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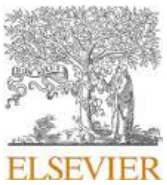

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Forensic toxicology backdates the use of coca plant (*Erythroxylum spp.*) in Europe to the early 1600s

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ABSTRACT

Cocaine hydrochloride salts are one of the most commonly used drugs of our days, yet there is very little hard evidence regarding when people started consuming such an extensively popular drug in Europe. In this paper, we report the exceptional finding of *Erythroxylum* spp. in human remains dated to the 1600's in Milan, Italy.

Toxicological analyses were performed on preserved human brains revealing the first evidence of *Erythroxylum* spp. use in Europe before the 19th century, backdating our understanding of the presence of the plant by almost two centuries. Specifically, the alkaloid of cocaine was detected in two separate biological samples and can be associated to *Erythroxylum* spp. consumption. Given that the plant was not listed inside the detailed hospital pharmacopeia, it may not have been given as a medicinal remedy but may have been used for other purposes. This study demonstrates the importance and the potential of the application of toxicological analyses to archaeological contexts and allows to backdate the arrival of the *Erythroxylum* spp. in Europe by almost two hundred years.

1. Introduction

This study deals with archaeotoxicological analyses on human remains of the 1600s in the context of an important hospital in Milan (Italy). The context of the archaeological site, the archaeotoxicological findings already detected in this site, and the history of the plant encountered during the analytical research performed on the human remains of the 1600s are presented below.

1.1. Historical and archaeotoxicological contexts

During the 17th century, the *Ospedale Maggiore* was a pioneering hospital in Europe, specialized in the medical treatment of acute illnesses among impoverished and disadvantaged individuals residing in the city. The Church of the *Beata Vergine Annunciata* was situated adjacent to the hospital, which was located within the hospital grounds, and contained a sepulcher, the Ca' Granda crypt (Agosti G., 2017). The crypt of the church was intended as the place of burial of the deceased patients of the hospital, for almost the entire 17th century (Biehler--Gomez et al., 2021; Carlessi and Kluzer, 2013; Mattia et al., 2022; Vaglienti and Cattaneo, 2013). The crypt contains 14 underground chambers, which were constructed for the purpose of depositing the bodies of the deceased. It is estimated that these chambers contain approximately 2.9 million bones, which represent over 10,000 individuals who perished in the late Renaissance and Modern hospital (Mattia et al., 2022) This represents an exceptional context from an archaeological, historical, and even toxicological point of view.

Part of the work that has been carried out on these human remains excavated from the crypt of the Ca' Granda hospital was conducetd in order to clarify the toxicological habits of the Milanese population of this

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period of time. Specifically, biological samples of preserved brain tissue and bone have been analyzed, revealing the presence of *Papaver somniferum* (Giordano et al., 2023a). In another study conducted on the same human remains, femoral bone samples were collected and analyzed revealing the presence of *Cannabis* spp (Giordano et al., 2023b). This plant was not present inside the pharmacopeia of the hospital suggesting a usage for recreational purposes, as self-medication, or it may have been administered as part of a medical remedy by healers not practicing in the hospital, or, a final hypothesis, is that it may result from occupational exposure (Giordano et al., 2023b).

1.2. Historical overview of *Erythroxylum spp.*

Cocaine hydrochloride salts are one of the most commonly used drugs of our days: it is an addictive stimulant extracted from the *Erythroxylum coca* plant native to South America. Nowadays, this drug is involved in 1/5 of overdose deaths across the world and had a retail value in 2020 of 1.5 billion dollars in the second largest illegal drug market. Yet, there is very little hard evidence of when people started consuming this plant in Europe.

