A novel tool for assessing microbiomes in cultural heritage documents

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Abstract. This mini-review reports a novel way for exploring the world Cultural Heritage, in the absence of damage or contamination of the items under investigation, called the EVA technique. It is based on films of ethylene vinyl acetate (EVA) impregnated with strong anion and cation exchangers and hydrophobic resins, C₈ and C₁₈. When in contact with any surface these films can harvest nano-moles of macromolecules as well as metabolites, which can then be identified by standard instrumentation (e.g. mass spectrometry). Some applications related to microbial contaminations are reported, such as the findings of Koch bacterium in Chekhov’s shirt and in a letter by Orwell to a Russian journal editor as well as the Y. pestis and anthrax bacteria in the death registries of Milan’s lazaretto in the 1630 plague bout. Novel findings are offered too, such as the identifications of three different strains of Aspergillus in the Aleppo Codex and the detection of melanin (produced by A. niger) in the Dead Sea Scrolls. It is hoped that the present methodology could open the doors of museums, state archives and private collections for detecting biological traces left by artists, literates and men of culture in their masterpieces.

1. Introduction
Non-invasive instruments and techniques are preferred for analysis of precious and unique objects, especially in the cultural and archaeological fields. Aware of that, we developed a methodology based on a plastic film of ethylene vinyl acetate (EVA) studded with strong cation/anion exchangers and with C₈ and C₁₈ hydrophobic resins for capturing a variety of macromolecules (proteins) and metabolites. The method was validated on frescoes, canvasses, bones, parchment and linen by scanning electron microscope and LED multispectral images, which confirmed that no damage or change affected the support surfaces and no residues were left from the application of the extractive film [1, 2]. The EVA technique is based on chemical principles and it can be modified to suit different scenarios. His very first application concerned the analyses of the original manuscript of Maestro I Margarita by Bulgakov [3, 4]. The four types of beads (two ionically charged, two hydrophobic) act by interacting with different analytes via non-covalent interactions, namely ion-ion, hydrophobic attraction and hydrogen bonding. Basically, then, EVA is a peculiar chromatographic technique that could be classified as a mixed-bed column. Mixed-bed chromatography is based on a mixture of sorbents supporting various ligands that are packed in a single column and where each of them plays a
role for adsorbing and releasing single proteins, protein groups or metabolites. Thus our EVA film can be assimilated to these mixed-bed packed columns except that they are in a form of a solid-state cartridge. The capture mechanisms are thus purely chromatographic and follow the same basic rules. In addition to the standard EVA film just discussed, a variety of different films can be produced, for instance EVA diskettes containing different metal chelators, so as to capture from various surfaces either free metals, or metals contained in pigments, or metallo-proteins. This peculiar film was applied to analyse the surface of a manuscript by Kepler dedicated to the Greek astronomer Hipparcus [5]. Tandem diskettes aimed at capturing genetic material from bones (in the past this was done by chipping away a piece of the bone and pulverizing it) have also been produced. The first one contains diffusible reagents that can penetrate in the bones for releasing the trapped DNA (a metal chelator and a protease for digesting histones enveloping the DNA). The second one comprises covalently attached histones that are aimed at capturing the freed DNA as it diffuses to the bone surface. It is thus shown that the EVA technology comprises a family of films manufactured so as to be targeted to the specific capture of different analytes. Why did we trap chromatographic bead into plastic foils? The primary scope was to have access to any item while avoiding any damage and contamination as it would have happened if free beads had been applied, impeding their full recovery. The other reason was the fact that the EVA polymer is hydrophobic so it does not get wet when immersed in water. It becomes merely humid due to the strong anion and cation exchangers coordinating hydration water and experiencing a minimum reswelling. This hydration water is not released in the surroundings but is needed for ion-ion interactions among the resins and analytes, such as proteins. This prevents water damage to the surface of items under study.

Since the present report is aimed at detection of microbiota on the surface of items present in museums, public and private collections, we will just survey here projects targeted to the detection of bacteria on such items and their historical significance or their damaging action on these components of cultural heritage.

