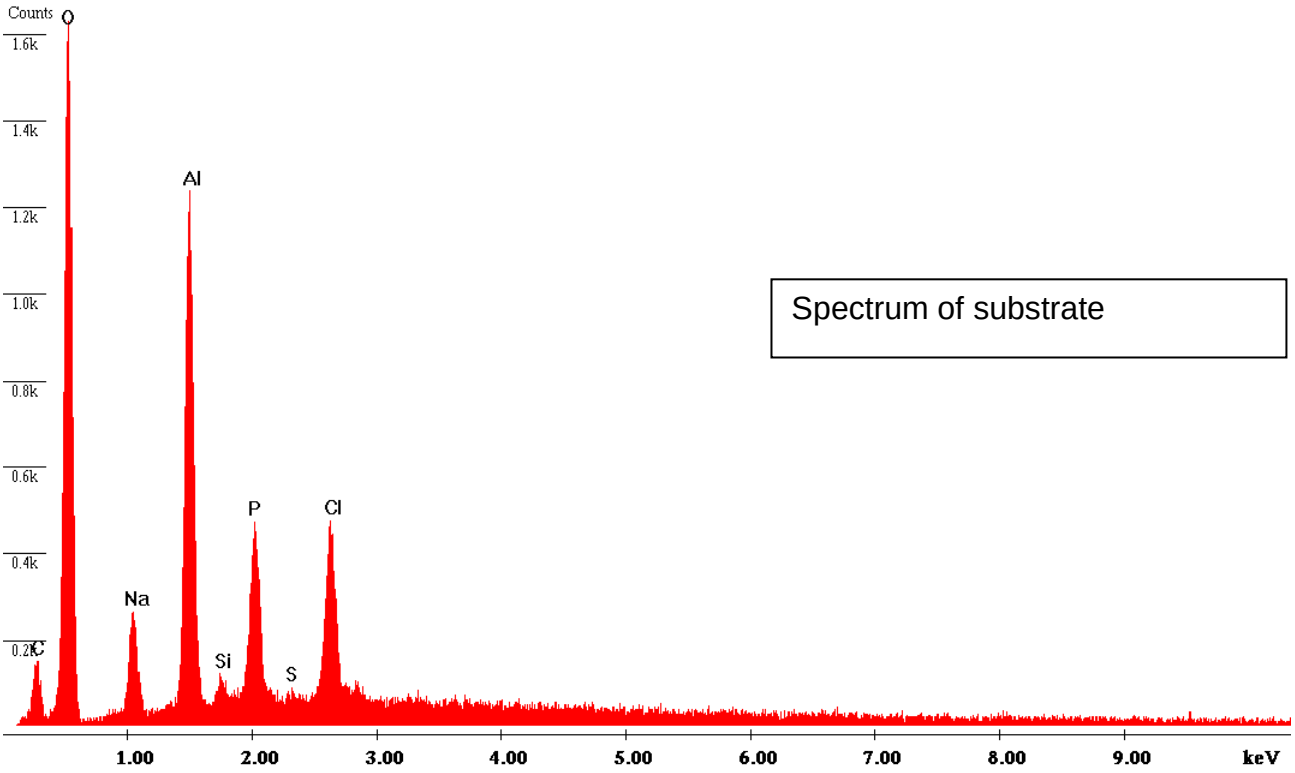


Image 7. The image shows another area of the vaccine droplet (10582x) where a 4-micron cluster of particles is visible. The qualitative composition of Oxygen, Aluminum, Tungsten, Phosphorous, Chlorine, Sodium, Calcium, Sulfur, Iron is the same as those of Analyses 5 and 6.



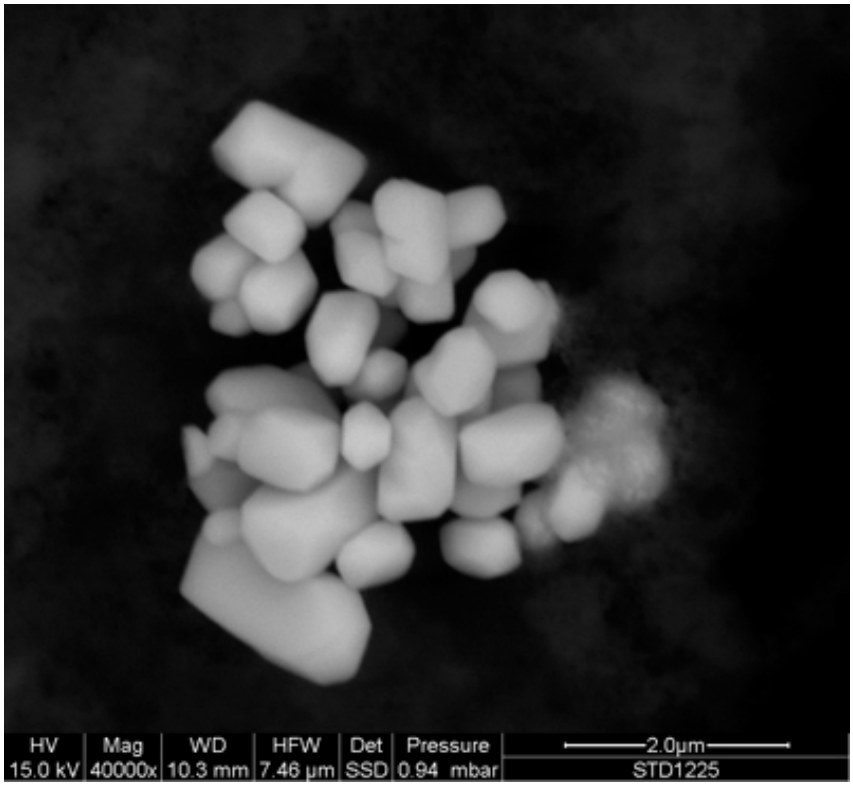


Spectrum of the debris



Spectrum of substrate

Image 8. The image shows at high magnification (10103x) a portion of the sample where a debris is visible. The EDS spectra show the debris chemistry (obtained by subtraction of the substrate signal) and that of the substrate (008std) . The debris is composed of Tungsten, Calcium, Iron, Calcium. The substrate (vaccine droplet) contains Oxygen-Aluminum-Chlorine-Phosphorus-Sodium-Silicon and Sulfur.



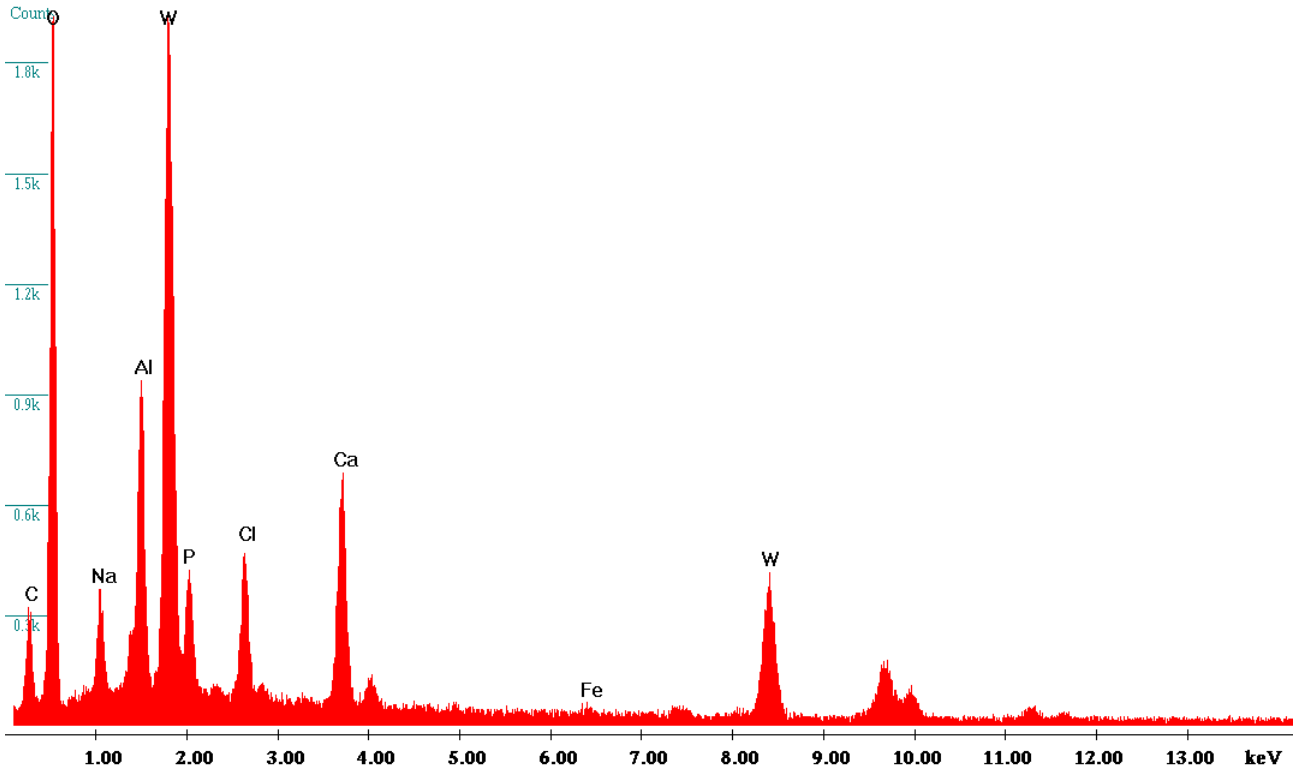
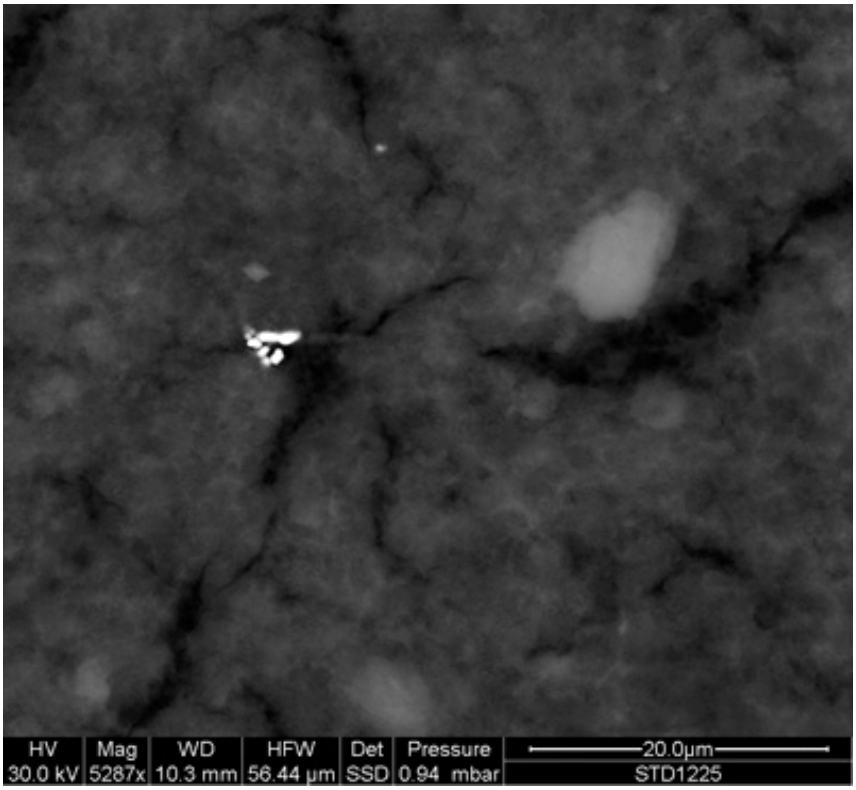


Image 9. A high-magnification (40000x) image of the Tungsten debris. A cluster of 0.3-1.8-micron particles is visible whose qualitative composition is the same as the one of Analyses, 5, 6 and 7. The particles are mainly composed of Tungsten.



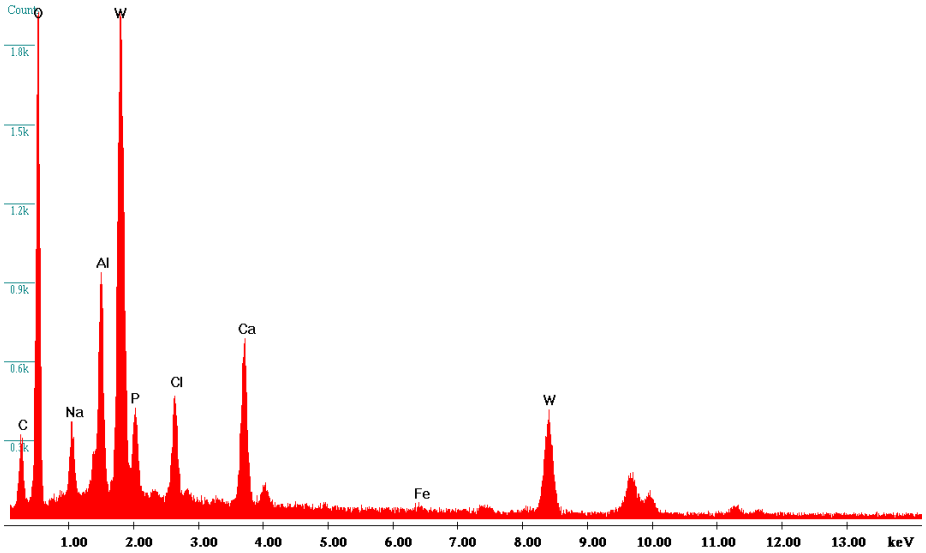


Image 10. The image shows further metallic debris mainly composed of Tungsten. (5287x).

