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Insights into mixed carbon–iron gall ink and its degradation in eighteenth-century manuscripts

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ABSTRACT

The study of mixed ink recipes has a broad interest since along the millennial historical transition from carbon-based inks to iron-gallate inks, mixed carbon-iron gallate might represent a long transitional phase. The knowledge of the impact and interaction of chemical and microbiological agents with the tangible archivistic heritage strengthens our ability to preserve historical documents over time. Herein, we propose a multi-analytical approach including optical and electron microscopies, vibrational spectroscopies, liquid chromatography with mass spectrometric detection. Moreover, microorganism isolation and characterization were carried out on selected samples. Our case study (Vanvitellian letters) demonstrates that, in the presence of carbon black, integration of spectroscopic technique with HPLC-MS/MS analysis is essential for the proper characterization of iron-gallate complex and its degradation products. Therefore, we propose and apply a protocol in which a Raman-based pre-screening easily detects carbon black in ink, and, in case of carbon black presence, HPLC-MS/MS is required to verify gallic acid/oxidation sub-products, to correctly assign the ink to the mixed recipe. Both ink formulation details and warnings on the diagnostics of mixed carbon-iron gall inks arise from the present study, calling for further investigation into their degradation mechanisms.

1. Introduction

Archival heritage, including manuscripts and written documents, is an integral part of cultural identity, and its preservation requires a thorough understanding of the chemical composition and degradation behavior of its constituent materials [1,2].

Typically, manuscripts and written documents result in a mixture of inorganic and organic compounds, that must be characterized from a physico-chemical point of view to predict any intrinsic risk of degradation [2]. Both support and ink chemical components can be significantly affected by the conservation environment, via the local atmosphere (water, oxygen, pollutants), via contact with microbiological agents (mostly fungi and bacteria), and due to changes in physico-chemical parameters (e.g., temperature, light exposure, pH) [1,3].

The chemical characterization of manuscripts relies on a very variable combination of elemental and molecular techniques. Despite methodological advances [4], systematic studies that simultaneously integrate elemental and molecular techniques remain extremely limited [5]. Most research adopts a single analytical technique, failing to correlate elemental composition, molecular structure, and degradation profiles. X-ray fluorescence (XRF) [6], scanning electron microscopy (SEM-EDS) [7], and inductively coupled plasma mass spectrometry (ICP-MS) [8] are commonly used for the elemental analysis of metals present in inks, with different degrees of invasiveness. Vibrational spectroscopies, such as Raman and Fourier-transform infrared spectroscopy (FTIR) [7,9–13], are employed for the molecular characterization of inks, in particular iron-gall complexes, and support materials, as well as their degradation products. Finally, gas chromatography –

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mass spectrometry (GC–MS) [14–16] provides the information on the polyphenolic compounds from iron-gall inks and liquid chromatography coupled with tandem mass spectrometry (HPLC-MS/MS) [17] provide additional information on the molecular markers of gallic acid degradation allowing the investigation of degradation mechanisms of gallotannins [18,19]. Although the ageing of paper has been widely studied, the detailed molecular characterization of historical inks and their preparation methods is still limited, mainly due to the variability of historical recipes and the complexity of the matrices [20]. In recent years, increasing attention has been given to multi-analytical approaches; however, these are often focused on either chemical or biological aspects, rarely combining both.

An additional layer of complexity arises from the susceptibility of both paper support and ink to microbial degradation. Fungi and bacteria colonizing the surface produce organic acids, cellulases, and hydrogen peroxide, thereby accelerating hydrolysis and oxidation mechanisms already catalyzed by ferrous ions [21]. Consequently, some degradation products may have either chemical or biological origins, making it essential to integrate chemical characterization with microbiological investigation.

In this context, the present work aims to overcome the limitations of conventional methodologies by proposing a multi-analytical protocol that, for the first time, synergistically combines SEM-EDS, micro-Raman spectroscopy, and HPLC-MS/MS for the study of historical iron-gall inks. This integrated approach proves particularly effective for the unambiguous determination of the composition of complex matrices and their degradation products, by exploiting the complementary strengths of each technique, and overcoming possible misleading conclusions arising from the exclusive adoption of single analytical approaches. Chemical analysis is further complemented by a microbiological survey, with the aim of distinguishing biodeterioration phenomena from purely chemical degradation processes and providing a comprehensive risk assessment for manuscript preservation.

A real case study, the historical letters of Luigi Vanvitelli (1700–1773), demonstrates the efficacy of the proposed chemical and microbiological workflow for the characterization of the inks. The approach overcomes key limitations of existing methodologies in the classification of common carbon-based historical inks, and it addresses the need of minimal sampling and the difficulty of linking chemical composition to biological degradation.

1.1. Brief history of inks

Inks are liquid or semi liquid materials used for writing. From a chemical point of view, they can be classified as complex mixtures composed of pigments suspended in a liquid carrier with binders and other additives [2]. Valuable reference on ink composition and preparation can be found in historical documents and in scientific reviews [1,22,23]. A simplified chronology of inks is sketched in Fig. 1.

The earliest use of a liquid which can be described as ink is found in documents on Egyptian papyrus, dating from 3500 B·C; however, the adoption of fluid ink has been attributed to a Chinese origin in 2700 BCE According to historians, the inventor of Chinese ink, also called Indian ink, was Tien Tcheu and its base, as for the early Egyptian and other inks, is carbon [24]. It has the advantage of being easily procurable, while at the same time indestructible except by fire and was probably prepared in the form of vegetable or animal charcoal (carbon black, ivory black, bone black) [25,26]. The fine carbon particles were suspended in gum, oil or other substances to make its application easier. Later, the discovery of natural oils, rosin and petroleum gave an opportunity to produce a different type of ink from black sooty residue obtained upon combustion of these carbonaceous materials in a limited supply of oxygen. This material is known as lampblack ink and can be manufactured following the Chinese and the European processes. The former involves terra-cotta lamps with polished interiors where oils are burned, and their smoke is collected. From time to time the cones are replaced by fresh ones, and the deposited soot removed by means of a feather. The latter involves the use of a furnace to burn the organic