2. Death registries of the plague bout in 1630 in Milano

We have screened the death registries of the lazaretto (stored in the State Archives in Milano) written during the plague bout of 1630 that wiped out half the population of Lombardy and of the city of Milano (statistics of the epoch suggested a death toll between 200 to 300 thousands). The captured material by the EVA diskettes applied to the lower right margins of 12 different pages of the registries was analysed by mass spectrometry. The findings were quite extraordinary: we could identify >600 proteins, among which >25 proteins from Yersinia pestis, two from a secondary, opportunistic infection due to anthrax, several vegetable proteins representing the meagre meals of the scribes and even keratins from rats that probably scurried on the pages by night in search of bread crumbs [6]. Since these last findings were obtained with only two proteins (ATP synthase subunit beta and adenine deaminase BA_3032/GBAA_3032/BAS2818), it might be argued that these data on anthrax infection would be statistically irrelevant. Indeed their validity was confirmed by correlating our data with historical records. The death registries were carefully written, by giving any possible data on the dying persons, such as the parish they belonged to, the sector of town, the age, the sex and the diagnosis (issued by barbitonsores, i.e. barbers). Plague diagnosis was easy, since the buboes were visible. Yet about 5% did not carry signs of plague but died rapidly of very high fever, as typical of anthrax infection. Assuming that the ratio between the >25 Y. pestis proteins and only 2 proteins for the presumptive anthrax infection would respect the relative abundances of the two bugs, we could sustain the thesis of this extra cause of death. There is more to it: we also identified two proteins (DNA ligase and gamma-glutamyl phosphate reductase) from a rare bacterium Methylibium petroleiphilum, able to digest petrol derivatives. What would this bug do on the surface of the death registries? A barber (Gian Giacomo Mora) had devised a concoction comprising six different powdered medicinal herbs, today known to contain bacteriostatic peptides, dispersed in petrol and olive oil. He gave this ointment to the lazaretto clerks, with recommendations to coat hands and feet, in the hope this would prevent contagion. It probably did work to some extent and, in any event, traces of this material were left on the page margins, which often had a greenish-blackish tinge. Figure 1 gives the Gene Ontology
analyses of cell components and molecular function terms distribution of the \textit{Y Pestis} proteins detected. It is noted that the two most abundant families are components of membranes.

![Gene Ontology analyses of cell components and molecular function terms distribution of the \textit{Y Pestis} proteins, classified according to the QuickGO software.](image)

3. **Chekhov’s shirt worn in his death bed**

Chekhov is one of the three seminal figures in the birth of early modernism in the theatre. His four classical pieces, \textit{The Seagull}, written in 1896, followed by \textit{Uncle Vanya} and by his last two plays, \textit{Three Sisters} and \textit{The Cherry Orchard} are still among the most represented ones in major theatres around the world. Together with these theatre pieces, Chekhov is widely known for having written a vast number of stories, short and long, no less than 201 at the latest count. Among them a few humorous ones, such as "Oh! the Public!", "The Orator", and "A Transgression" up to one of the most moving, "Misery". Among the longer stories perhaps one of the most notorious is "The Steppe", which contains the most famous thunderstorm in literature. Although his literary production is amply dealt upon by literates and historians, a lesser accent has been put on the fact that he was a medical doctor and regarded this profession as the main scope of his life, in which he devoted most of his efforts, going out of his way to help the poor and needy. Although there is no record that the two doctors, Chekhov and Koch, ever met face to face, Chekhov met Koch’s deadly messenger, the \textit{Mycobacterium tuberculosis}, which the German doctor had isolated and described in 1882. Only three years after this discovery, in 1885, Chekhov found himself coughing blood, and in 1886 the attacks aggravated. Over the years his health worsened and by May 1904, Chekhov was terminally ill with tuberculosis. He died on 15\textsuperscript{th} of July 1904 at the age of forty-four, having lived a short but very intense life.