A section of the sample at high magnification (5287x) is shown where a few particles can be seen clustered.

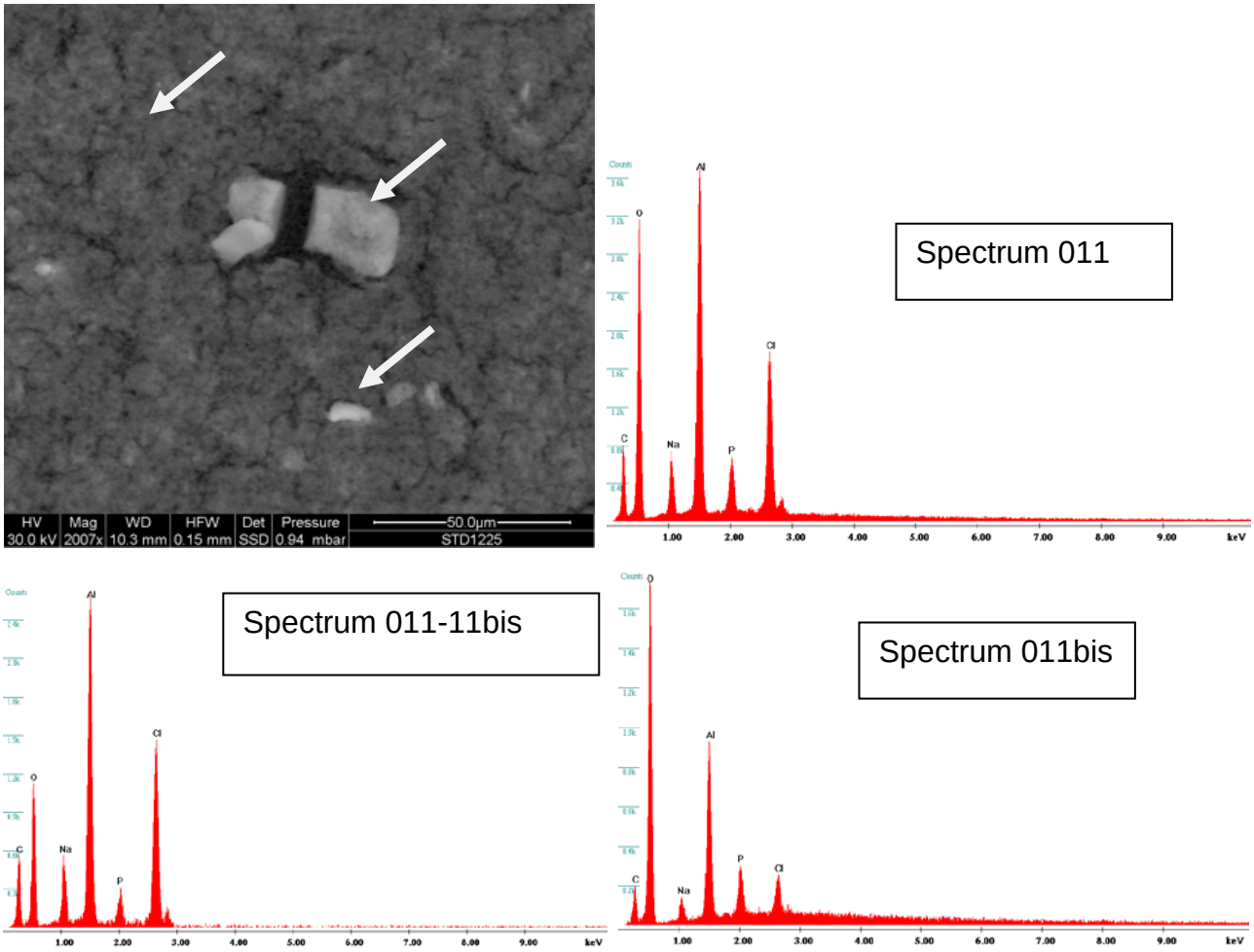


Image 11. The images show debris in the vaccine substrate (high magnification (2.007x) . The spectra identify different zones.

011 - Alluminum, Oxygen, Chlorine, Carbon, Sodium, Phosphorous

011- 11 bis Aluminum, Chlorine, Oxygen, Carbon, Sodium, Phosphorus,

011bis Oxygen, Aluminum, Phosphorus, Chlorine, Carbon, Sodium

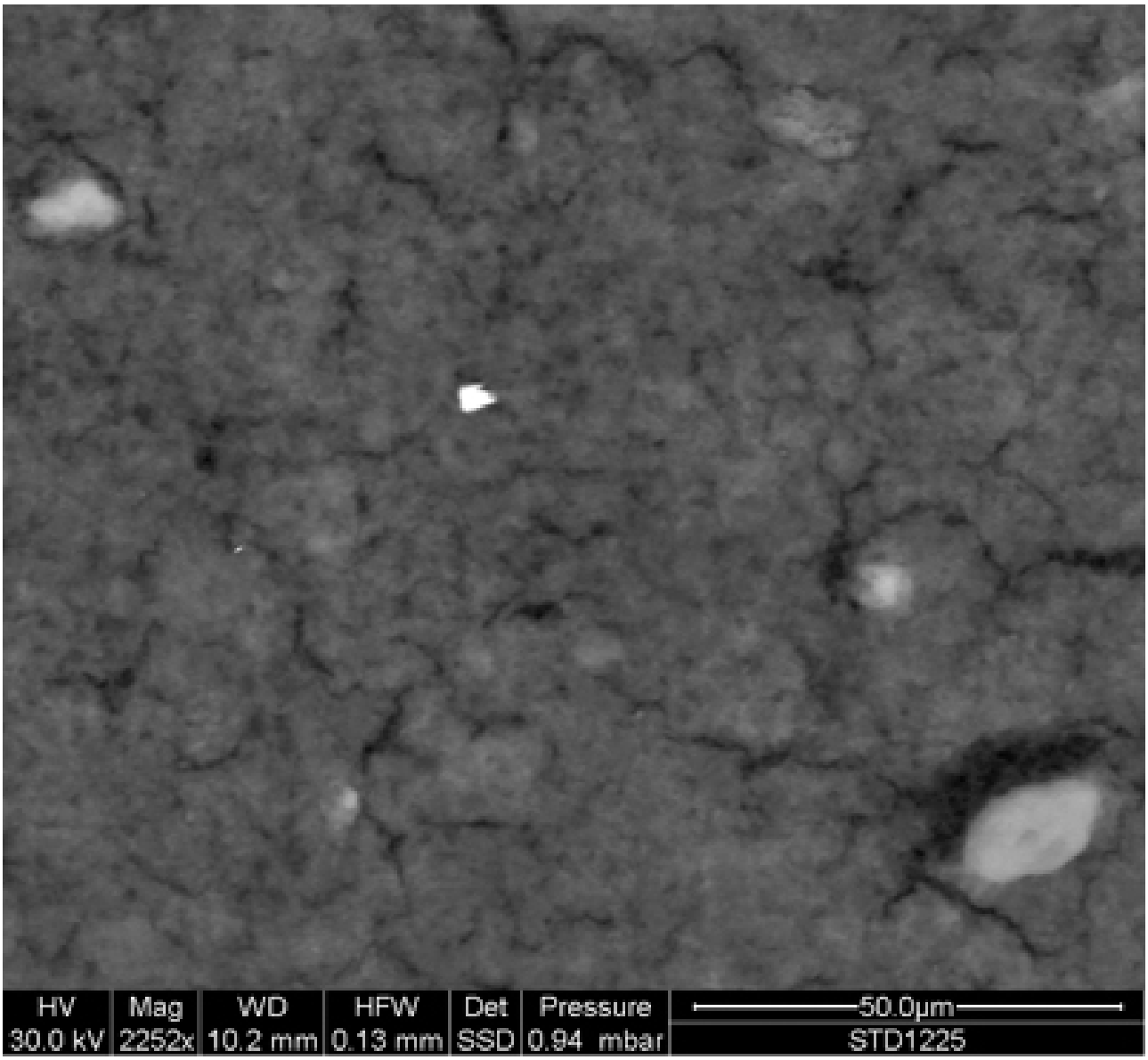


Image 12. The image shows an area of the sample at high magnification (2252x) where a bright debris is visible.

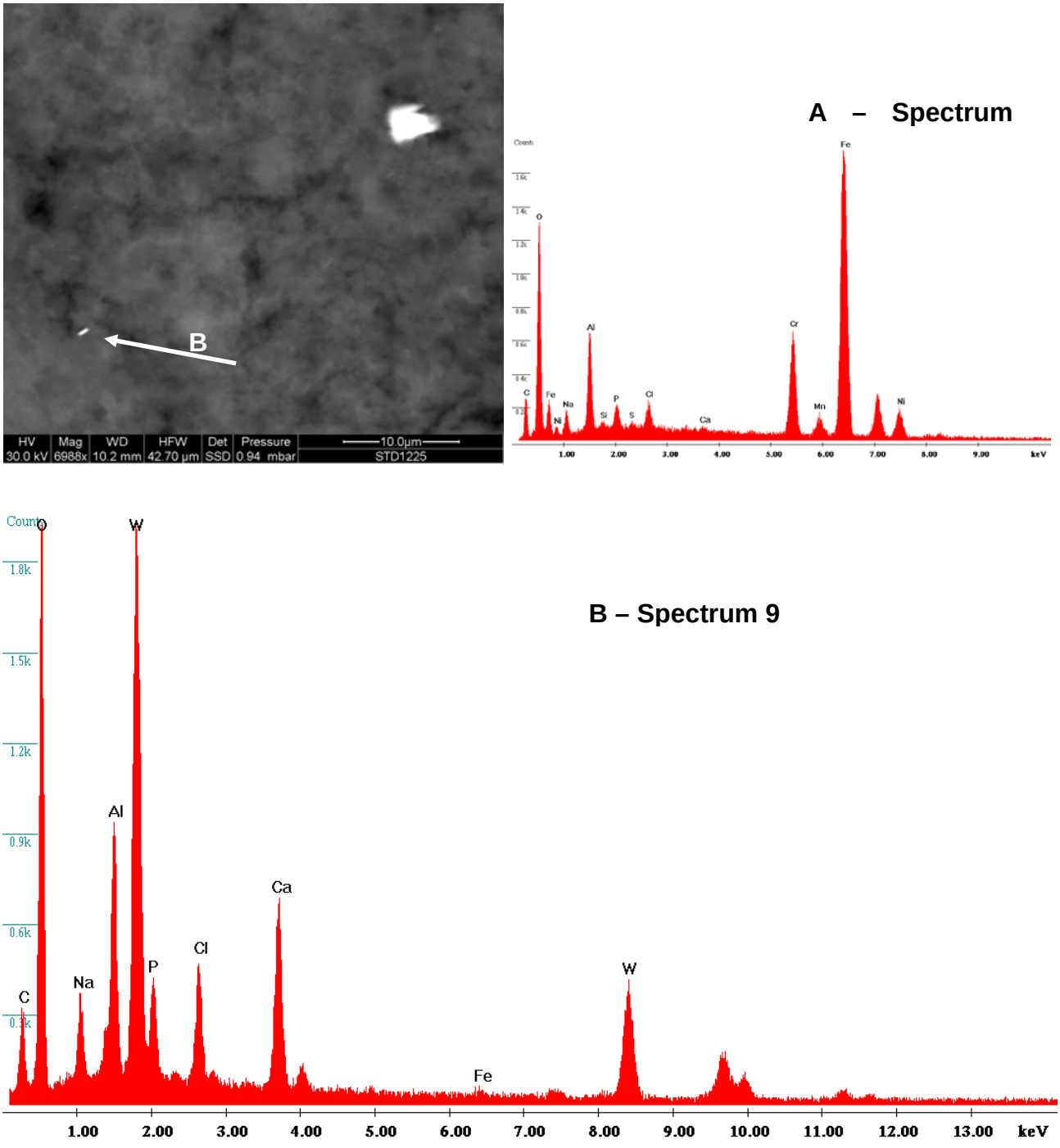
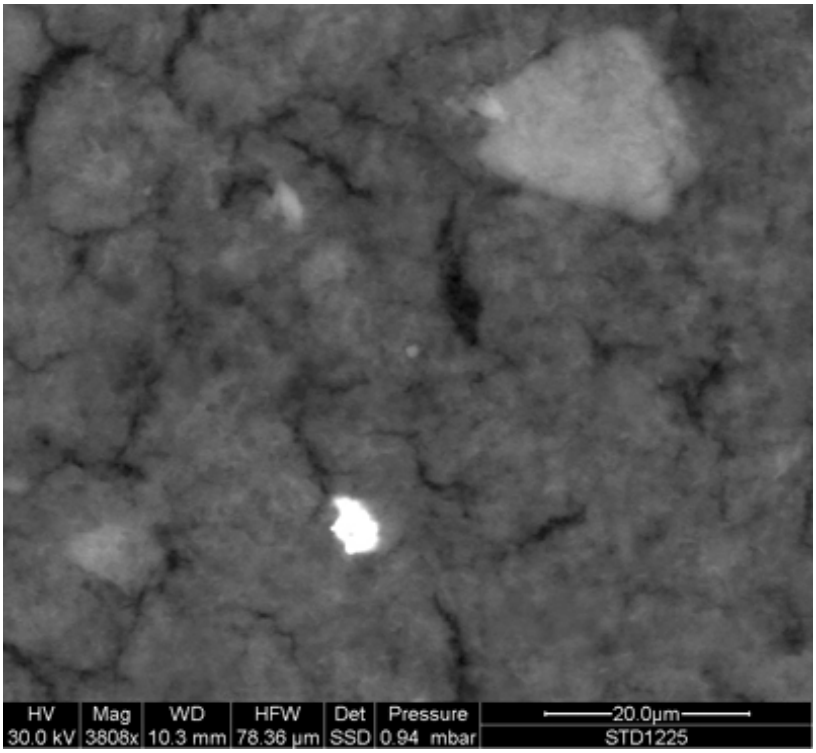


Image 13. The high-magnification (3.413x) photograph of the image of Analysis 12 shows an area where two particles, sized 5 and 0.8 microns respectively, can be seen. The 5-micron one (A) is composed of Iron, Oxygen, Aluminum, Chromium, Nickel, Chlorine, Phosphorous, Sodium, Silicon, Manganese, namely Stainless steel; while the 0.8-micron one (B) is composed of Oxygen, Tungsten, Aluminum, Calcium, Chlorine, Sodium, Phosphorous, Carbon and Iron, namely Tungsten as the other already identified..