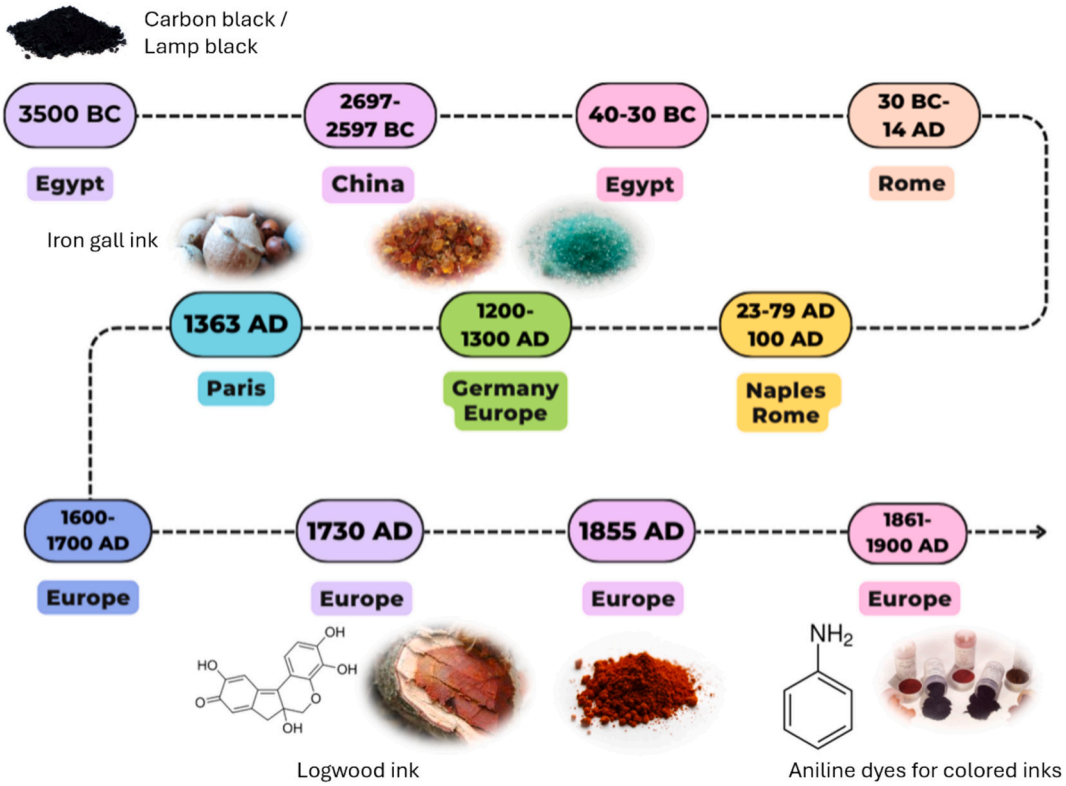

Fig. 1. Timeline of the spread of ink formulations over the centuries.

materials, then the dense smoke is conducted through a cylindrical chamber covered with sacking or sheep skin where the lamp black is deposited and recovered through an iron cone that fits exactly the chamber and is used to scratch the residue [22,27]. By the 4th and 3rd century BCE, the use of Arabic gum as binder for black carbon-based inks was already well established. This was not only because it helped to keep the fine insoluble carbon particles in suspension but also because it modified the viscosity of the ink making its application easier, assisting the writing procedure. However, the addition should be controlled, since too much Arabic gum resulted in the tendency for the writing to flake off from its substrate upon drying.

In medieval times, iron gall inks gradually replaced carbon black ink [28]. Iron gall ink has recently been detected in the *Vercelli Gospels,* which are the oldest existing translation of the Four Gospels from Greek to Latin, dating back to the 4th century CE [29], but he first written reference to an iron gall ink and its preparation comes from the 11th century *Diversarum artium schedula* written by a monk named Theophilus. Iron gall was the first ink type to be obtained by a chemical reaction and, despite the variability of its method of preparation, the historical recipes contain three main components: an extract of gall nuts, an iron salt and a binder. Galls are structures made up of plant tissue that develop in response to stimuli produced by insects. Gall nuts are spherical protuberances resulting from the egg-laying of wasps on oak trees. Their extract is rich in hydrolysable tannins that react with iron (II) leading to the formation of an iron gallotannate complex, that is subsequently oxidized by atmospheric oxygen resulting in complexes between Fe^{3+} and phenolic compounds that have dark violet color. In the 18th century, scribes started to use dyestuff as logwood and indigo to strengthen the color of the ink [20]. However, this approach became way more used from the 19th century on when a new way of producing ink showed up. It consisted in slowing down the ink oxidation as much as possible and letting the process happen directly on paper fibers. For this reason, logwood or indigo were added to the formulations to impart an intense color to the otherwise pale ink, already during writing. As the demand for ink increased, the next development in its manufacture in the second half of the 19th century was inclusion of the newly synthesized and cheap aniline dyes, to be used both for black and colored inks [30].

2. Materials and methods

2.1. Vanvitellian letters

The chemical characterization of written documents from historical libraries and archive collections can provide new insights into the composition of the inks and their homemade production. In this paper, letters written by Luigi Vanvitelli have been investigated (Figs. S1). The correspondence of Luigi Vanvitelli (1700–1773), stored in the Palatine Library of the Museum Royal Palace of Caserta, is composed of 1624 documents. The letters are almost all addressed to his brother, Urbano Vanvitelli, abbot of the church of “San Giovanni dei Fiorentini” in Rome and cover a period that goes from 1751 to 1768. This collection represents a valuable source of information on his life, cultural activity, and historical context.

2.2. Sampling

The sampling was performed at the Museum Royal Palace of Caserta. The fragments of inked paper were collected from the parts that were already or partially detached from the ancient manuscripts to preserve as much as possible their integrity. The sampling was curated by Dr. Gennaro Tortino, conservator and responsible for the Archives and Libraries of the museum; it was performed using a scalpel on erratic fragments partially detached from the letters surface, ensuring ethanol cleaning of the blade after every recovery. Particularly, 35 letters (both written and sent to the architect) from 1751 were chosen as a temporally homogeneous set for our diagnostic study. For the characterization of ink and paper composition, 39 samples were collected with a scalpel from the letters' surface, divided in: *i)* paper, *ii)* oxidized ink, *iii)* inked paper, *iv)* sealwax. All samples were examined using Optical Microscopy. A subset of seventeen samples was further analyzed using Raman Micro-Spectroscopy and Scanning Electron Microscopy-Energy Dispersive-X-Ray Spectroscopy. Additionally, two inked paper samples were selected for analysis by High Performance Liquid Chromatography coupled with Quadrupole-Time of Flight tandem mass spectrometry analysis.