We have learned that his house-museum in Melikhovo (a small village 50 miles south of Moscow) stores several letters he wrote during the last few month of his life, together with a shirt that he wore on the day of his death. In some of these items there appear to be brownish stains, indicating possible blood spots. Five different letters and post cards as well as the shirt worn by Anton Chekhov on his death bed have been examined by applying to these surfaces our EVA diskettes. Three different eluates (under acidic and basic conditions and with acetonitrile) were analyzed by high resolution mass spectrometry. The environmental microbiota present on samples and the \textit{M. tuberculosis} strain (found only on the collar of the shirt he wore in his death-bed) were described by a meta-proteomics approach. Eight identified \textit{M. tuberculosis} proteins (see table 1) confirmed the presence of the bacterium and the cause of Chekhov death, in addition to several sequenced peptides belonging to other bacterial species [7]. The human plasma proteins and human keratins, detected on a tiny blood spot on the shirt, demonstrated the power of the combined approach. We hypothesized that Chekhov
touched the collar of his shirt with fingers wet with saliva, thus leaving on it traces of this bacterium. Per se such findings are not exceptional, since they barely confirm historical facts. What is unique is that the bacterium was still present in a 115-year old garment.

Table 1  M. tuberculosis identified proteins in Chekhov’s shirt

| Accession Numbers | Protein name | Peptide sequence |
|-------------------|--------------|------------------|
| A0A045H4D1        | 1,4-dihydroxy-2-naphthoate octaprenyltransferase | DTGLAMLVLWALAVAGALAFGQLS |
| A0A0U0S6V7        | 1-deoxy-D-xylulose-5-phosphate synthase | LLVTLEDNGVNGGAGSAVAALR |
| A0A0T9XK77        | Acetolactate synthase large subunit ilvB2 | VTWAVLNDGQMSASAGPVSGR |
| A0A045JBU3        | Lipoprotein LpqB | QFLTESASNAWDDAGSALLIDHVVFVETR |
|                   |               | FPGAINDLQLSR |
| A0A045IMV5        | Protein of uncharacterized function (DUF1490) | MVWHGFLAK |
| A0A045J451        | Transcriptional regulator | LVPGYTASGDAQVDETAAEIGLGR |
| A0A0U0R9R8        | Transporter MMPL8 | LAELLGVSRPAVR |
| A0A1K6RJ8I        | 8-amino-7-oxononanoate synthase | LAGGANLMASK |
|                   |               | QIAVHTGDIDK |
|                   |               | MPTGLGYDFLRPVEDSGINDLK |
|                   |               | VLAAAEMYMATGLAR |

4. Orwell's letter to the Russian editor Dynamov

While talking of TBC a curious finding was obtained in the case of Eric Blair (George Orwell as *n teenage de plume*), the author of the famous novels *The Animal Factory* and *Nineteen Eighty-Four*, among his other books, including a report on the Spanish Civil War called *Homage to Catalonia*. In December 1936 he joined the International Brigades fighting Franco’s push and in May 1937 he was severely wounded in the neck by a rifle bullet. Hospitalized in Barcelona, he was infected with TBC. In July he ran away from Spain, escaping purges activated by the Communist Party and reached home in England. There he wrote a letter to Mr. Dynamov, Editor of Soviet journal “Foreign Literature”, warning him that, if he wanted to publish his novel “*The Road to Wigan Pier*”, he should be aware that Stalin might have prosecuted him for this collaboration. We discovered this document, registered as “the one and only Orwell’s letter to the Soviet Union”, in the Russian State Archive of Literature and Arts in Moscow. We applied five EVA diskettes to its surface, four in the corners and one directly over his signature. As luck goes, here too we identified the *M. tuberculosis*, a remarkable finding considering that the letter was typewritten (Figure 2). The bacterium was found in the two upper corners and in the signature, but not in the two lower corners of the letter, which served as negative controls. The only possible explanation is that Orwell touched the upper part of the document, while releasing it from the typewriter, with saliva-wetted fingers. The other explanation is that, since Orwell was at the peak of his pathology, the relative abundance of the bacterium was considerably higher than the levels that could be found in periods of a quiescence of the pathology. In figure 2 the left part is a picture of Orwell’s letter to Dynamov. The four red dots in the four corners represent the areas of placement of the four Eva diskettes. The red arrow indicates the positioning of the fifth diskette, which was placed directly over the original Orwell signature. The right part shows the meta-proteomic approach to the attribution of one protein (from a total of 8 identified) specifically to the Kock bacillus while excluding others, such as *africanum, bovis, micoti* and *mungi*. These data further reinforce the notion that the EVA methodology is a formidable tool for exploring documents stored in archives, public libraries and/or private collections while ensuring that no damage nor contamination is done to the items under investigation. Here too the novelty is not so much the finding of *M. tuberculosis* infection, known to have occurred to Orwell, but that traces of this bacterium would still be present in
a eighty-years old document. These data are also unique if one considers that, during this long storage, most proteins had been hydrolyzed by the acidic environment of the paper, leaving mostly peptides trapped on the page fibers.