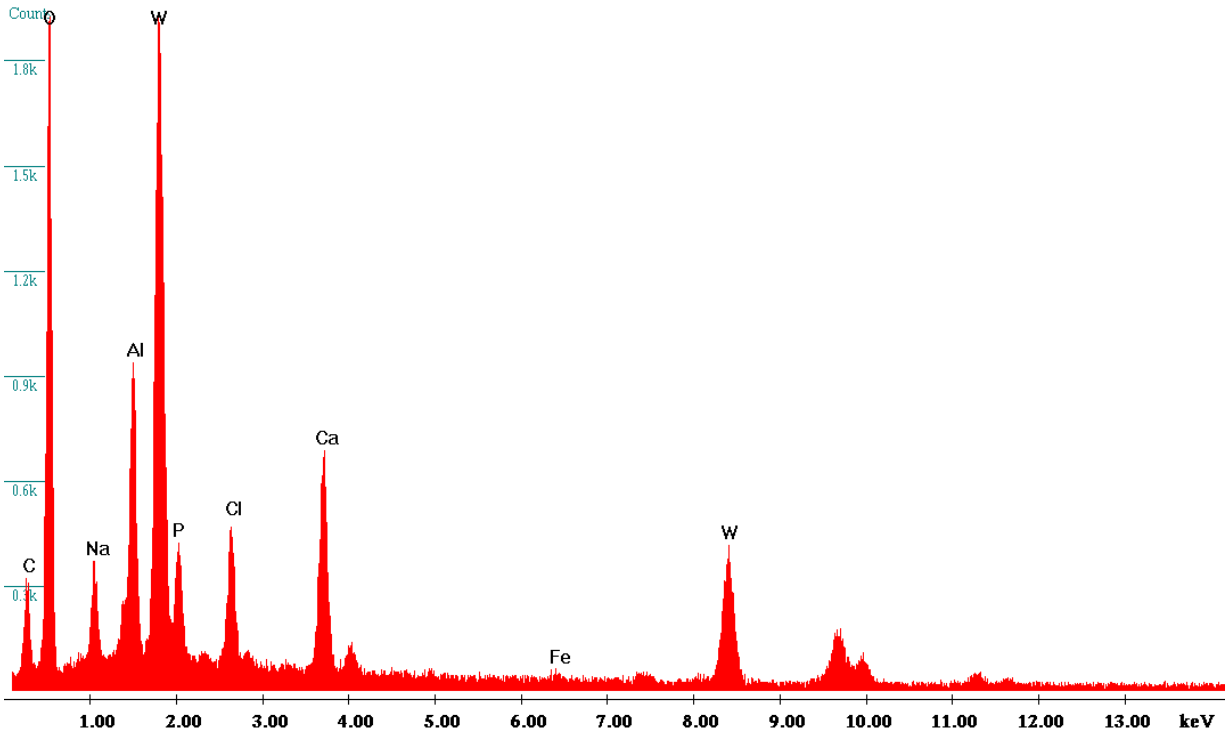


Image 14. The high-magnification (3808x) image shows a 7-micron detritus made of Oxygen, Tungsten, Aluminum, Calcium, Chlorine, Sodium, Phosphorous, Carbon and Iron. Again Tungsten is embedded in the Aluminum phosphatic precipitates.

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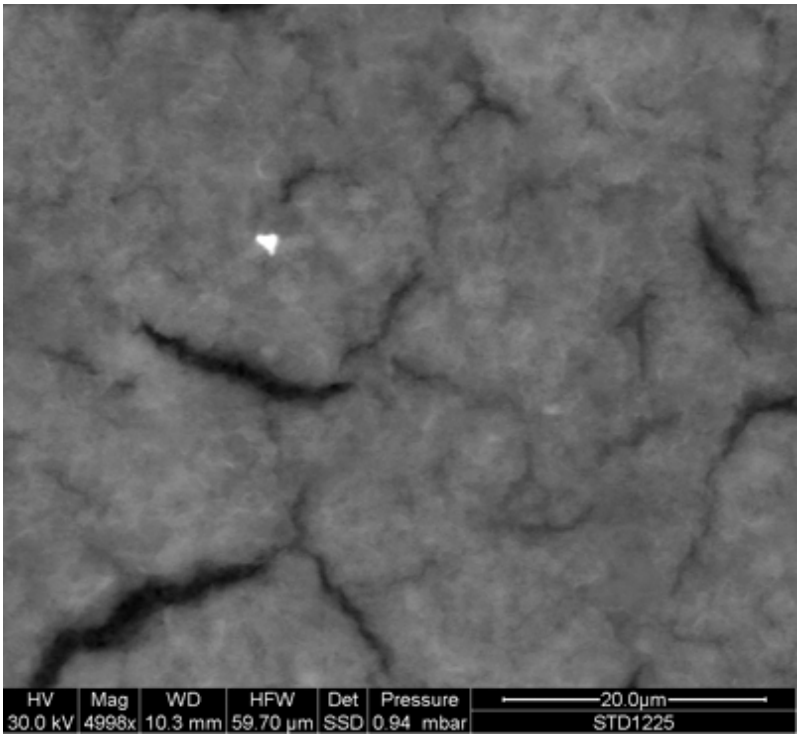

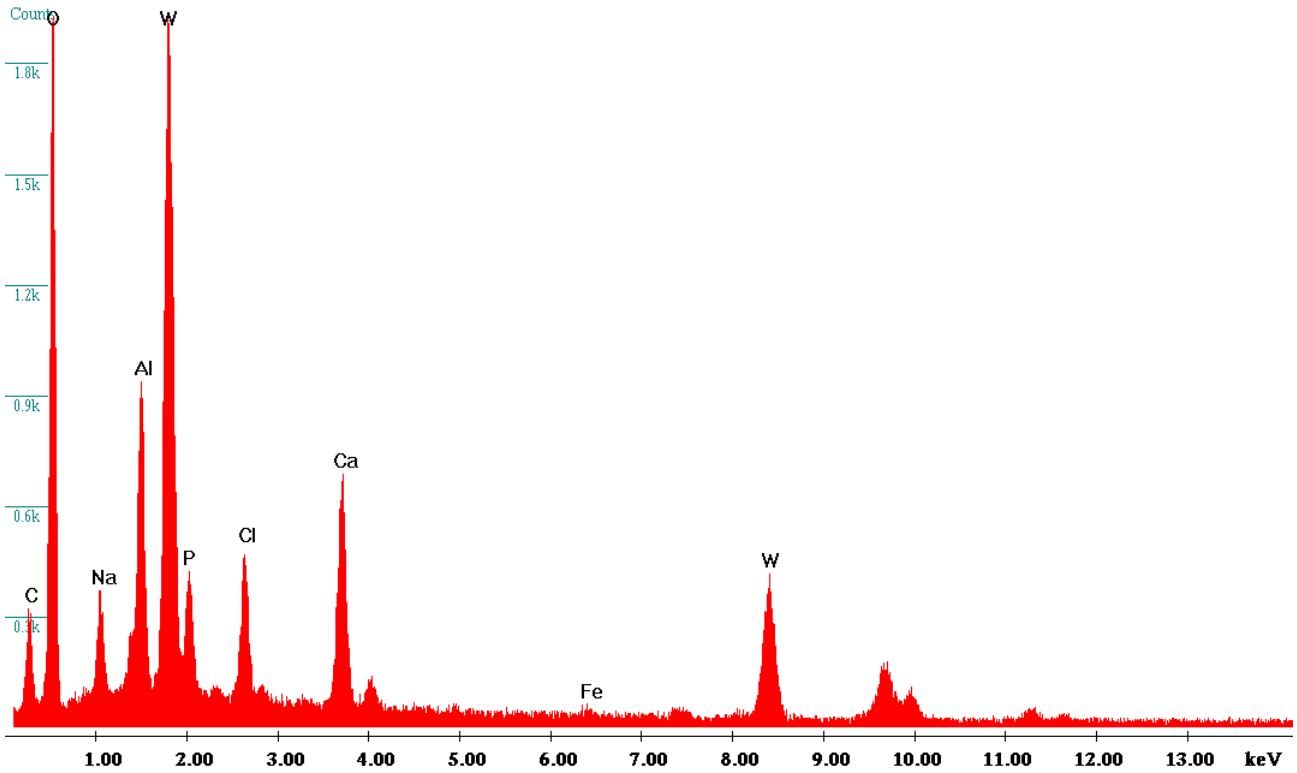

Image 15. A 1.5-micron particle is shown in the high-magnification (4998x) image. Its composition is Oxygen, Tungsten, Aluminum, Calcium, Chlorine, Sodium, Phosphorous, Carbon and Iron, a qualitative combination often observed throughout the sample.

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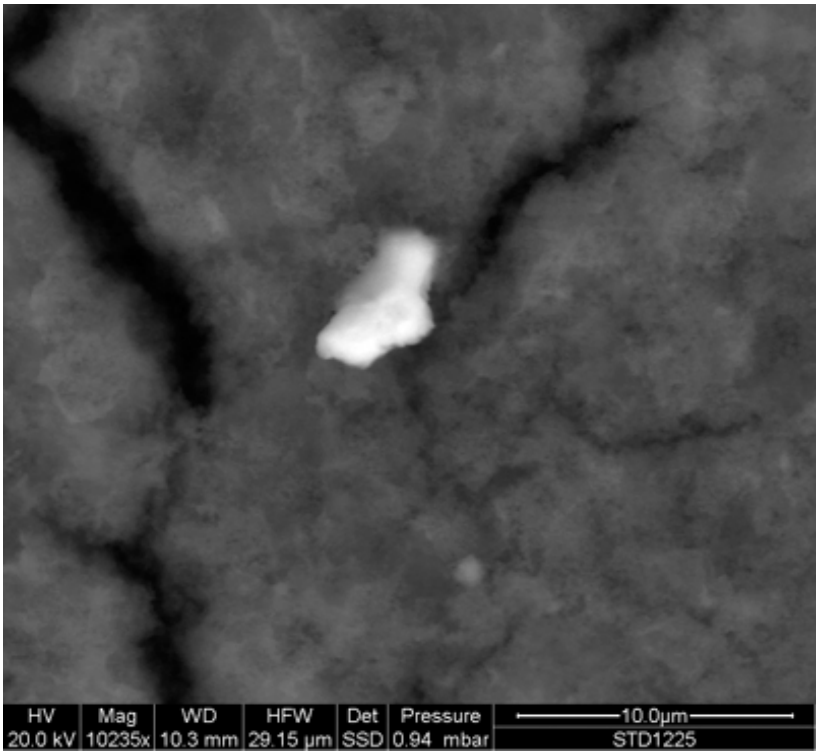