2.3. Optical microscopy

All samples were analyzed via a macroscopic observation on 3D materials with a stereomicroscope. The stereomicroscope used is the AXIO ZOOM V16, powered by the Museum Center of Natural and Physical Sciences of University Federico II. The lighting takes place from a CL 9000 LED CAN cold source for the stereomicroscope. Light reflected and transmitted in the bright field was used for the work carried out. The AxioCam ICc5 camera was used for image acquisition. The software used for image acquisitions is Axiovision SE64 Ref 4.9.1 with *Z*-Stack, Extended Focus, and Panorama modules.

2.4. Scanning electron microscopy-energy dispersive – X-ray spectroscopy (SEM-EDS)

Quantitative microchemical analyses of paper supports and inks were carried out by FE-SEM coupled with energy-dispersive X-ray spectroscopy (EDS) [31,32]. Morphological observations and quantitative chemical analyses were performed using a Zeiss Merlin VP Compact field emission scanning electron microscope (FE-SEM), equipped with a charge compensation system and an Oxford Instruments X-max 50 energy-dispersive X-ray spectroscopy (EDS) detector. The microscope, featuring a Gemini II column, is equipped with three secondary electron detectors (SE2 – conventional detector; VPSE – variable pressure detector; InlensDuo – low voltage detector) and two backscattered electron detectors (AsB and InlensDuo). Dedicated software was used for both chemical data processing and image analysis. For semi-quantitative analyses, small paper and ink fragments were carefully mounted onto conductive carbon adhesive stubs, ensuring complete adhesion of the specimens to obtain the flattest possible analytical surface and to minimize geometrical effects during X-ray acquisition. The mounted samples were positioned at the same working height as the cobalt calibration standard used for instrumental calibration. Prior to analysis, all samples were coated with a thin conductive carbon layer. Quantitative elemental analyses were performed after calibration using the cobalt reference standard. A total of 20 analytical points were acquired for each sample. The resulting elemental data were subsequently processed through stoichiometric recalculations in order to identify the mineral phases and reconstruct their chemical composition.

2.5. Raman micro-spectroscopy

The Raman spectra were collected at room temperature using a confocal micro-Raman spectrometer (Jasco, NRS-3100), working with the 514 nm excitation line from an Ar^{+} laser injected into an integrated Olympus microscope and focused to a spot size of 2 μm by a 100× or 20× objective. The laser power at the sample was 2 mW. A holographic notch filter was used to reject the excitation laser line. Raman scattering was dispersed through a monochromator (2400 grooves/mm grating) and collected by a Peltier-cooled 1024 × 128 pixel CCD photon detector (Andor DU401BVI). The typical average spectral resolution was 4 cm^{-1}. The spectra acquisition times varied from 20 to 60 s and spectra were acquired three times in different sample zones to improve representativity. The assignment of Raman bands was performed by comparing data with the literature and available online databases (e.g., RRUFF

library) [33].

2.6. High performance liquid chromatography coupled with quadrupole-time of flight tandem mass spectrometry (HPLC-ESI-Q-ToF)

A HPLC 1260 Infinity, coupled with a Quadrupole-Time of Flight tandem mass spectrometer 6530 Infinity Q-ToF detector by a Jet Stream ESI interface (Agilent Technologies, (USA), was used. The data were processed with Mass Hunter Qualitative Analysis software. ESI conditions were: drying gas (N_2; purity>98%), temperature 350 °C, flow 10 L/min; capillary voltage 4.5 KV; nebulizer gas pressure 35 psig; sheath gas (N_2; purity>98%) temperature 375 °C, flow 11 L/min; fragmentor voltage was 175 V. High resolution MS and MS/MS acquisition range was set from 100 to 1000 *m/z* both in negative and positive mode with a scan rate of 1.04 spectra/s; the voltage in the collision cell was 30 V. The chromatographic separation was performed at 30 °C on an analytical reverse phase column Poroshell 120 EC-C18 column (3.0 mm × 75 mm, 2.7 μm particle size) with InfinityLab Poroshell 120 EC-C18 guard column (3.0 mm × 5.0 mm, 2.7 μm particle size), injection volume of 10 μL for the HPLC-ESI-Q-ToF analysis of samples. The elution gradient, at 0.4 mL/min, is reported in Table 1 (eluent A is formic acid 0.1% *v*/v in H_2O and eluent B is formic acid 0.1% v/v in CH_3CN). The elution program was as follows: 0–2.6 min, 95% A; 2.6–15.6 min linear gradient to 50% A; 15.6–20.8 linear gradient to 30%A; 20.8–27.0 min linear gradient to 100% B, held for 8 min. *Re*-equilibration took 11 min. Further details on the method can be found in Ferretti et al. [17]

For the characterization of the organic inks, the sample was subjected to a mild extraction with EDTA-DMF solution. The treatment consists in the following steps: addition of 50 μL of 0.1% Na_2EDTA in H_2O/DMF (1:1, *v*/v) solution, then extraction in ultrasonic bath at 60 °C for 1 h, finally centrifugation at 3000 rpm for 2 min and separation of the supernatant from the solid sample using a micropipette. The extraction method has been optimised as described in our previous publication, on reference mock-ups prepared by casting iron-gall ink on paper [2]. The solvents and reagents used for the extraction methods were: ethylenediaminetetraacetic acid disodium salt (EDTA, Fluka, USA), dimethylformamide (DMF; 99.8% purity, J.T. Baker, USA), acetonitrile (ACN; HPLC grade, Sigma Aldrich, St. Louis, MO, USA). The eluents used for HPLC-ESI-Q-ToF were: water and acetonitrile, both LC-MS grade (Sigma Aldrich, St. Louis, MO, USA) for HPLC-ESI-Q-ToF analysis.