Figure 2. Left: George Orwell’s letter to Dynamov. Right: Identification of one specific protein (8-amino-7-oxononanoate synthase) of *M. tuberculosis* via a meta-proteomics approach.

5. **Aleppo Codex**

The Aleppo Codex is a medieval bound manuscript of the Hebrew Bible that was written in the city of Tiberias, in what is currently northern Israel, in the 10th century A.C., and endorsed for its accuracy by Maimonides. Together with the Leningrad Codex, it contains the Ben-Asher masoretic tradition, but the Aleppo Codex lacks most of the Torah section and many other parts. The Karaite Jewish community of Jerusalem purchased the codex about a hundred years after it was made. During the First Crusade, the synagogue was plundered and the codex was transferred to Egypt, whose Jews paid a high price for its ransom. It was preserved at the Karaite (then Rabbanite) synagogue in Old Cairo, where it was consulted by Maimonides, who described it as a text trusted by all Jewish scholars. It is rumoured that in 1375 one of Maimonides’ descendants brought it to Aleppo, Syria, leading to its present name. The Codex remained in Syria for five hundred years. In 1947, rioters enraged by the United Nations Partition Plan for Palestine burned down the synagogue where it was kept. The Codex disappeared, to re-emerge in 1958, when it was smuggled into Israel by a Syrian Jew, Murad Faham, and presented to the president of the state, Yitzhak Ben-Zvi, who entrusted it to the Ben-Zvi Institute and Hebrew University of Jerusalem. Since 2019 it is on display in the Shrine of the Book at the Israel Museum. Since parts of the margins of several pages were quite ruined and had a blackish tinge (see figure 3), we investigated via the EVA technique the causes of this degradation.
After eluting from the EVA diskettes the surface material captured and ms analyses, we identified three strains of *Aspergillus*, namely *A. fumigatus* (with 5 proteins: Fucose-specific lectin FleA, Heptaketide hydrolyase ayg1, Laccase abr2, Arginine biosynthesis bifunctional protein ArgJ, Beta-hexosaminidase), *A. pseudoglaucus* (with 5 proteins: MAT1-1, SLA2, Actin, MAT2-4, RNA polymerase II) and *A. amstelodami* (with 8 proteins: beta-tubulin, RNA polymerase II, Beta-1 and beta-2 tubulin, Calmodulin, euricin, Cct8, RNA polymerase II largest subunit, Cytochrome c oxidase subunit 1). Whereas contemporary microflora can proliferate in growth media in Petri dishes, antique microflora traces cannot propagate in growth media due to the absence of alive spores or intact microbes. We tried to grow the material captured without any success, indicating that the contamination was quite old, although not amenable to dating. For that, one could resort to studying amino acid racemization and/or epimerization, a method today in use for such a dating.

We have additionally developed techniques that give the possibility of taking extracts from single parchments fibres under a microscope. This technique would offer the possibility of sampling directly from individual fibres on any microscopic component of the parchment foils. Inks letters (written text) detachment from parchment is additionally a visible damage that occurred during the storage period of Aleppo Codex. This is result of some phase separation between the inks and the parchment substratum producing mechanical deformation manifestations. Some sort of peeling of dry inks from parchment is the result of segregation between the ink components (binders) and the parchment.