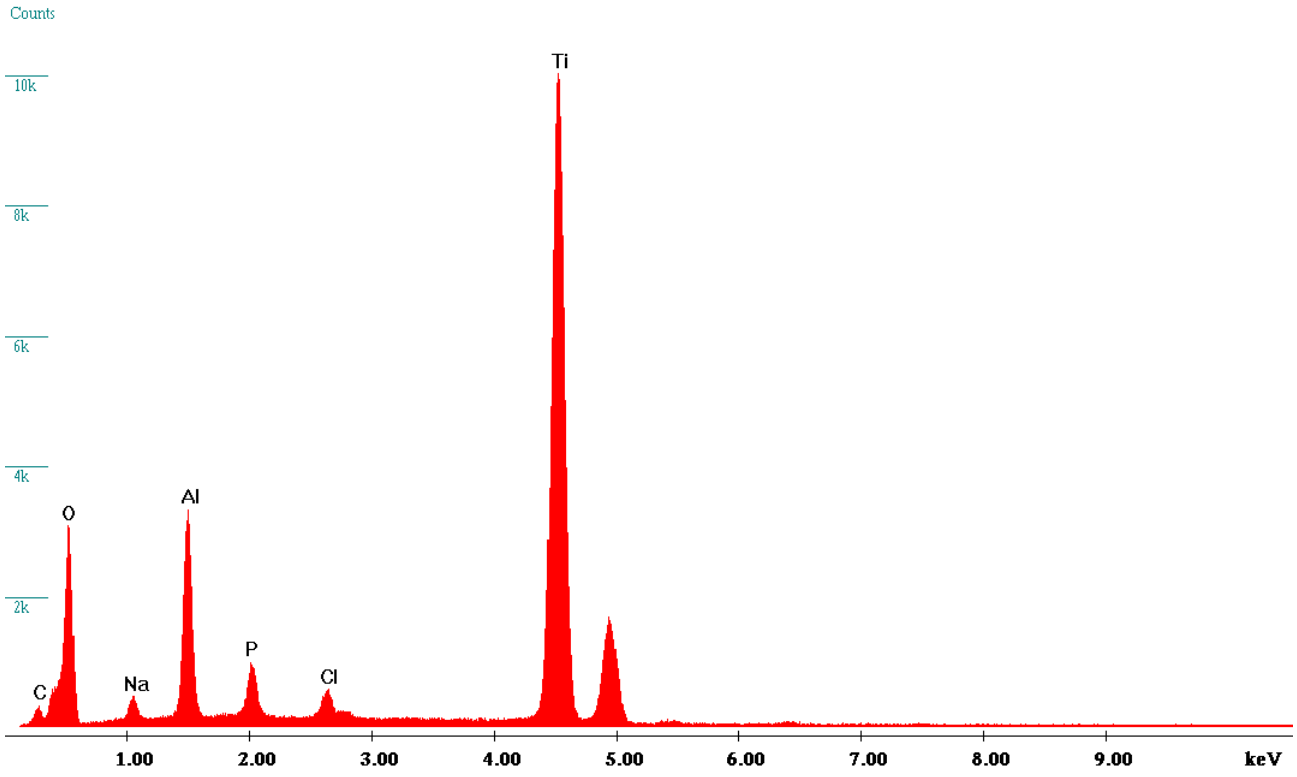
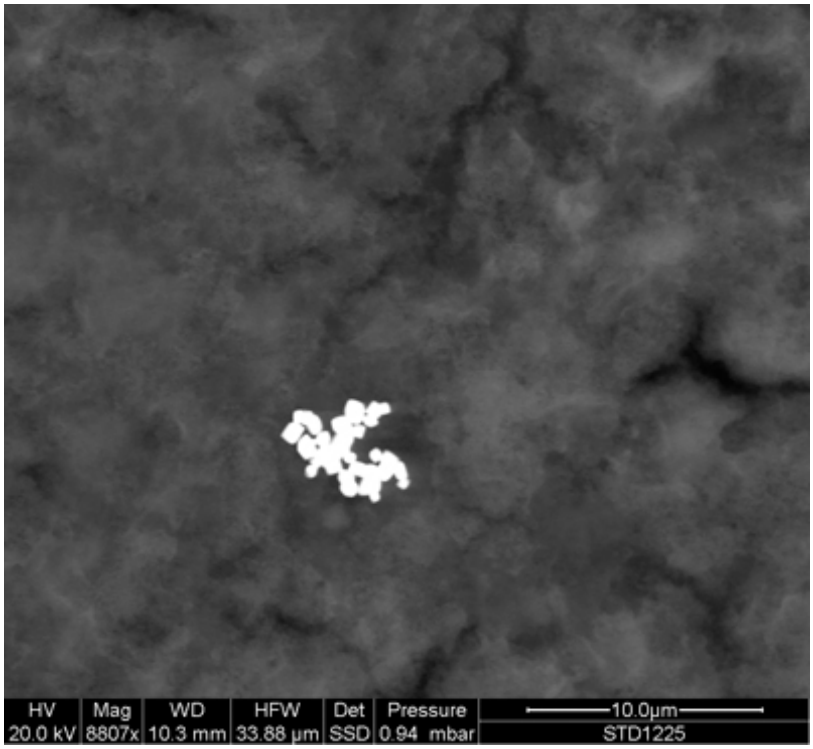


Image 16. The image shows a 5-micron particle (10235x) mainly composed of Titanium in the Aluminum background.



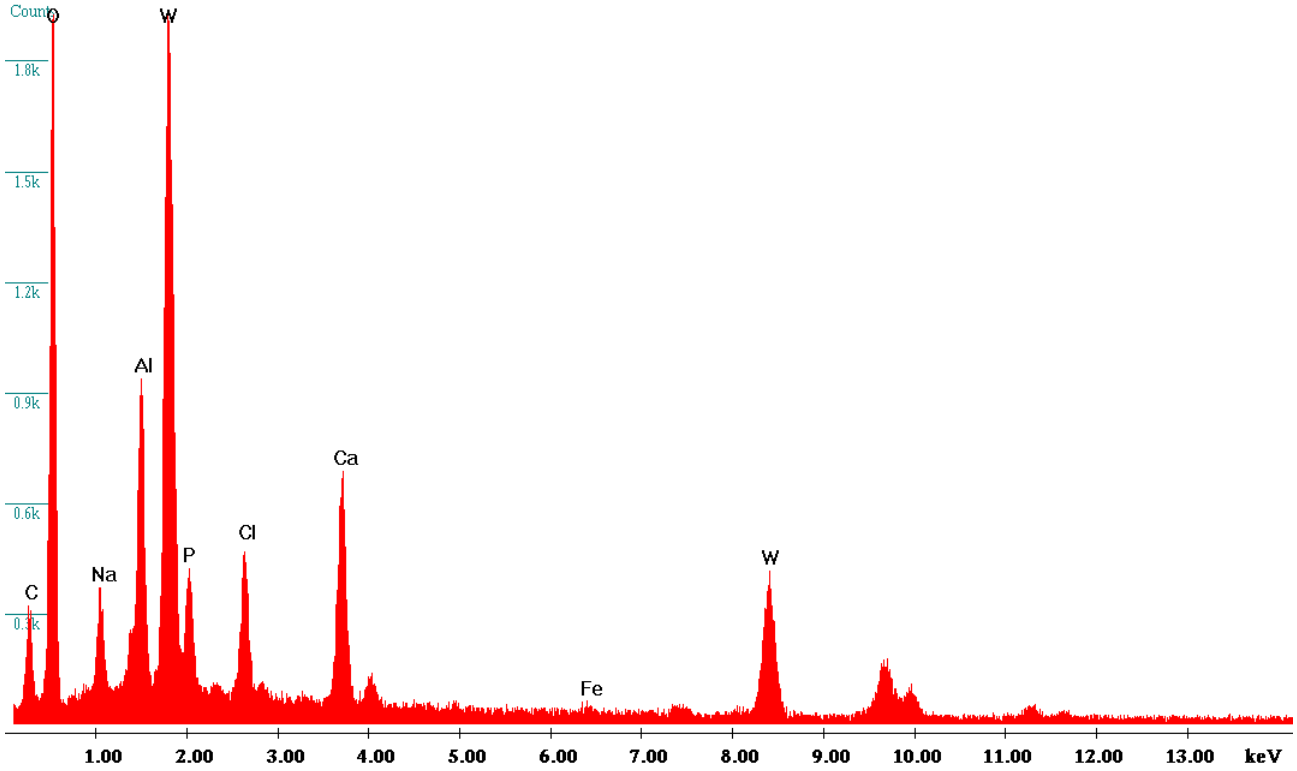
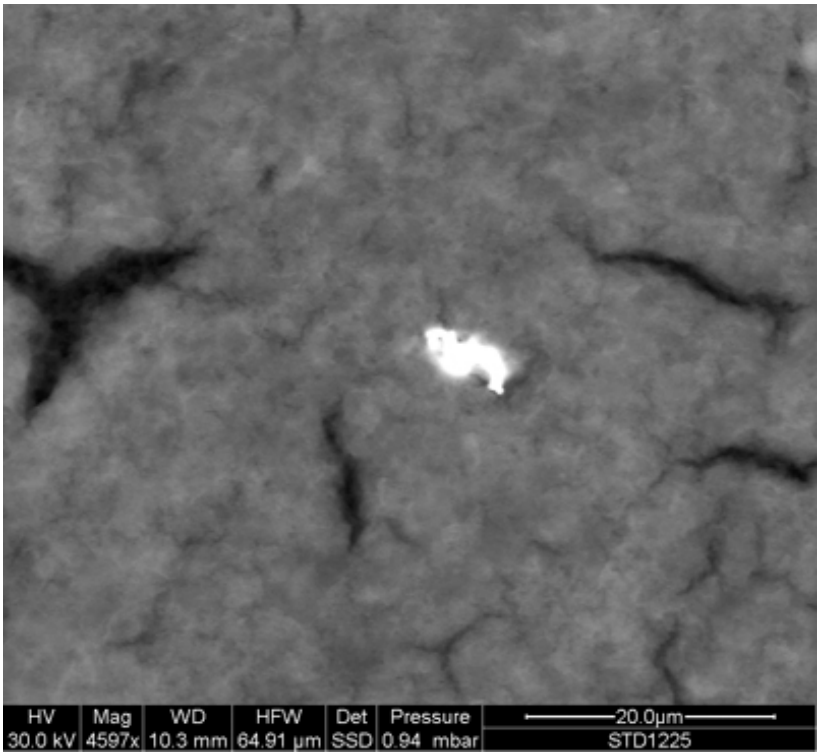


Image 17. Cluster of cubic particles, (8807x) with a size below 0.5 microns is shown. The composition of the particles is Oxygen, Tungsten, Aluminum, Calcium, Chlorine, Sodium, Phosphorous, Carbon and Iron: the qualitative composition of many of the particles found in the sample.



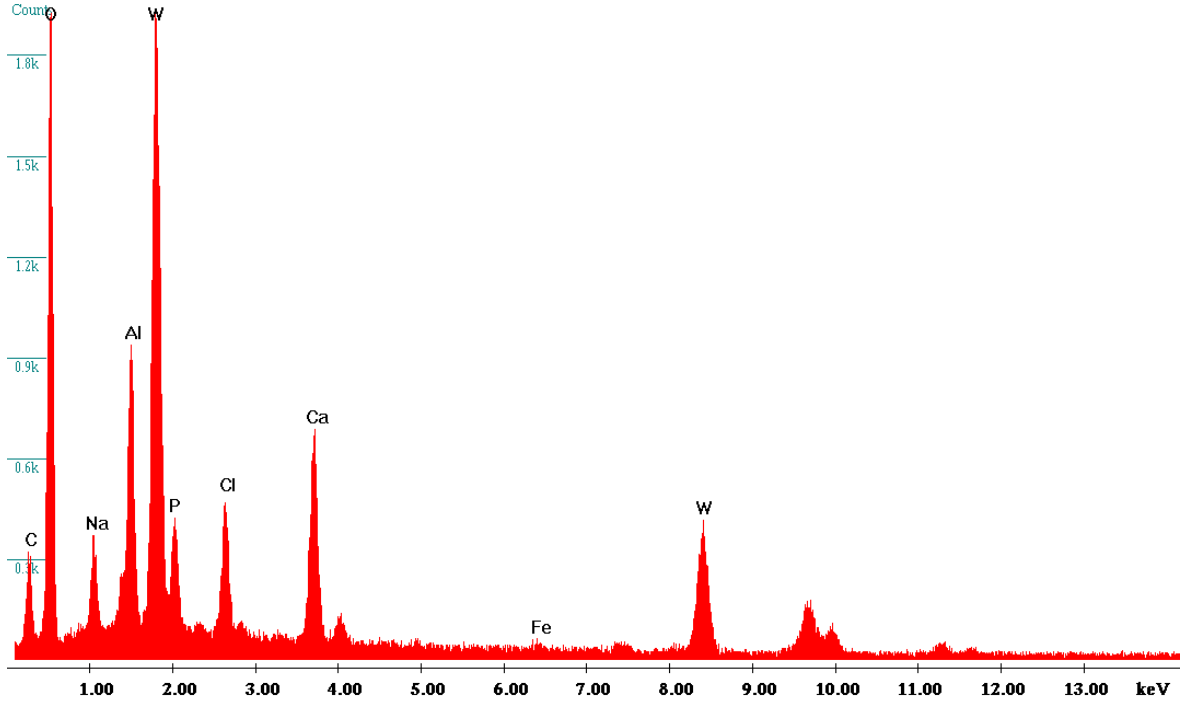
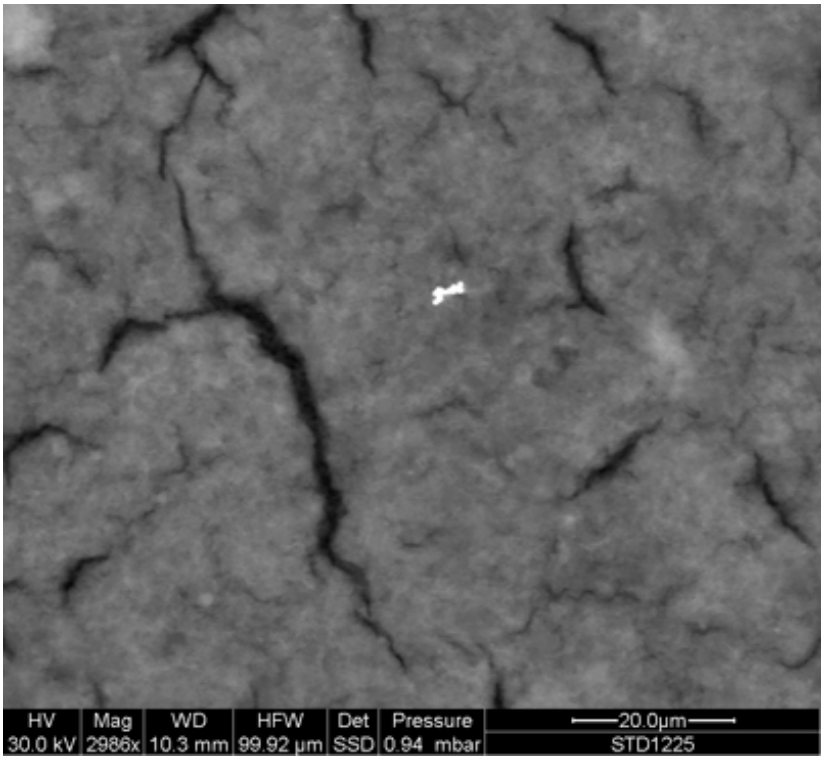


Image 18. Another aggregate of Tungsten debris is observed (4597x). Many of the particles composing it have a size smaller than 0.5 microns. The qualitative composition is the same as the one observed in many other particles in this specimen: Oxygen, Tungsten, Aluminum, Calcium, Chlorine, Sodium, Phosphorous, Carbon and Iron.