The figures of merit of the procedure were described in our publication on iron gall ink recipes [17]. In detail, stock and standard solutions (0.5, 1.0, 2.5, 5.0 and 10 μg/g) of gallic acid and ellagic acid were prepared in DMSO and calibration curves were obtained by integrating the Extract Ion Chromatograms (EICs) peak areas of gallic and ellagic acid for the solutions, analyzed in triplicate. Limit of detection (LOD) and limit of quantification (LOQ) were estimated using the standard solution with the lowest concentration level giving a visible signal and the calibration curve with q = 0 and were: 3×10^{-2} μg/g (LOD) and 8×10^{-2} μg/g (LOQ) for gallic acid and 2×10^{-1} μg/g (LOD) and 6×10^{-1} μg/g (LOQ) for ellagic acid [17].

2.7. Microbiological investigation

The assessment of microbiological degradation was conducted non-invasively using 14 sterile swabs, which were subsequently streaked onto Petri dishes containing five different culture media (PDA, MALT, YES, CYA, and CD). The plates were incubated at 22 ± 2 °C for 30 days. Genomic DNA was extracted from a small piece of mycelium using a modified Doyle & Doyle protocol [34]. Three independent PCR reactions were carried out, targeting the ITS region (ITS5-5′-GGAAGTAAAAGTCGTAACAAGG-3′ and ITS4-5′-TCCTCCGCTTATTGATATGC-3′) [35], β-tubulin (Bt2a_for - 5′-GGTAACCAAATCGGTGCTGCTTTC-3′ and Bt2a_rev - 5′-ACCCTCAGTGTAGTGACCCTTGGC-3′) [36], and calmodulin (CMD_for - 5′-CCGAGTACAAGGARGCCTTC-3′ and CMD_rev - 5′-CCGATRGAGGTCATRACGTGG-3′) [37]. Each PCR reaction was performed in a 50 μL reaction volume, containing 25 μL of EconoTaq PLUS 2× Master Mix (EconoTaq, LGC Biosearch Technologies, Teddington, Middlesex, UK), 0.5 μL of each primer, and 1 μL of DNA template. The amplification was conducted using an Applied Biosystems 2720 thermal cycler, and the resulting PCR products were visualized through 1.2% (*w/v*) agarose gel electrophoresis. Amplicons were then sequenced using the Sanger method. The obtained sequences were analyzed through BLAST (NCBI databases), confirming the identification of the isolate as for taxonomic identification *Aspergillus tubingensis* Mosseray, 1934. Finally, the sequences were deposited in GenBank, with the following Accession Numbers: ITS (PV199124), β-tubulin (PV390663), and calmodulin (PV390664).

3. Results and discussions

3.1. Optical microscopy and SEM-EDS analysis

Selected samples were analyzed by optical and electron microscopy for morphological and chemical investigation. Morphological observations revealed the presence of three main types of paper fragments: white fragments (Type 1, Fig. 2A), yellowish to brown fragments (Type 2; Fig. 2B), and very dark fragments containing visible ink residues (Type 3; Fig. 2C).

Type 1 fragments are characterized by cellulose fibers of varying sizes, clearly distinct from one another. A white polycrystalline powder is frequently observed on the surfaces and between the fibers; the microcrystals appear transparent and/or translucent. EDS analysis indicates that these microcrystals are attributable to silicates, sulfates bearing both K and Ca, and Ca—Mg carbonates or Ca oxide (Fig. 2D), with respective weight percentages of 1%, 0.4%, and 0.4%. The occurrence of these compounds in Type 1 fragments is consistent with materials used in paper production.

Type 2 fragments exhibit flattened cellulose fibers that aggregate into thicker bundles, associated with translucent to opaque microcrystals ranging in color from yellowish to brown and occasionally white. In addition to the compounds already observed in Type 1 (silicates, K—Ca sulfates, Ca—Mg carbonates or Ca oxide; Fig. 2E), EDS analysis also revealed the presence of K and Fe sulfates (Fig. 2E). In Type 2 fragments, the occurrence of iron and potassium sulfates may be attributed to degradation processes induced by prolonged contact with ink components due to sheet overlay. Such compounds, typically released during

Table 1
Molecular markers detected with HPLC-ESI-Q-ToF on samples collected from Vanvitellian letters.

Molecular marker	Label	t_R (min)	$[M-H]^-$	Raw formula	Sample A	Sample B
Gallic acid	GA	2.1	169.014	$C_7H_6O_5$	x	x
Digallic acid	diGA	7.6/8.5	321.019	$C_{14}H_{10}O_9$		x
Trigallic acid	triGA	10.5	473.032	$C_{21}H_{14}O_{13}$		x
Ellagic acid	EA	11.1	300.997	$C_{14}H_6O_8$	x	x
Degradation product of GA	m_{11}	8.8	239.016	$C_{10}H_8O_7$	x	x
Degradation product of tannins	d_3	10.2	247.025	$C_{12}H_8O_6$	x	x