The *Erythroxylum* spp. is a bush endemic of western South America (Karch, 2017). There, it has been cultivated and used for religious and cultural purposes for thousands of years, whereas it became known to the Old World only after the "discovery" of the Americas (Biondich and Joslin, 2016; Grinspoon and Bakalar, 1979; Karch, 2017). The first Europeans who came into contact with the plant were the Spaniards, upon their arrival in the Americas in the 15th century. When Amerigo Vespucci arrived in Venezuela in 1499, he noticed that the inhabitants of the New World chewed hard leaves of a plant together with lime and roasted shells (a practice they used for 500 years): the plant was the coca plant (Grzybowski, 2008; Karch, 2017; Lemery, 1737). The Spaniards, after the invasion of the New World, reported that the Inca Empire took control of all the coca plant crops of the Andes territory thanks to the expansion of the Empire and that all coca leaves collected were delivered to the Inca Royals and used for the religious practices of the entire population. Indeed, the Inca population considered it a miraculous and magical plant that had the power to take away hunger and thirst, produced exhilarating effects, could be used as medicine (as antiseptic and analgesic, to help in digestion, to cure asthma, stomach ache, chest pain and sores, reduce nose bleeding and vomit), and induced a sense of well-being (Bernabè Cobo, 1890; Grinspoon and Bakalar, 1979; Karch, 2017). The Incas knew that chewing coca led to a loss of sensitivity on the tongue; therefore, they hypothesized that this could slow down the decomposition of a body and used a coca tincture for mummification procedures (Karch, 2017). The Spaniards showed little interest in coca leaves up until later when they understood their immense commercial value; indeed, they began to financially prosper from *Erythroxylum* spp. by managing the production and commerce of the plant into the New World (Bernabe Cobo, 1890; Karch, 1999, 2017). They used coca leaves to enhance their ability to work in gold and silver mines as well as plantations (Grinspoon and Bakalar, 1979). However, historical sources report that *Erythroxylum* spp. was not exported as there was no demand in Europe (Karch, 1999, 2017). This is due to the information embargo that the Spaniards imposed on the New World in the 16th and 17th centuries, keeping Europeans ignorant about the *Erythroxylum* spp. (Grinspoon and Bakalar, 1979; Karch, 1999, 2017). Indeed, the Spaniards were far more interested in the trade of silver, gold, tobacco, and sugar as these were products in high demand in Europe, largely available, and very lucrative, rendering the expenses of the transatlantic voyages profitable (Karch, 1999, 2017). In spite of this embargo, some conquerors tried to export coca leaves, but these would ultimately undergo serious deterioration during transport preventing their introduction in Europe until the beginning of the 19th century (Karch, 2017; Plowman, 1984).

According to literature, the consumption of *Erythroxylum* spp. was limited to the New World until the 19th century, when it was synthesized as cocaine hydrochloride salts (Karch, 2017; Plowman, 1984). However, some authors suggest that this may not be the case considering that Spaniards already knew of the existence of this plant and its effects on the human body (Karch, 2017). In addition, people from the Old World were interested in the exotic plants present in the New World. For instance, Nicholas Monardes, a Spanish doctor of the 16th century, had access to ships returning from the Americas and personally grew New World plants (Karch, 2017). Moreover, Karch (2017) reports that a French botanist, Joseph de Jussieu, was able to ship samples of *Erythroxylum* spp. back to Paris during the 1750s (Grinspoon and Bakalar, 1979). Between the 16th-17th centuries a sea-trade of exotic plants was established between Milan, at the time under Spaniard domination, and the New World, to expand the naturalistic knowledge of the time, thus demonstrating a direct connection between the Italian city and the continent of origin of the plant (Crivelli, 2021).

In an innovative approach using archaeotoxicology, hard evidence from human remains could shed new light into the history of consumption of cocaine in Europe. Toxicological analyses performed on archaeological remains are rare in literature but constitute a substantial contribution to our knowledge on the history of medicine and on the lives and habits of past populations. Indeed, archaeotoxicological analyses have been performed on hair of pre-Columbian coca leaf chewers revealing the alkaloid of cocaine (Cartmell et al., 1991b; Springfield et al., 1993), benzoylecgonine alone, the inactive metabolite of cocaine (Brown, 2014; Cartmell et al., 1991a; Indriati and Buikstra, 2001) or both of them (Socha et al., 2022b).

Yet, to the best of our knowledge, the active component of cocaine that may be reassociated to the use of *Erythroxylum* spp. has never been detected in archaeological human remains in Europe before the Contemporary era (19th-21st century).