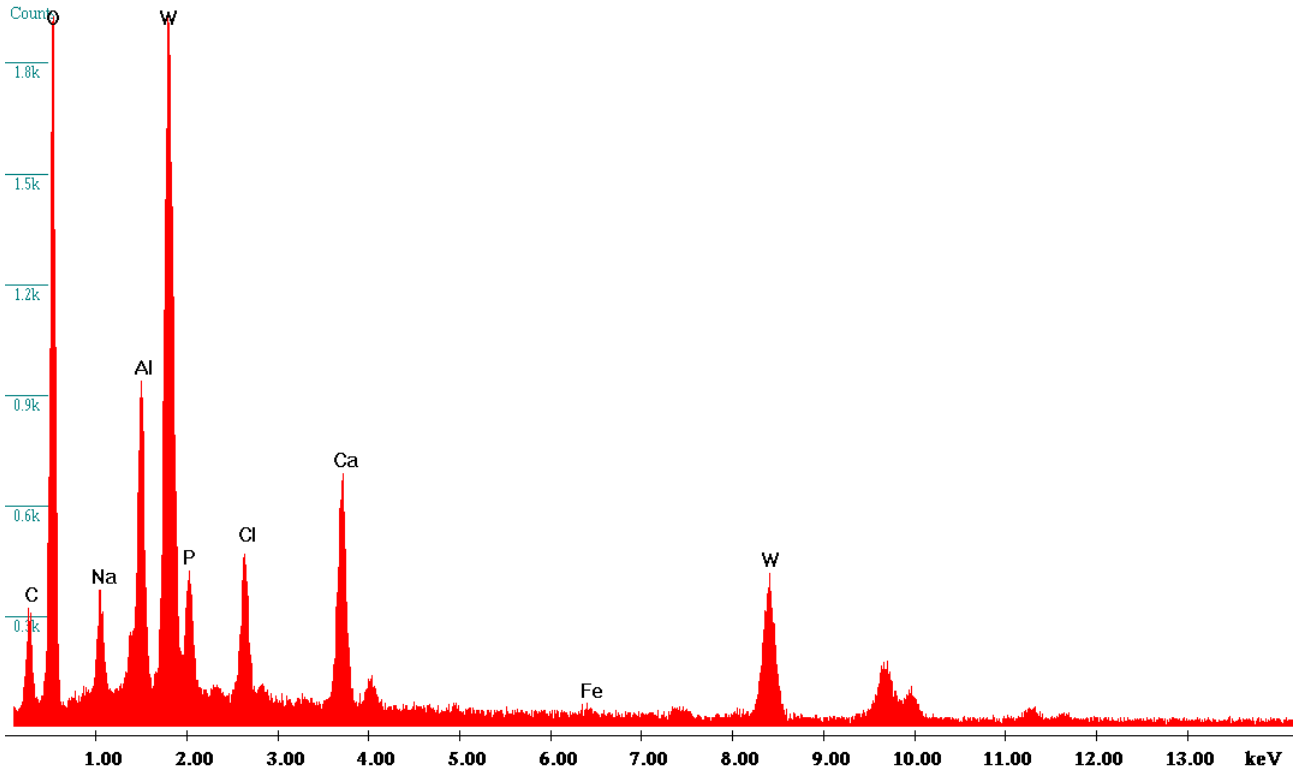
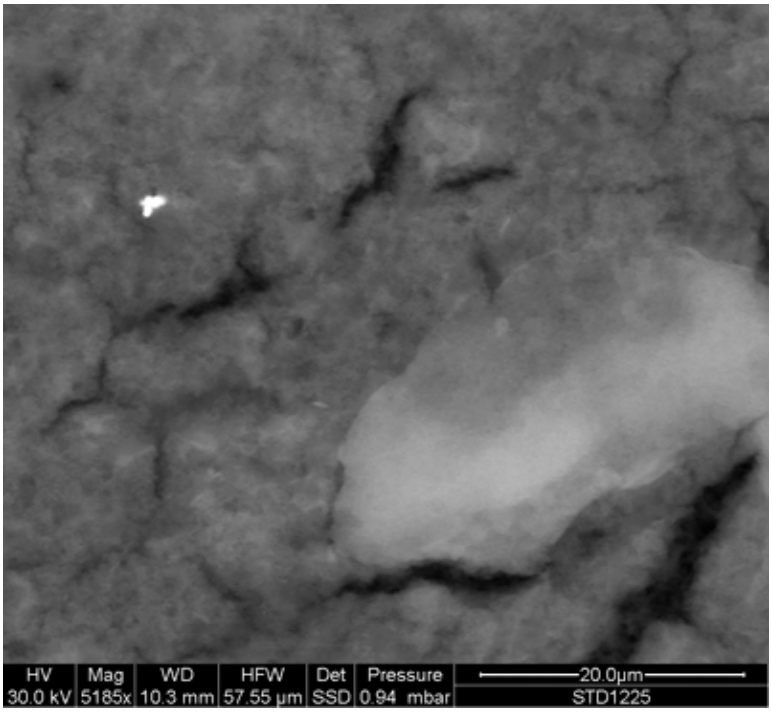


Image 19. A debris composed of smaller particles seen at high magnification (2986x) shows the much detected qualitative composition: Oxygen, Tungsten, Aluminum, Calcium, Chlorine, Sodium, Phosphorous, Carbon and Iron.



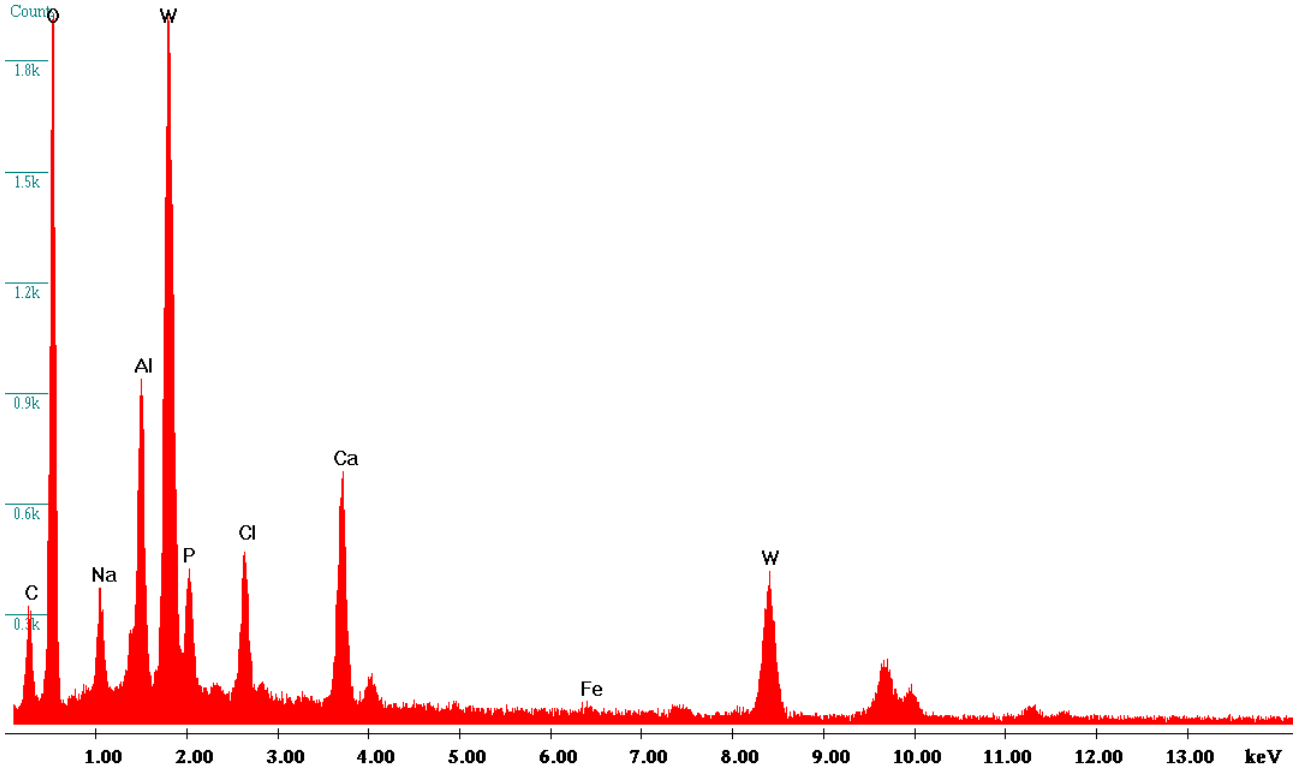
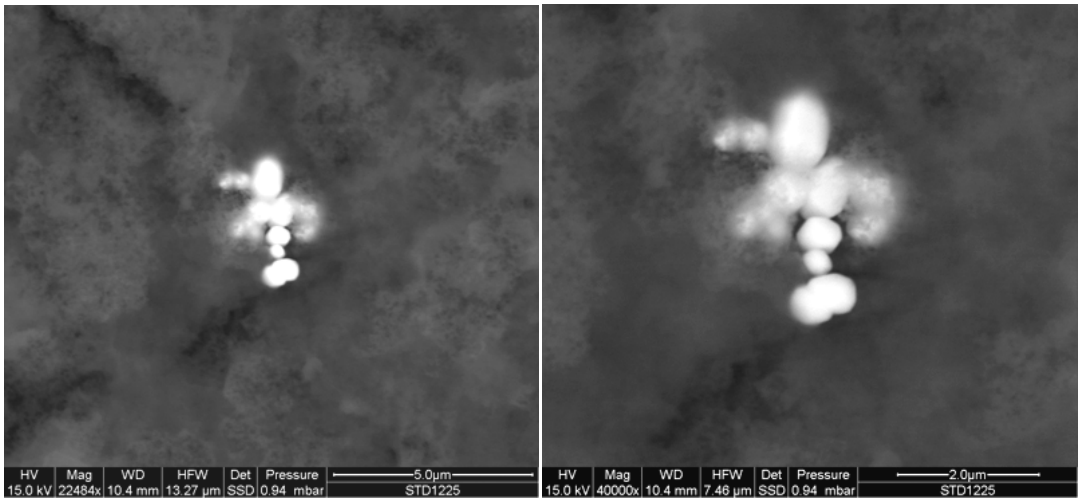


Image 20. At a magnification of 2986x, a debris sized below 1 micron and composed of much smaller particles can be seen. Its composition is qualitatively the same as very often detected in this sample: Oxygen, Tungsten, Aluminum, Calcium, Chlorine, Sodium, Phosphorous, Carbon and Iron.



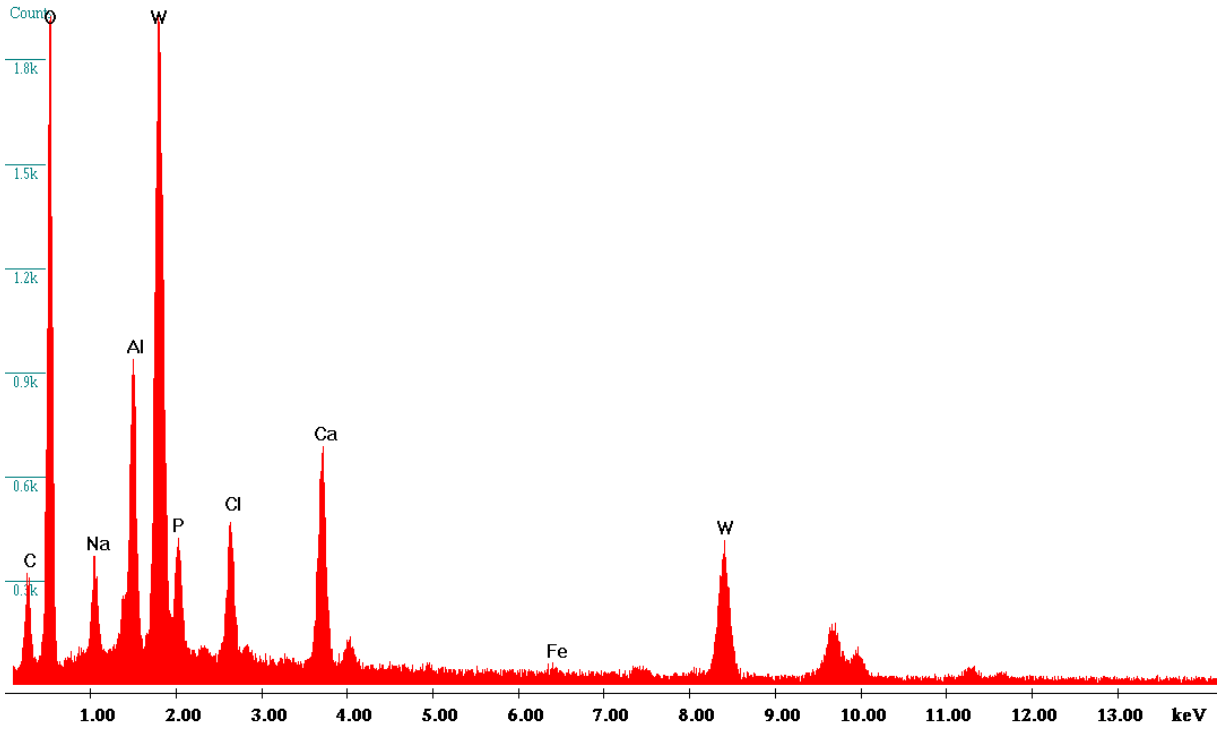
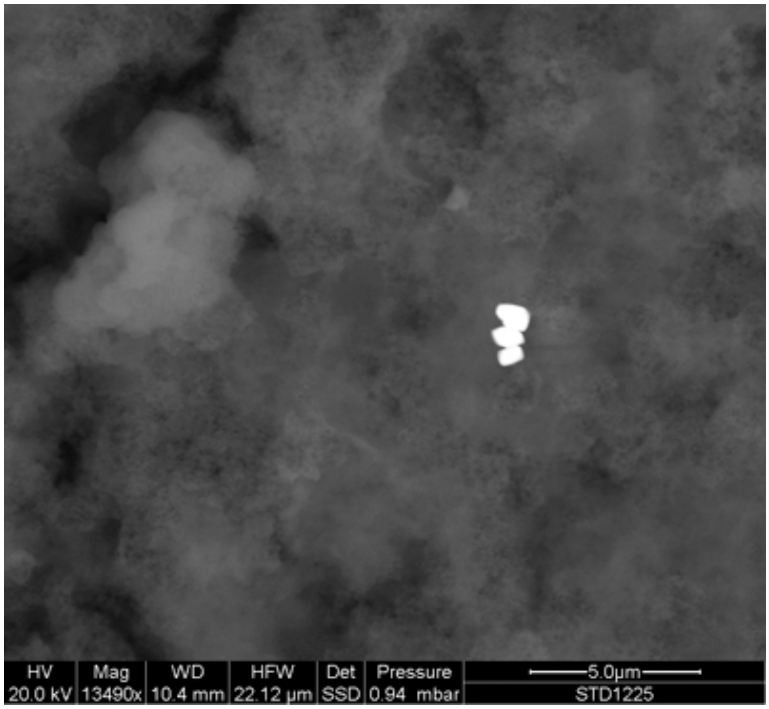


Image 21. At two different magnifications (22484x and 40000x), the images show an aggregate of Tungsten particles (sized 1 micron and below). The elemental composition is the “usual” one: Oxygen, Tungsten, Aluminum, Calcium, Chlorine, Sodium, Phosphorous, Carbon and Iron.