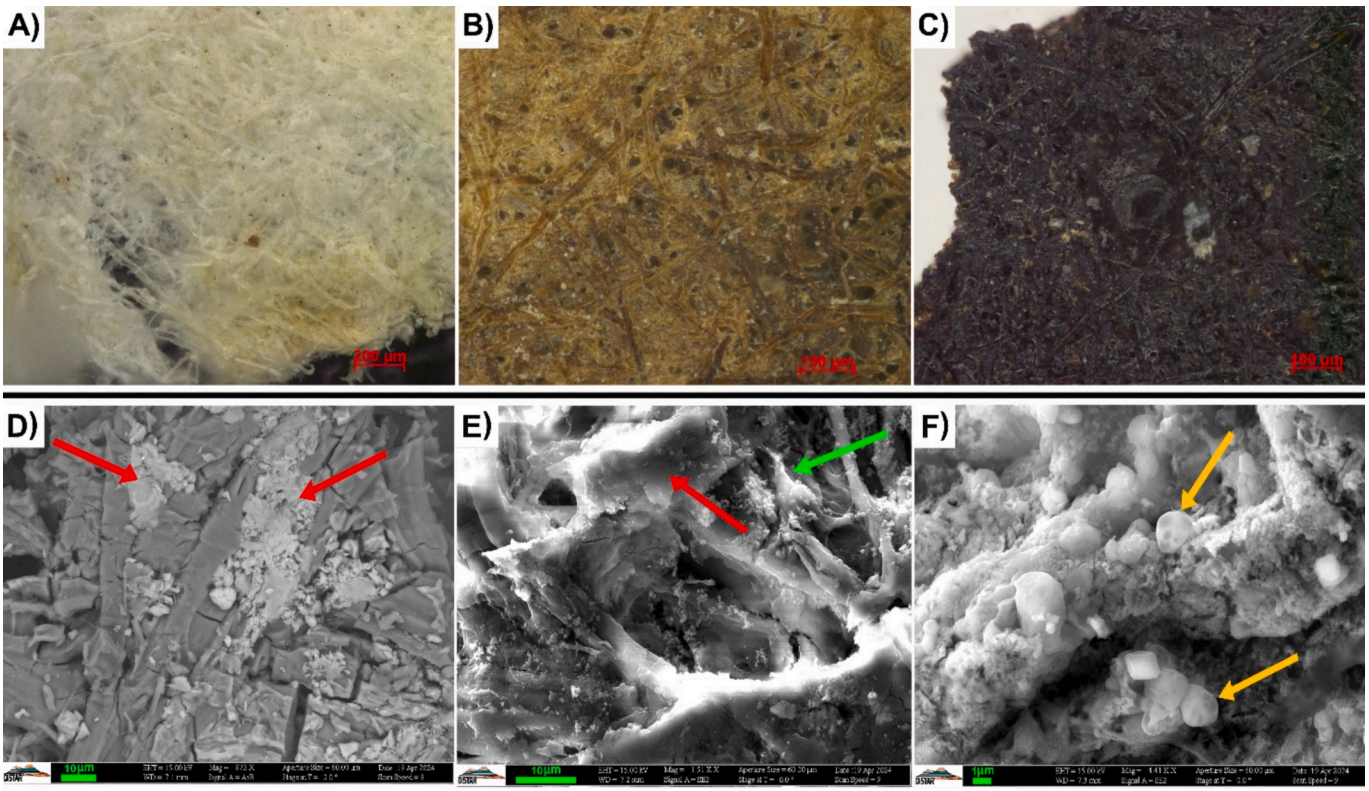

Fig. 2. Optical microscopy (OM) and scanning electron microscopy (SEM) images of a representative sample from the Vanvitelli letters. A) OM image of Type 1 fragments; B) OM image of Type 2 fragments; C) OM image of Type 3 fragments; D) SEM micrograph of Type 1 fragments: the red arrow indicates Ca and K sulfates and Ca—Mg carbonates or Ca oxide; E) SEM micrograph of Type 2 fragments: the green arrow indicates K and Fe sulfates; F) SEM micrograph of Type 3 fragments: the orange arrows indicate compounds containing S, Fe, K, Zn, and Cu. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the ageing of iron-gall inks, can migrate through adjacent sheets [33,34], especially in bound documents, leading to localized yellowing and chemical alteration of the paper fibers.

Type 3 fragments, where ink is clearly present, exhibit cellulose fibers with a morphology similar to that of Type 2, but dark, opaque microcrystals are also visible. SEM-EDS analysis indicates that the darker areas, attributable to the ink, consist of two main phases: one containing S, Fe, K, and Zn, and the other containing S, Fe, K, and Cu. EDS analysis provided an indicative average composition, in weight percent of metals, of 80% Fe, 15% Zn, and 5% Cu (Fig. 2F). The observed chemical composition is consistent with historical vitriols often used in ink synthesis [38].

In rare cases, white opaque crystals were identified within Type 3 fragments. EDS analysis suggested that these are likely calcium sulfate. The presence of this compound may be due to a pH decrease (expected for the formation of iron pyrogallate in iron gall ink). An excess of dissolved iron sulfate in solution can indeed cause the reaction of SO_4^{2-} ions with Ca^{2+} derived from the calcium carbonate used in paper fiber manufacturing. Ultimately, the application of iron sulfate-based inks on paper can lead to the displacement of calcium ions from carbonates to sulfate compounds, which precipitate white crystals. Other studies have confirmed the relationship between paper corrosion effects and acidity.

Just in one sample, large short-prismatic crystals with a greenish hue were observed (see Figs. S2A and B). These are attributed to minerals of volcanic origin; EDS analysis confirmed that they are clinopyroxenes (specifically augite), associated with the ink and partially covered by it. Therefore, it is plausible that the pen, ink, or inkwell Vanvitelli used for writing — items that he carried with him during his travels — were contaminated with Vesuvius-derived volcanic ash. (see more below in Raman section and Supplementary material).

3.2. Raman micro-spectroscopy

A vibrational spectroscopy study was carried out to identify the major chemical components of the inks. Raman micro-spectroscopy on Vanvitellian letters allowed us to identify the inorganic composition of the inks (Fig. 3A and B). The investigated fragments of inked paper did not feature any significative chemical differences. Ink molecular characterization resulted in a mixture of a) amorphous carbon (around 1340 and 1600 cm^{-1}) that is ubiquitous, b) iron and other sulphates, with peaks around 986 cm^{-1} (green trace in Fig. 3A), and c) sporadic weak bands (1490 cm^{-1} and 1424 cm^{-1}, 500–600 cm^{-1}, blue trace in Fig. 3A) possibly due to organometallic species related to iron-gallotannates [12,38]. According to experimental results, the ink used by Luigi Vanvitelli could be classified as a mixed ink, with the presence of carbon and iron complexes (possibly iron-gall). The evidence of this type of ink can be found also in Raman characterization of Sardinian documents dating from the 14th to the 16th centuries, particularly the 'Condaghe di San Martino', that revealed the presence of mixed ink, indicating a modified composition of iron gall ink and carbon-based pigments [39]. Moreover, Raman measurement on the *Nova Rethorica* manuscript by Francesco Maria da Ponticelli from the 18th century showed the presence of peaks related to amorphous carbon [23,40,41].

Further Raman features can possibly be related to degradation products. A common quite intense and sharp band can be related to calcium oxalates with peaks at 1475 cm^{-1} and 909 cm^{-1} (green trace in Fig. 3B), as a major degradation product, related to both paper fabrication and iron gall ink degradation [3,42], or induced by microorganisms. The presence of calcium and other metal ions on the surface, as confirmed by SEM-EDS analysis, can be explained as degradation of calcium carbonate (added in paper manufacturing) converting into calcium oxalate compounds.