1.3. Aim of the study

This paper will show that during the toxicological analyses carried out for the investigation on the pharmacological habits of the population of the city of Milan represented within the crypt (that is, the poor and the laborers), well-preserved brains revealed the presence of alkaloids characteristics of the *Erythroxylum* spp. These laboratory analyses allowed not only to backdate the arrival of the *Erythroxylum* spp. by almost two centuries in Europe, but also demonstrate that some Milanese citizens came into contact with the New World plant and chewed its leaves.

2. Material and methods

2.1. Study sample

Eight crania and eight residues of brain tissue (C1 to C8 and B1 to B8, respectively) were collected from the stratigraphic unit 4 of chamber O of the Ca' Granda crypt. In addition, a ninth well-preserved brain tissue (B9) was found without its cranium. Due to the comingled human remains situation, it is usual to find disarticulated human remains and as a consequence it is not unusual to find preserved brain tissue no longer positioned inside its cranium.

The anthropological results are summarized in Table 1. The skeletal remains were evaluated to determine age-at-death, biological sex, and ancestry, as well as pathological indications. This was done in accordance with standard anthropological methods (Alqahtani, 2019; Hackett, 1975, 1976; Hefner, 2009; Hefner and Ousley, 2014; Mann, 1987; Mann et al., 1987; Trotter, 1970; Walker, 2008). The finding of tertiary syphilis (Fig. 1) was already published in a previous paper (Giordano et al., 2023a).

2.1.1. Sample collection and preservation

Details on sample collection and preservation are reported in our previous paper (Giordano et al., 2023a). A sterilized scalpel was utilized

Table 1
Summary of anthropological results. "ND" = not determinable; "/" = absent.

CRANIAL SAMPLE	SEX	AGE (YEARS)	PATHOLOGICAL SIGNS	TRAUMA	POPULATION AFFINITY	STATURE
C1	Male	ND	/	/	ND	ND
C2	Male	30–45	Tertiary syphilis: *caries sicca* on the right parietal bone (stage 3)	/	ND	165.9 ± 4.05 cm
C3	Female	Young adult	/	/	European	ND
C4	Female	ND	/	/	ND	ND
C5	Female	Young adult	/	Ante-mortem trauma on left parietal bone	European	ND
C6	ND	ND	/	/	ND	ND
C7	ND	11–12 years	/	/	ND	ND
C8	Male	ND	/	/	ND	ND