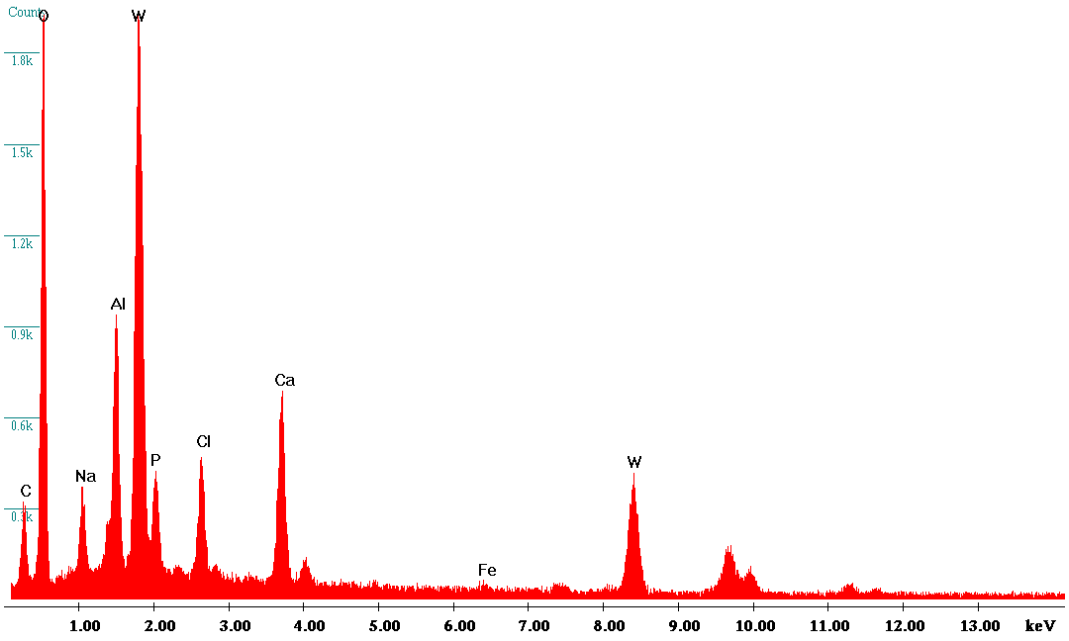
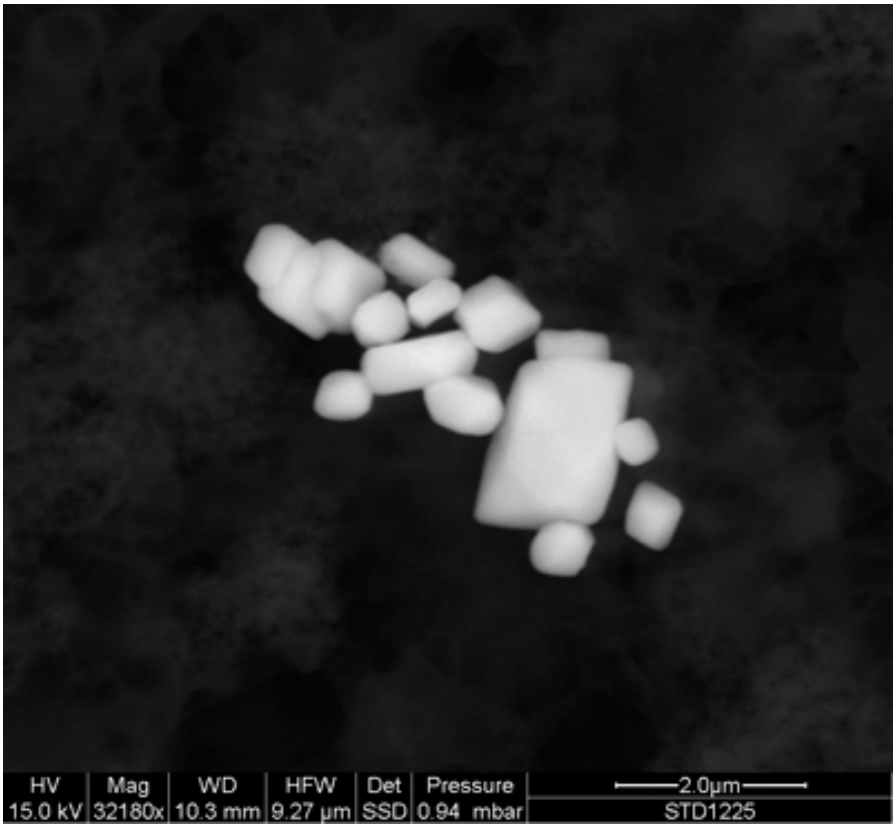


Image 22. At 13490x, three particles sized 1 micron can be seen. The composition is the same as that of many other particles observed in this sample: Oxygen, Tungsten, Aluminum, Calcium, Chlorine, Sodium, Phosphorous, Carbon and Iron.



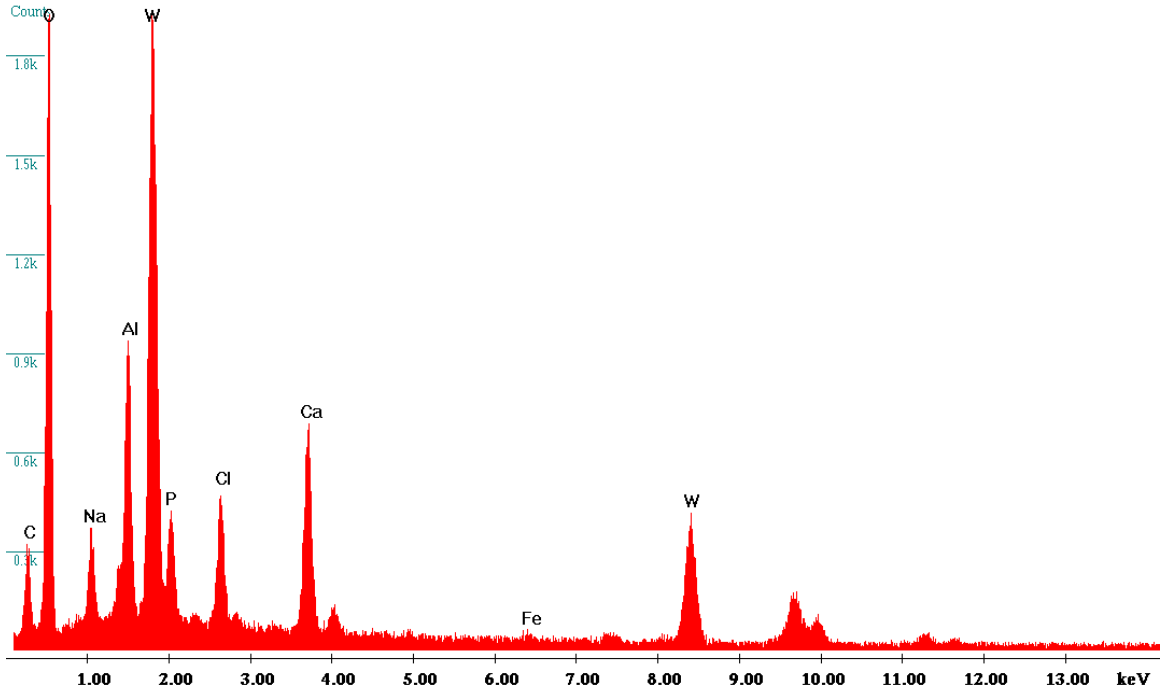
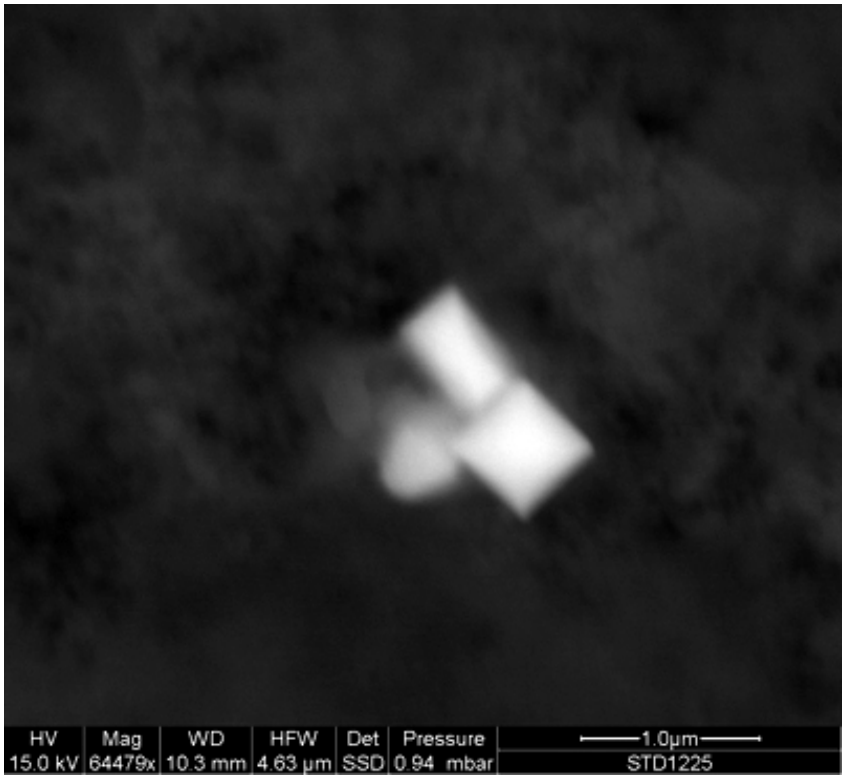


Image 23. The high-magnification (32180x) image shows an aggregate of Tungsten particles, sized 0.4-1 micron. Their composition is Oxygen, Tungsten, Aluminum, Calcium, Chlorine, Sodium, Phosphorous, Carbon and Iron.