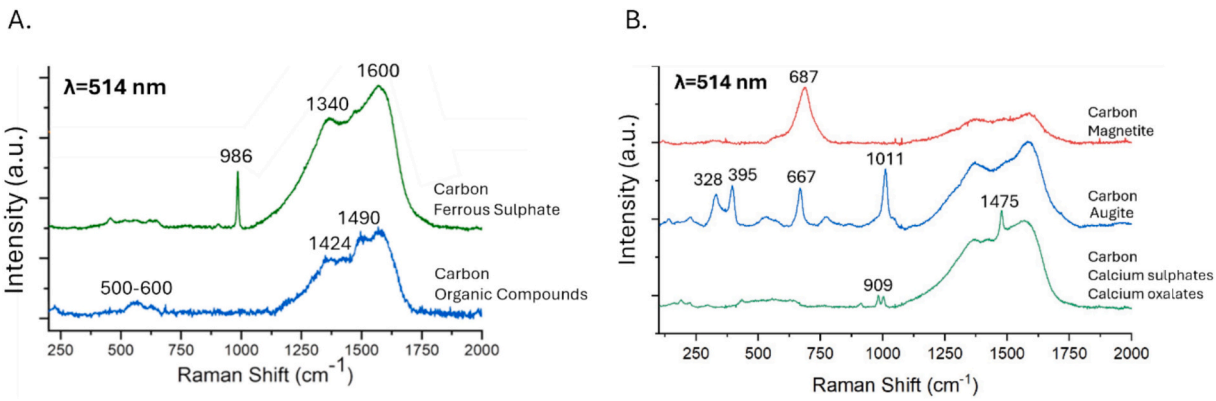

Fig. 3. Raman spectra of ink major components (A) and evidence of major ink components along with potential degradation products or volcanic contamination (B). Laser line 514 nm (2 mW at the sample).

It is worth noting that while performing the diagnostic investigation, some components, not directly associated with possible ink ingredients, were found (Fig. 3B). They correspond to both degradation products (e.g. oxalates, magnetite, calcium sulphate) and products of contamination as volcanic ash (augite) [41–43]. It is noteworthy that volcanic minerals, such as augite [44,45], are present in the capital letter of one of Vanvitelli's writings. This Raman evidence (Fig. 3B), along with the above optical microscopy observation (Figs. S2A nd B), can be explained by the fact that Vanvitelli was still residing in Naples in 1751, a year characterized by intense volcanic activity of Mount Vesuvius, which involved not only lava flows but also the emission of volcanic ash [46]. Therefore, it is plausible that the pen, ink, or container he used for writing — items that Vanvitelli carried during his travels — were contaminated with Vesuvius-derived volcanic ash, even though the letter was composed in Foligno. Furthermore, given that Vanvitelli depicted the 1754 eruption in a drawing preserved at the Royal Palace of Caserta (catalog number 1500052562, inventory number 1817), it is possible that he conducted field observations in the Vesuvius area in 1751, further supporting the hypothesis of contamination of his writing materials [46]. Indeed, the hypothesis of volcanic contamination is consistent with the fact that it was observed only at the beginning of the letter, possibly with a pen still dirty.

In order to prove the robustness of Raman detection of iron gallates (if not degraded) in carbon rich matrices we have also prepared ink mockup for both iron-gall ink and carbon-iron gall inks, using a recipe accepted in literature [1], and consistent with our case study. Indeed, Fig. S3 proves how the Raman spectroscopy can provide a sensitive iron-gallate detection in the presence of abundant 16% in weight amorphous carbon (trace IGI + carbon black in Fig. S3), with features equivalent to those of iron gall complex in the absence of carbon black (trace IGI in Fig. S3), showing robustness in carbon-rich matrices. Despite this Raman ability, real historical inks (even with only 270-year-old as the case under study) may not show detectable iron gall complex Raman features (Fig. 3A) because of degradation, thus providing a false-negative about the absence of iron-gall complex in the ink.

3.3. HPLC-ESI-Q-ToF

HPLC-ESI-Q-ToF analysis was performed on two samples belonging to two different letters collected from two different areas of the letters, one from an inscription (sample A) and the second from an uppercase letter (sample B). The chromatograms acquired in negative ionization mode features the peaks attributed to gallic and ellagic acid, main molecular markers of polyphenols in iron gall inks (Fig. 4), and to degradation products of gallic acid (m_{11}) and polyphenols (d_3) arising during ageing of these writing materials [17].

The amount of gallic acid detected in the extract of sample A was at LOD level, while 2.16 ppm of ellagic acid corresponding to approximately 108 ng in the sample were quantified. For sample B, gallic acid was below LOD, while ellagic acid was detected above LOD but below LOQ (see Materials and Methods Section 2.6 and [17]). It is important to highlight that the extraction procedure used here was optimised on mock-ups prepared with iron-gall inks and not with mixed inks, and that the presence of carbon black may have negatively affected the recovery of the molecular markers.

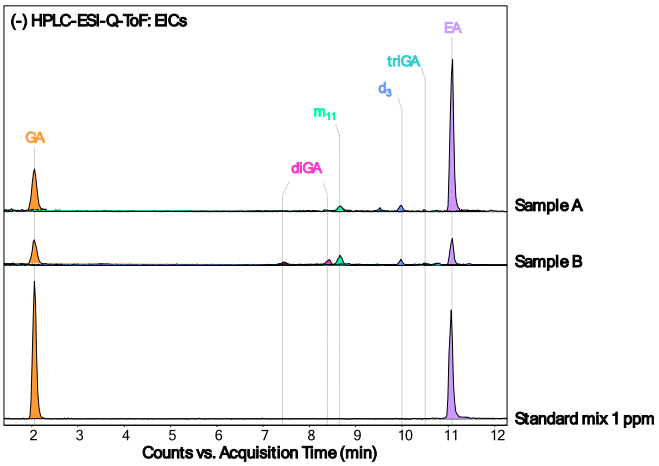

Fig. 4. HPLC-ESI-Q-ToF Extract Ion Chromatograms (EICs) acquired for the EDTA-DMF extracts of samples A and B and a standard solution of 1 ppm gallic and ellagic acid. Gallic acid (GA), digallic acid (diGA), ageing product of gallic acid (m_{11}), ageing product of polyphenols (d_3), trigallic acid (triGA), and ellagic acid (EA). Negative ionization mode. All chromatograms are presented in the same scale and are stacked for purpose of clarity.