6. Dead Sea Scrolls analysis

The Dead Sea Scrolls are ancient Jewish religious manuscripts found in the Qumran Caves in the Judaean Desert, on the northern shore of the Dead Sea. Scholarly consensus dates them from the last three centuries BC and the first century AC. The texts have great historical, religious, and linguistic significance because they include the second-oldest known surviving manuscripts of works later included in the Hebrew Bible canon, along with deuterocanonical and extra-biblical manuscripts which preserve evidence of the diversity of religious thought in late Second Temple Judaism. Almost all of the Dead Sea Scrolls are currently in the collection of the Government of the State of Israel and they are housed in the Shrine of the Book on the grounds of the Israel Museum. Many thousands of written fragments have been discovered in the Dead Sea area. They represent the remnants of larger manuscripts damaged by natural causes or through human interference, with the vast majority holding only small scraps of text. However, a small number of well-preserved, almost intact manuscripts have survived – fewer than a dozen among those from the Qumran Caves.
Figure 4. Pictures of three out of the nine fragments of Dead Sea Scrolls analysed by both EVA technology as well as zeolite extraction.

We have analysed several fragments of the Scrolls (catalog number: Plate 1032, Frag 10, B-366325; 3 of them are visible in figure 4) by extracting surface material via both the EVA technology and zeolite crystals application. In a first application, we have analysed the captured material via Energy-Dispersive X-Ray Spectroscopy (EDS), which has produced an energy spectrum that has permitted to determine the relative abundance of specific elements, as shown in figure 5. These elements are no doubt due to sand impregnating the parchment of the Scrolls. Additionally, in darker areas, we have analysed the eluted material via HPLC coupled to mass spectrometry and identified melanin, produced by a few strains of *Aspergillus*. These data, together with those of the Aleppo codex, confirm that the biggest offenders of ancient documents are moulds such as various types of *Aspergillus*, contaminants that museum curators should be aware of and take appropriate steps for prevention.

Other general findings are here listed. Mold’s protein fragments: Alpha-galactosidase A and Arabinanolytic transcriptional activator araR. Plant acids: Stearic, palmitic, azelaic, oleic acids. Plant terpenes: triacontanol, catechin, lupeol. Plant proteins fragments: Alpha-galactosidase, Calmodulin, Ribulose bisphosphate carboxylase large chain, Rhamnogalacturonate lyase and different glycoproteins consisting of various combinations of pentosyl and hexosyl units. Another interesting aspect we are now exploring is ink composition in the Scrolls. Antic and medieval ink components (staining compounds and binders) based on carbon, tannins and other ingredients of plant’s dyes can also be characterized by extraction and LC-MS analysis. A very interesting direction is the identification of long term metabolomics of inks-collagen interaction. Glycoproteins and carbohydrates are part of plant-based inks which were used for some types of antic inks (before iron inks) preparation. These glycoproteins react with collagen and proteins in the scroll and create specific compositions similar to Maillard reaction products (manuscript in preparation).
Figure 5. Identification and relative abundances of various elements found on the parchment surface, of sand origin, as detected and quantified via a JEOL EDS system.

7. Conclusions
We hope this brief survey will give to readers an idea of the power of the EVA technology and of what it can achieve in exploring documents present in museums as well as public and private collection. Although we have limited this excursus to microbiota present in paper pages and/or parchment, we have shown that this method can be applied with success to canvasses, frescoes, even sculptures present in museum and in open air [1, 2]. An interesting variant for biomolecule extraction could be the use of inorganic ceramics based on zeolites. These inorganic porous ceramics are an interesting additional extraction tool for collecting colloidal particles and compounds based on mixtures of organic ion exchangers and organic sorbents not so efficiently adsorbed by the EVA diskettes. The major reason for using inorganic extraction tools will be to avoid influence on carbon dating methods (work in progress). For an ample survey of any possible methodology that can be utilized in Cultural Heritage screening, see [8].

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