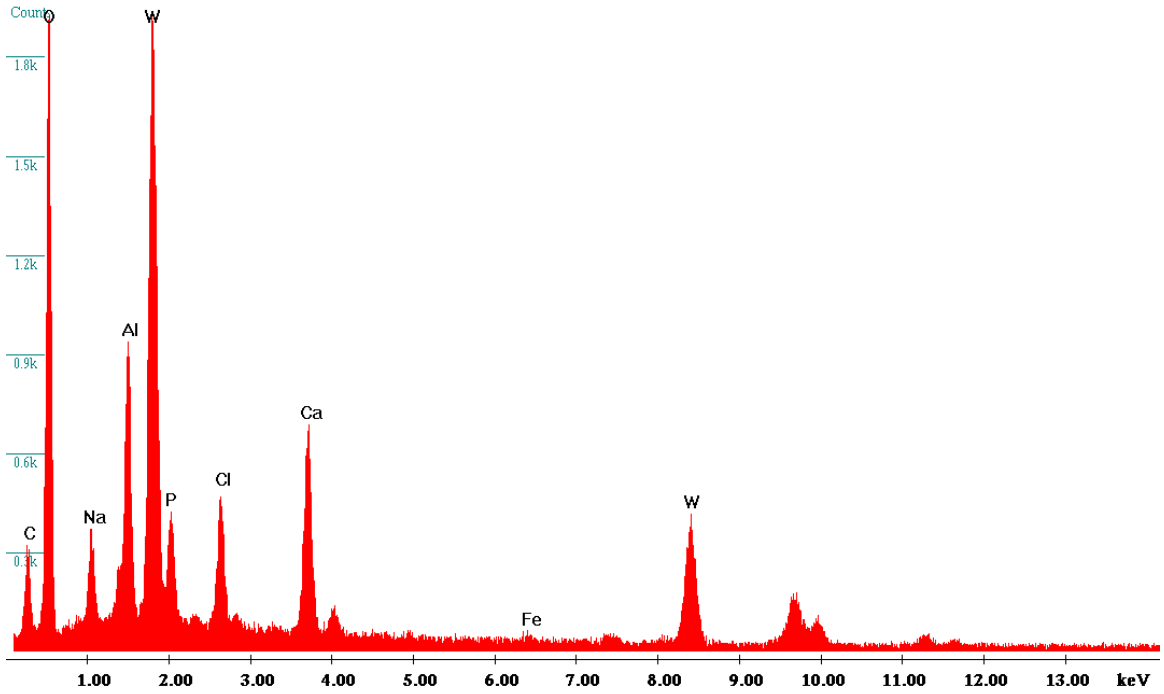
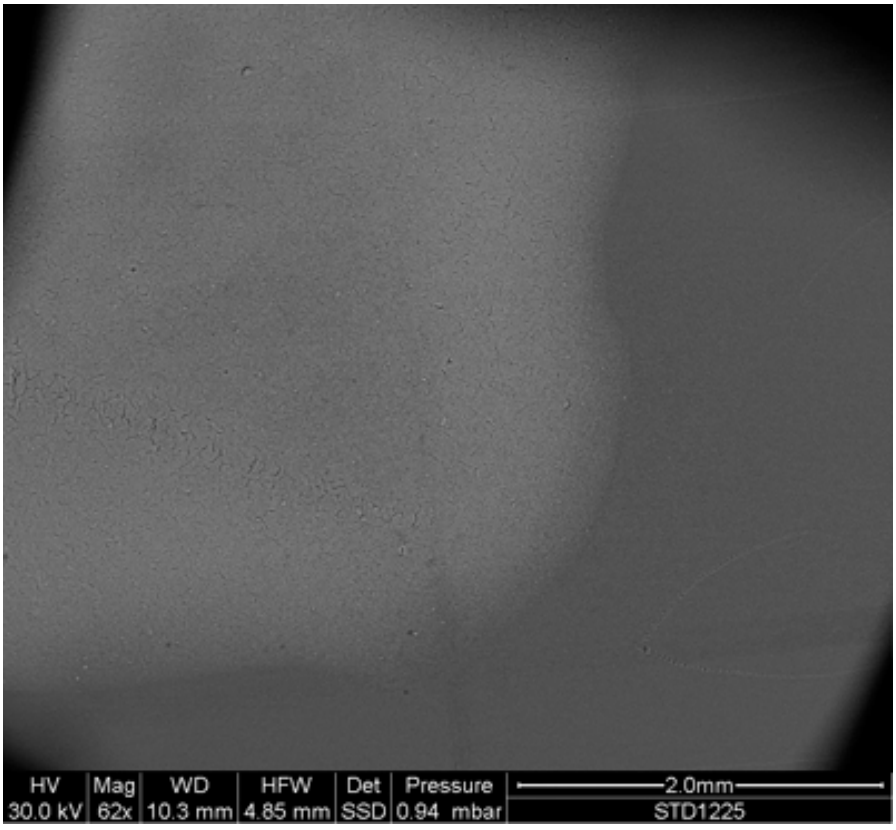


Image 24. At high magnification (64479x) particles with cubic morphology are visible. They have the same composition as many others found in this sample: Oxygen, Tungsten, Aluminum, Calcium, Chlorine, Sodium, Phosphorous, Carbon and Iron.



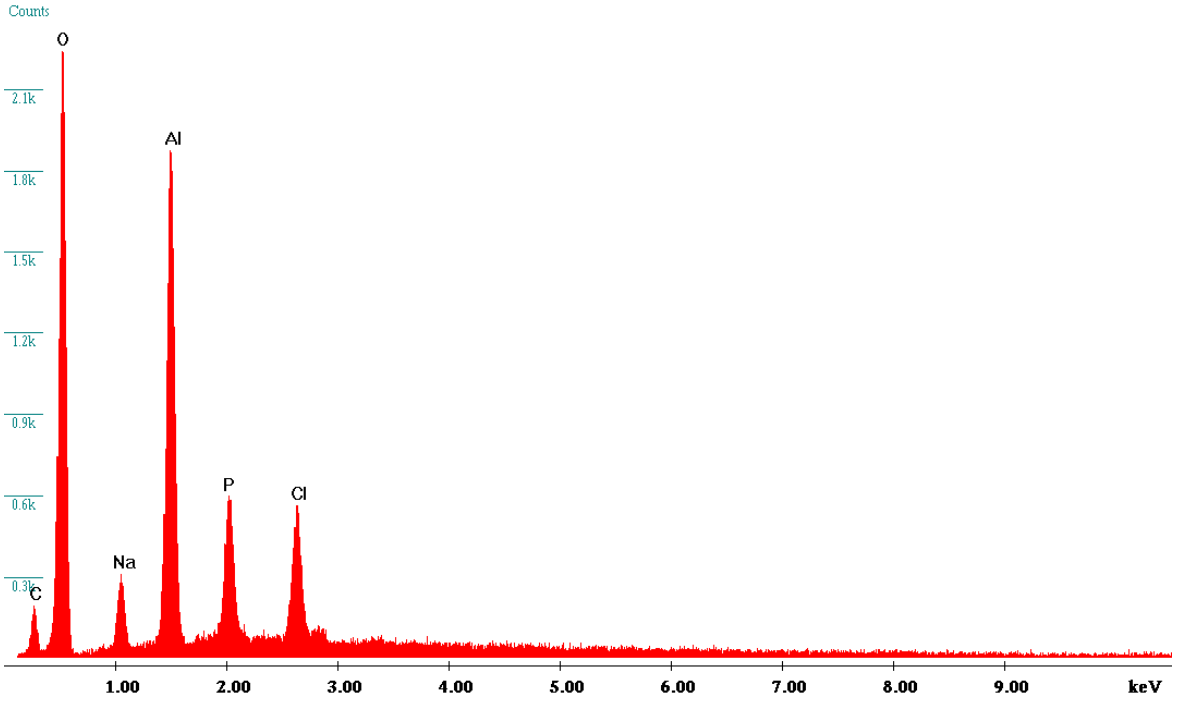
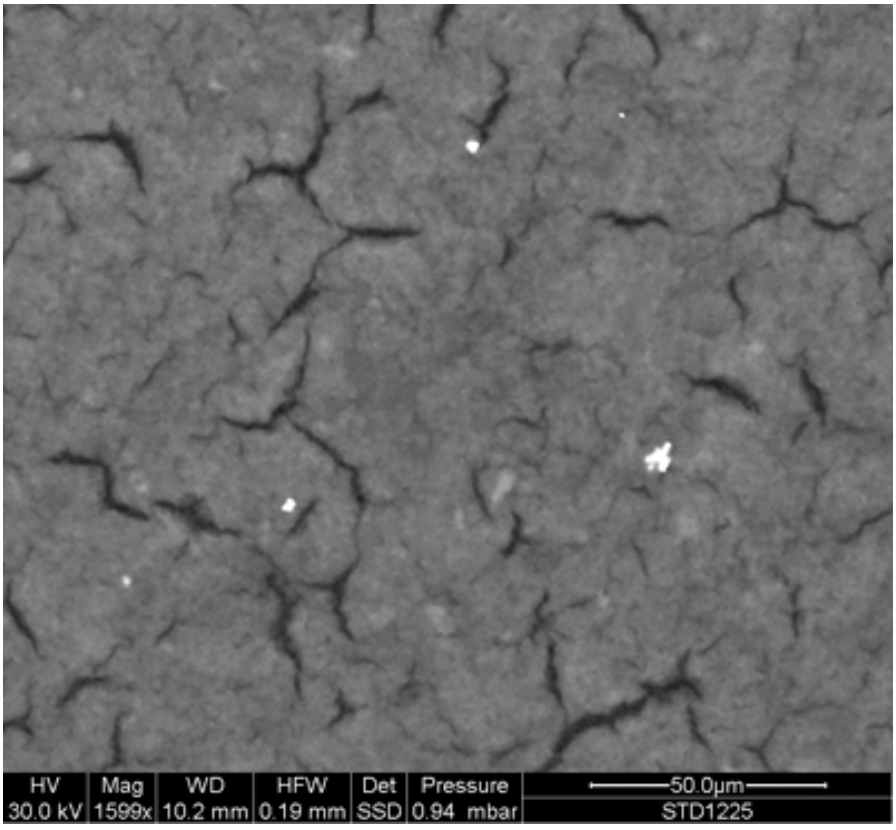


Image 25. The image shows another area of the vaccine droplet where only the Aluminum precipitates are present. The EDS spectrum identifies the drug's composition: Oxygen, Aluminum, Phosphorus, Chlorine, Sodium, Carbon.



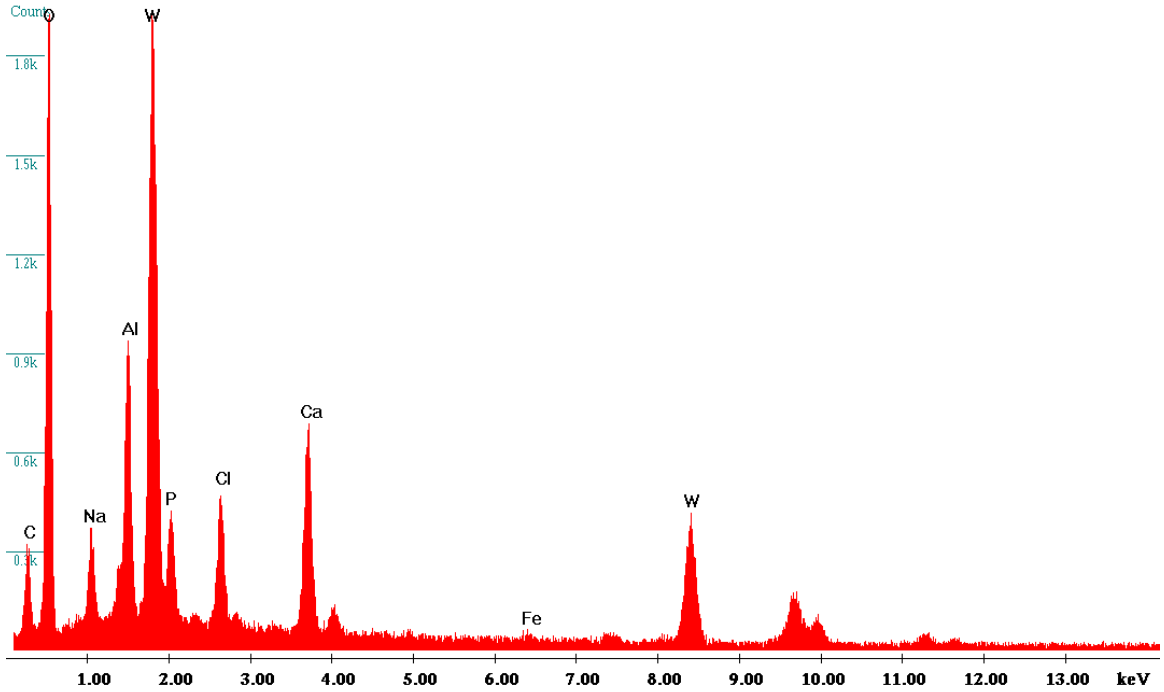
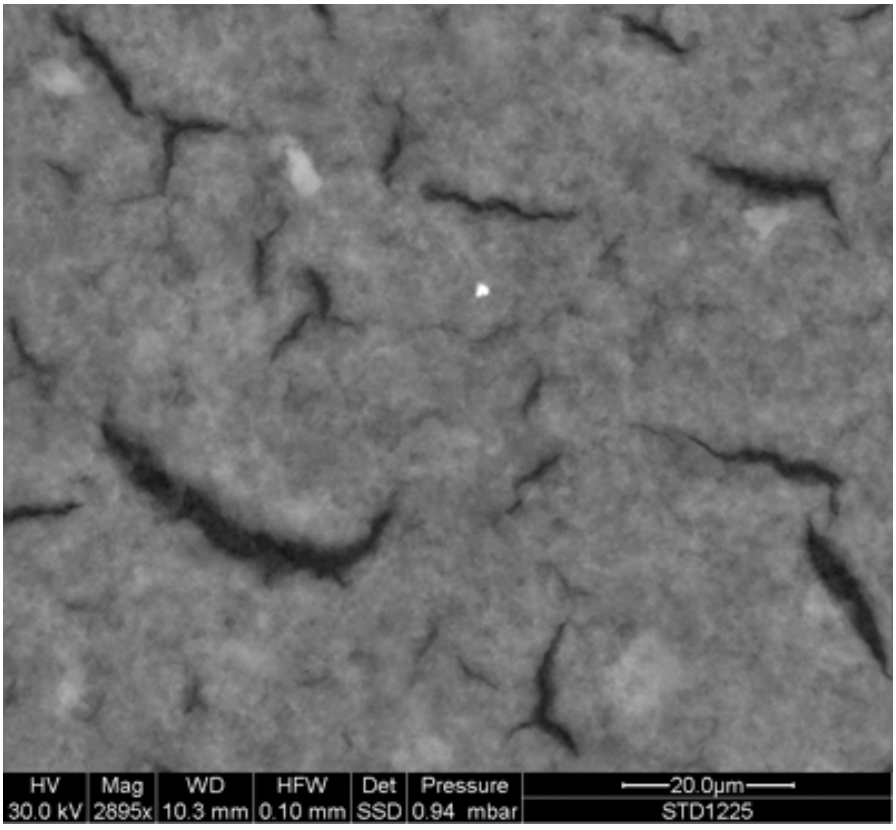


Image 26. The image shows other Tungsten-based debris (1599x).They are composed of Oxygen, Tungsten, Aluminum, Calcium, Chlorine, Sodium, Phosphorous, Carbon and Iron.