Moreover, sample B contains digallic and trigallic acids, which may originate either from the oak gall decoction used in ink preparation or as side-products formed during the auto-oxidation of gallic acid to ellagic acid throughout ink ageing [2,17,18]. All the detected species are reported in Table 1. Taken together, the molecular markers identified in both samples unequivocally confirm the presence of iron gall ink, which eluded clear Raman identification owing to the low abundance of iron gallate in the sample and its degradation (Fig. 3A, trace blue). Our work proves a previously unrecognized analytical failure Raman mode in identifying iron gall in carbon-rich matrices. Only the coupling with a sensitive HPLC-MS/MS provides a false negative reduction, unveiling the gallic acid presence. Moreover, despite the expected heterogeneity of the real case study and mockups, the Raman spectra were well reproduceable in at least five different spots of the sample.

3.4. Microbiological analysis and biodeterioration

To assess the potential occurrence of biodeteriogenic microorganisms, a screening analysis was carried out on the selected letters. Only one of the sampled swabs produced a fungal colony, which was initially examined under a stereomicroscope and an optical microscope to evaluate the morphology of its reproductive structures. The colony appeared black with a velvety texture, and microscopic observations revealed biseriate conidiophores with globose vesicles. Conidia were rough-walled, ranging from 3 to 5 μm in diameter (Fig. 5).

For further confirmation, molecular identification was performed by extracting and amplifying its DNA. The obtained sequence confirmed the presence of *Aspergillus tubingensis* [47,48]. This species belongs to the genus *Aspergillus*, a well-known group of fungi frequently associated with biodeterioration processes in cultural heritage materials. This is mainly due to their ability to produce acids and enzymes capable of degrading organic substrates, including cellulose [49]. The extremely limited presence of microorganisms is related to the microclimatic parameters of the Palatine Library optimised for paper conservation.

3.5. Ink composition and degradation

The integrated analytical approach, combining elemental and molecular techniques (SEM-EDS, Raman micro-spectroscopy, and HPLC-ESI-Q-ToF), demonstrates that the ink is a mixed formulation containing both carbon black (as identified by Raman micro-spectroscopy) and iron gall ink (as identified by HPLC-MS/MS and SEM-EDS), and its degradation products.

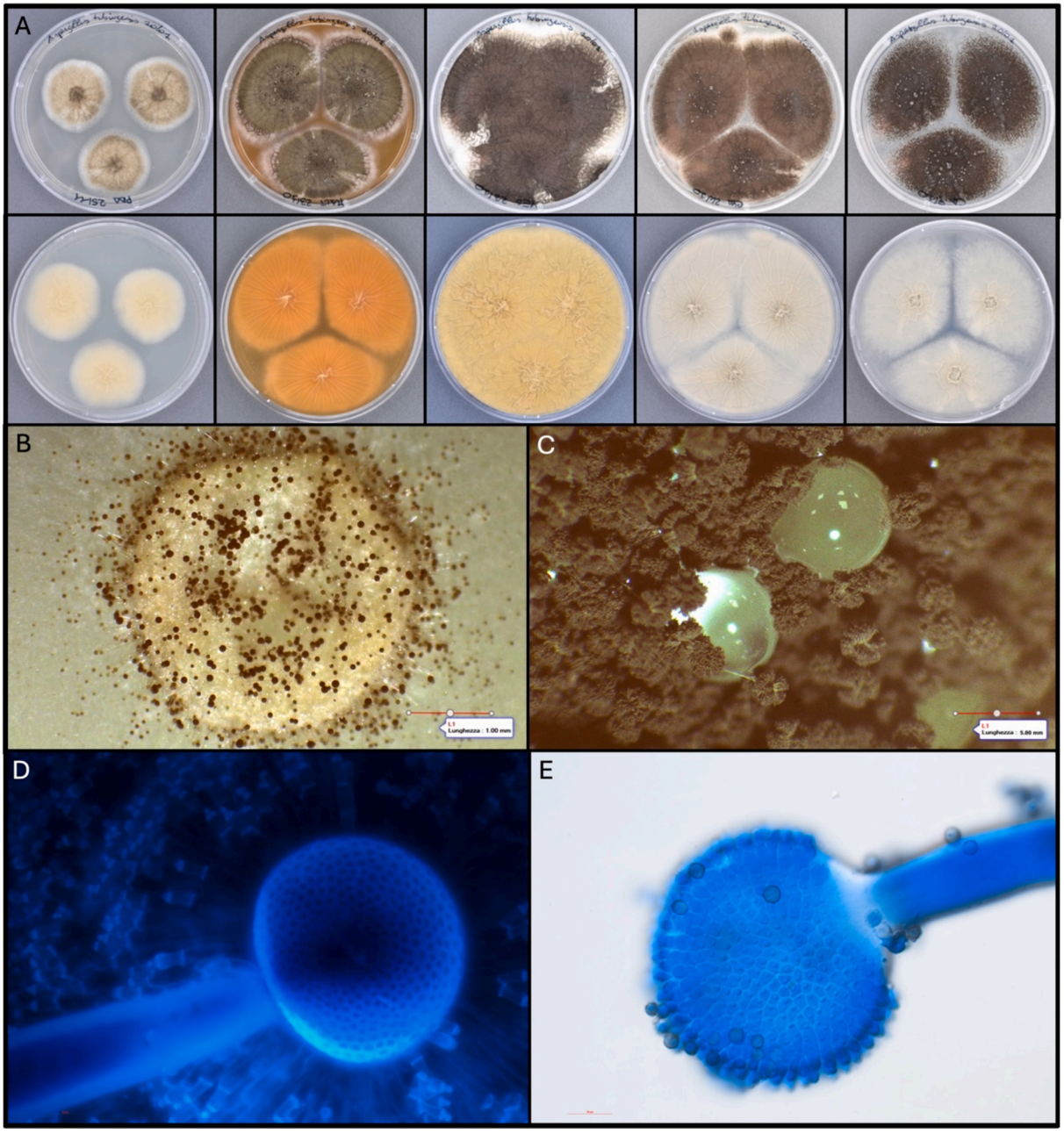

Fig. 5. Morphological characteristics of *Aspergillus tubingensis*: (A) Colonies (top and bottom view) on PDA, MALT, YES, CYA, and CD after 7 days of incubation at 25 °C; (B) colony on CD after 3 days of incubation at 25 °C; (C) detail of conidiophores with exudates on CD after 7 days of incubation; (D) conidiophore stained with Calcofluor, magnification 100×; (E) conidiophore with conidia, stained with Lactophenol Blue, magnification 100×. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