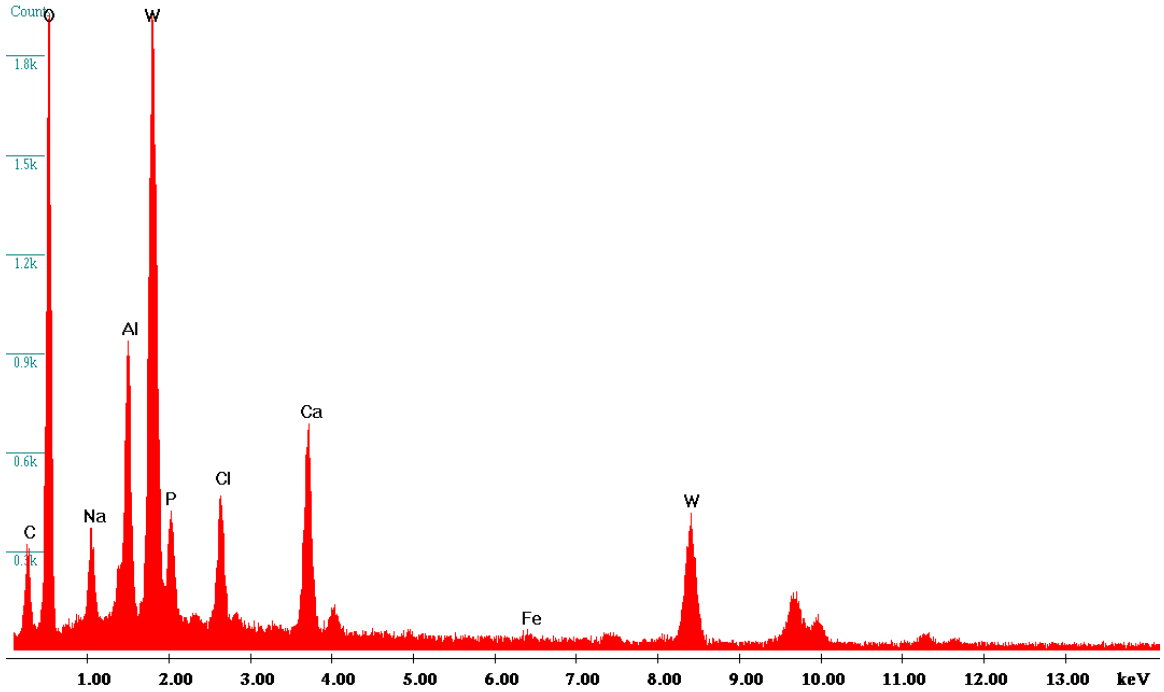


Image 27. The photograph at high magnification (2895x) shows a debris slightly larger than 1 micron whose composition is Oxygen, Tungsten, Aluminum, Calcium, Chlorine, Sodium, Phosphorous, Carbon and Iron.

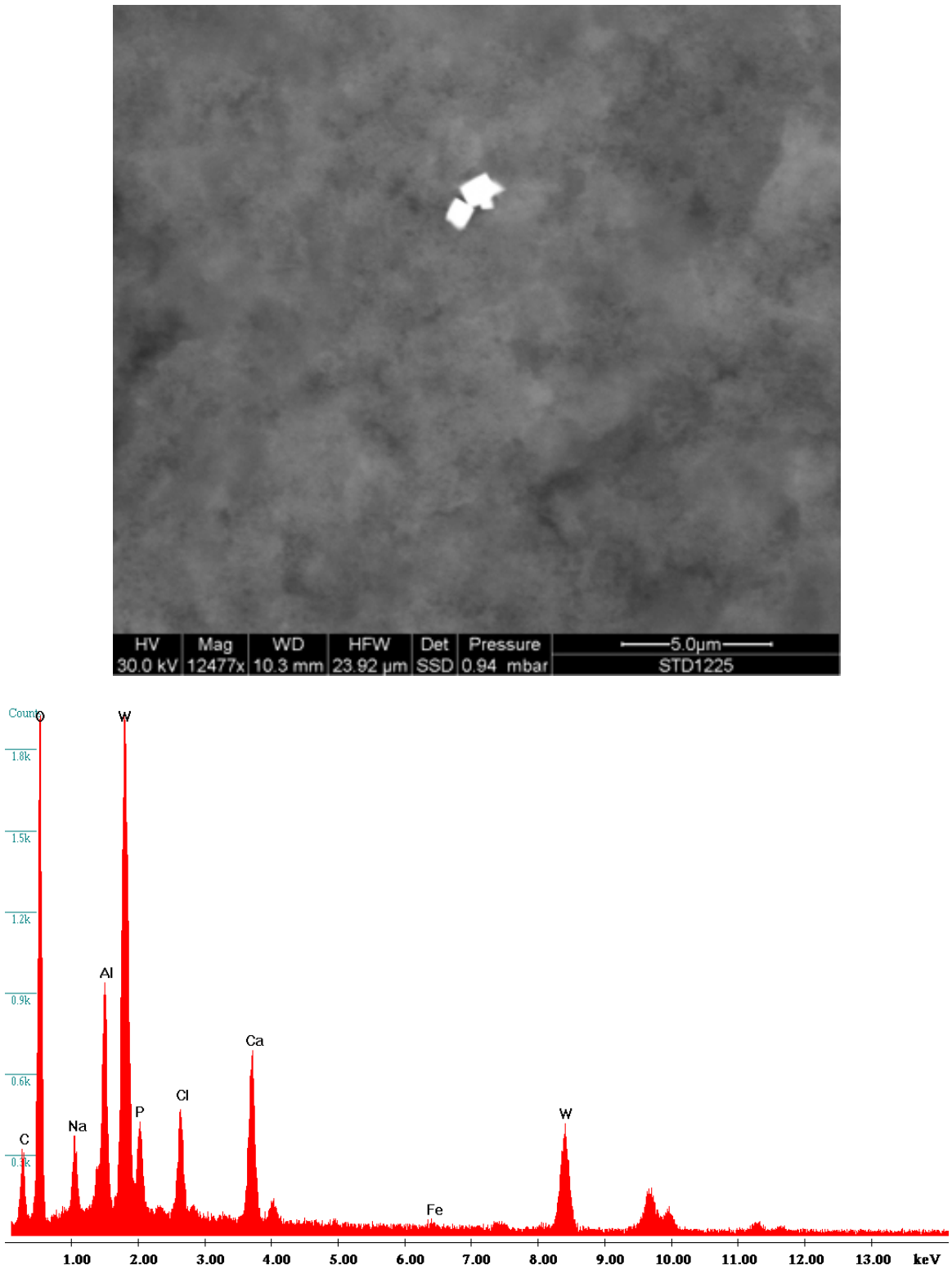


Image 28. At high magnification (12477x) three particles are shown with a squared morphology, only one of which is larger than 1 micron. Like many other particles detected in the sample, also they are made of Oxygen, Tungsten, Aluminum, Calcium, Chlorine, Sodium, Phosphorous, Carbon and Iron.

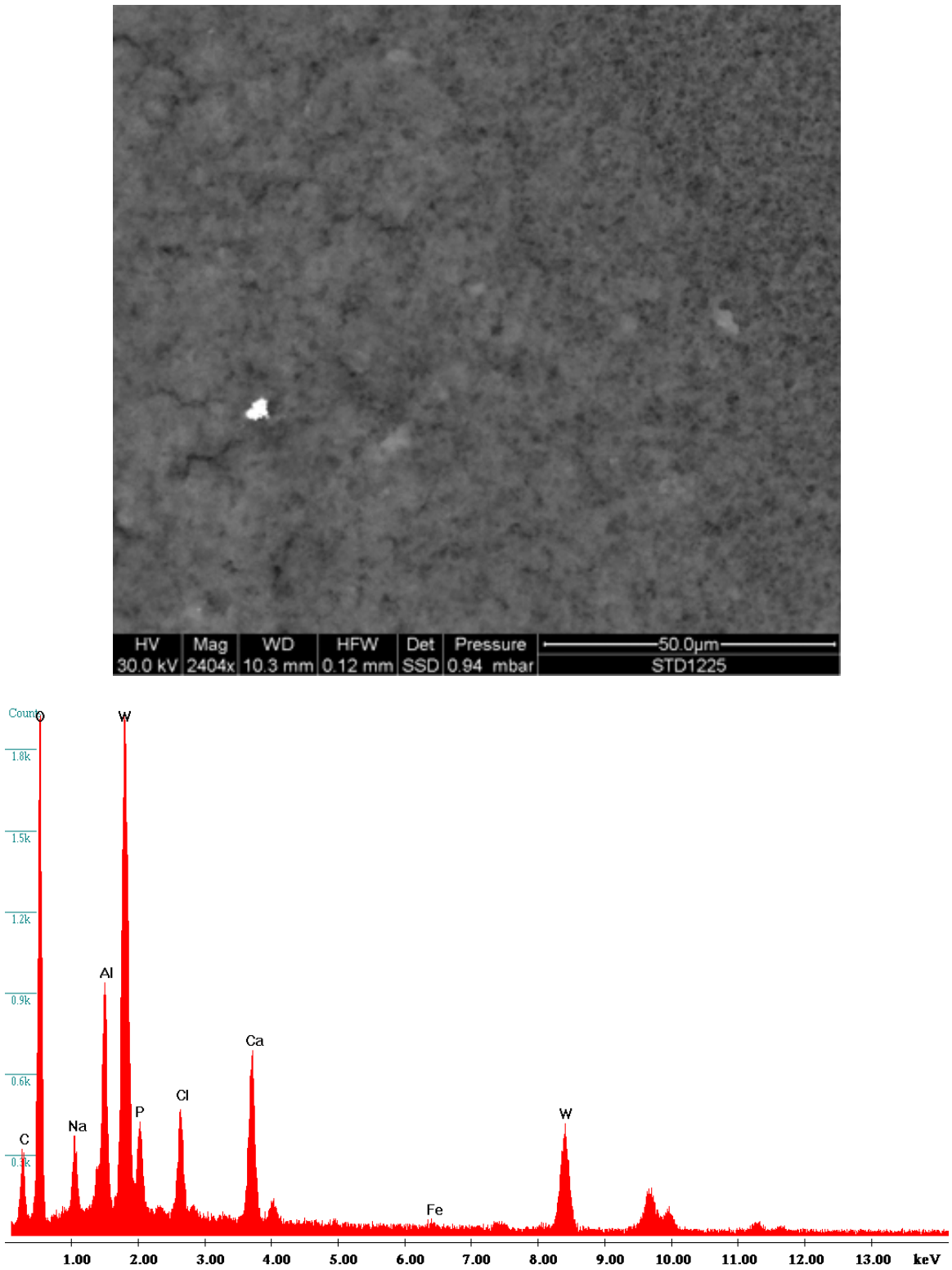


Image 29. At a magnification of 2404x a group of particles can be seen: Their composition detected at EDS, like that of many other particles found in this sample, is Oxygen, Tungsten, Aluminum, Calcium, Chlorine, Sodium, Phosphorous, Carbon and Iron.

6. DISCUSSION AND CONCLUSIONS

The sample of vaccine Infanrix checked contains a relevant number of solid, inorganic particles. We identified 3 different compositions: Stainless steel (Iron-Chromium-Nickel-Manganese-Silicon), Titanium, and mostly Tungsten. The composition obtained subtracting the background of Aluminum-Phosphorus-Sodium-Chlorine is Tungsten-Calcium-Sulfur-Iron. The particles detected are micron- and submicron- and nano-sized, and some have a square morphology. They might be the result of a technological (or nanotechnological) process. Their aggregation is typical of nanoparticles, a phenomenon due to their high surface energy. The presence of those particles is not listed among the ingredients reported in the data sheet and, in any case, it is not clear why they are there.
Judging by their composition, they are not soluble either in water and in fats, are not biodegradable and are not compatible with the human organism.

As far as pathogenicity is concerned, their most important feature is their small size, often below one micron. It is a well-known fact that the smaller the particle, the more capable it is of penetrating tissues and even cell nuclei where they can interfere with the DNA (3). Besides being bodies foreign to human histology and, because of that, triggering factors of inflammatory reactions (chronic reactions, being they non biodegradable), due to their small size the ratio between external surface and volume is very high and, as a consequence, their reactivity is particularly elevated.

Apart from some particles of different composition (for example one with a very high presence of Titanium), the vast majority of them is composed of Oxygen, Tungsten, Aluminum, Calcium, Chlorine, Sodium, Phosphorous, Carbon and Iron.

While the presence of Oxygen, Aluminum, Calcium, Chlorine, Sodium, Phosphorous and Carbon could find an explanation (though certainly not in particulate form) as rightful components of the vaccine, Titanium, Stainless steel and Tungsten are a puzzle. Tungsten, in particular, has been detected with a very high frequency.

It must be emphasized that the pathogenicity of micro-, and, above all, nanoparticles is only partially dependent on dose and composition. References show the impact of nanoparticles on human body and cells (4-28). Among other factors, their capability of inducing an adverse reaction to the organism is related to their size, their

shape and the tissue or organ the blood flow had carried them to, a variable actually impossible to predict.

The World Health Organization defines particulate matter sized 2.5 micron or less as class-1 carcinogen, namely, they can induce cancer (51).

As a matter of fact, it is impossible to predict where the blood flow will carry those particles. Thus, their possible adverse effects depend on the target they hit and the dilution at which they hit it. When dilution is high enough, the effects are very often not clinically visible.

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