The semi-quantitative EDS analysis provided the average composition in percentual terms of the three vitriols of Fe, Zn and Cu as 80, 15 and 5%, used as starting compounds. Raman evidence of abundant ferrous sulphate suggests a significant molar excess of this component with respect to gallic molar content, possibly contributing with iron-related oxidation path of the manuscript. Furthermore, SEM-EDS and Raman spectroscopy detect the presence of other inorganic compounds associated with degradation phenomena, arising from the complex interactions among the iron gall ink, the paper substrate, and the surrounding environment. These compounds include oxalates, potassium, iron and calcium sulphates, as well as possible contaminants present on the manuscripts, such as volcanic minerals (e.g., augite).

The claim for mixed iron-gall has been reported in several works [11,39,49,50], although the unambiguous identification of all the components is somehow impaired because of the complex nature of the ink and the degradation of gallates. The herein proposed analytical strategy, based on the combination of independent techniques will eventually benefit from the possibility of detecting also the degradation products of gallates, thus indirectly identifying the organic component of the iron-gall ink even in mixture with carbon black [9,29,51–53]. The critical pursuit of multidisciplinary analyses (as summarized in Fig. S4) might definitively help in getting a definitive assignment of the ink recipe.

4. Conclusions

This study provides a novel contribution to the field of cultural heritage science by proposing a combined SEM-EDS, micro-Raman spectroscopy and HPLC-MS/MS approach for the deep characterization of historical inks, enabling complementary molecular and elemental insights into their complex composition and alteration products. In particular, the methodological approach proved to be efficient in characterizing the occurrence of a mixed carbon–iron gall ink formulation in 18th-century manuscripts, an ink recipe that remains “rarely” reported in the literature and difficult to be unambiguously assessed by any single technique.

Our study falls in a research frame headed to shed light historical transition from carbon-based inks to iron-gallate inks, where mixed carbon-iron gallate represents a long transitional phase. Our proposed multi-analytical approach proved to be effective in identifying all the single components of a mixed formulation combining carbon black and iron gall, and, moreover, provides multiple clues on degradation mechanism and volcanic contamination. Indeed, the chemical investigation of the letters exhibits distinct degradation phenomena associated not only with the gallic fraction of the ink (ellagic acid, di-and tri-gallic acid and another degradation product of gallic acid, designated as m_{11}) but also with reactions between the paper substrate and the iron gall components (oxalates and calcium sulphates). Collaterally, the presence of volcanic minerals (e.g., augite) is interpreted as environmental contamination, likely related to Vanvitelli's documented exposure to effusive volcanic activity of Mount Vesuvius. Environmental contamination by volcanic minerals is also hypothesized (augite), attributed to the frequent visits of Vanvitelli for drawing the Vesuvio eruptions during 1751–1754. Microbiological investigation indicates minimal biological impact under current conservation conditions, with only limited fungal presence attributable to *Aspergillus tubingensis*.

From a methodological perspective, Raman spectroscopy, although theoretically suitable for the identification of iron gall inks, proved insufficient for an unequivocal detection of iron gallate complexes in carbon-iron gall ink. HPLC-MS/MS analysis proved essential for identifying the characteristic iron gall ink markers undetected by Raman spectroscopy, whose limited sensitivity in this case was compounded by the extensive degradation of iron-gallate complexes. The degradation products arising from such processes were likewise identified chromatographically. Mockups ageing experiments (in progress) will investigate any possible role of carbon black in catalyzing degradation of iron gallate complex. If confirmed, this would imply a potentially much less rare use of carbon-iron gall ink in the human history. These considerations once more emphasize how the multifaceted nature of cultural heritage objects demands that the investigation of such complex materials extend well beyond the capabilities of any single analytical technique.

Our methodological approach suggests for mixed inks the necessity of a Raman pre-screening followed, if necessary, by HPLC-MS/MS (rarely adopted in literature for mixed inks). Such a combination allows to clearly identify carbon black (via Raman spectroscopy) and indirectly identify iron gall inks (via HPLC-MS/MS), namely its degradation products (such as ellagic acid), in order to avoid misleading interpretations. Ultimately, our work proves a previously unrecognized analytical failure Raman mode in identifying iron gall in carbon-rich matrices. Only the coupling with a sensitive HPLC-MS/MS provides a false negative reduction, unveiling the gallic acid presence.

Altogether, a fundamental takehome message of our study is that when the Raman pre-screening identifies carbon black in an ink, iron gallate complex might be hardly detectable, but HPLC-MS/MS analysis will independently provide the data needed to definitively assign the ink.

Furthermore, the interpretation of degradation processes is inherently complex, as the detected compounds may arise from overlapping contributions of ink composition, paper manufacturing materials, environmental exposure, and possible biological activity. As such, causal relationships between specific degradation pathways and observed chemical markers cannot be herein definitively established, but they cannot be generally ruled out.

CRediT authorship contribution statement

Miriam Alberico: Writing – review & editing, Writing – original draft, Visualization, Investigation, Formal analysis. **Brunella Cipolletta:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis. **Manuela Rossi:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation, Formal analysis. **Silvia Pizzimenti:** Writing – review & editing, Writing – original draft, Visualization, Methodology. **Alessandro Grieco:** Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis. **Maria Francesca Sannino:** Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis. **Antonino Pollio:** Writing – review & editing, Writing – original draft, Supervision, Resources, Methodology. **Adele Ferretti:** Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis. **Ilaria Degano:** Writing – review & editing, Writing – original draft, Supervision, Resources, Methodology. **Leila Birolo:** Writing – review & editing, Writing – original draft, Supervision, Resources, Methodology. **Andrea Carpentieri:** Writing – review & editing, Writing – original draft, Supervision, Resources, Methodology. **Alessandro Vergara:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.microc.2026.118752.

Data availability

Data will be made available on request.

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