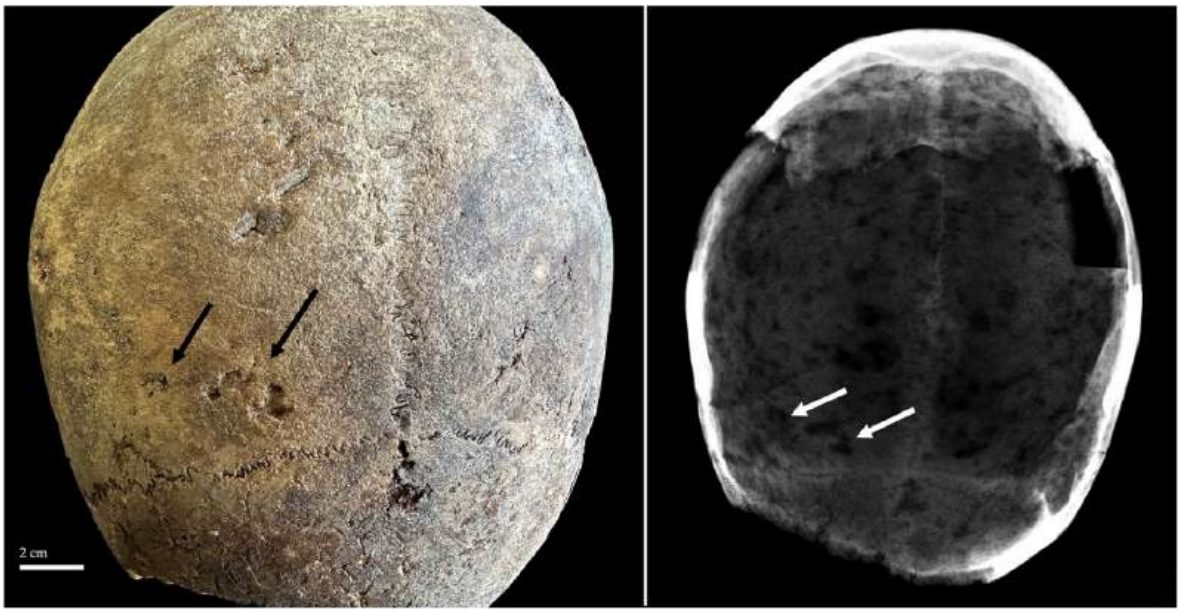

Fig. 1. Signs of caries sicca on cranium C2, both photograph and X-rays image.

for the collection of brain samples. The sampling site section chosen for histological analysis exhibited clearly visible and well-preserved encephalic convolutions, with the assumption that these areas would contain more intact structures (Dinis-Oliveira et al., 2016).

All brain specimens presented portions of cerebral hemispheres with gyri and grooves that could be recognized; they had similar morphology, consistency, and color. Different specimens of one brain (B4) were sampled for histological analysis which confirmed that the biological soft tissues were actually brain tissue, as reported in Giordano et al. (2023a). Even for the histological investigation, the selection of the specimens was based on the state of preservation of the brain remains: indeed, the well-preserved and well-visible encephalic convolutions hypothesized more preserved structures. Three samples were obtained from the frontal, parietal, and occipital brain regions, as described in our previous study (Giordano et al., 2023a).

Additionally, a bone sample from C2 was sent to Beta Analytic Radiocarbon Dating Laboratory (Miami, Florida) for radiocarbon dating using in-house NEC accelerator mass spectrometry and Thermo Isotope Ratio Mass Spectrometry (IRMS). The bone sample, weighing approximately 5 g and showing no signs of diagenesis or taphonomic processes in the cortical bone, was selected for radiocarbon analysis based on its weight and excellent preservation. The calibrated results reported with 95.4% probability: (55.4%) 1539–1635 cal AD (411-351 cal BP) or (40%) 1460–1540 cal AD (490-420 cal BP), as previously published (Giordano et al., 2023a). Considering that all the human remains were found in the same stratigraphic unit, we can assume that the radiocarbon dating can be applied on all the subjects considered in this study.

2.2. Toxicological analyses

2.2.1. Instrumentation

A standard 12-port vacuum manifold and Bond Elut™ Certify cartridges (130 mg, Agilent) were employed for Solid-Phase Extraction (SPE) procedures. Analyses were conducted using a Thermo Scientific™ TSQ Fortis™ II Triple-Quadrupole Mass Spectrometer.

2.2.2. Chemicals and reagents

All standard molecules utilized in this study, including the Internal Standard (IS) SKF 525-A (Proadifen hydrochloride, analytical standard, >95%, 100 mg), were obtained from Sigma-Aldrich and LGC Standards and stored at −20 °C. Solvents such as methanol were sourced from Sigma-Aldrich, while buffer solution pH 6, dichloromethane, 2-propanol, acetic acid, ammonia solution, and water for UV, HPLC, ACS were procured from PanReac AppliChem ITW Reagents.

2.2.3. Sample preparation

Samples were collected from the brain tissues of individuals of the 17th Century Ca' Granda crypt of Milan. The samples preparation procedure is reported in Table 2 (Di Candia et al., 2022; Giordano et al., 2023a).

Table 2
Steps for the preparation of the samples.

STEPS FOR THE SAMPLE PREPARATION	
BRAIN SAMPLES	- Powdered with a scalpel −0.5 g of powder put in 8 mL of pH 6 phosphate buffer solution −100 ng of Internal Standard SKF 525-A (proadifen hydrochloride) were added - Solutions agitated on a Vortex mixer (Heidolph, REAX top) - The samples were left on a rotating wheel (Falc F205) for 48 h - Centrifuged for 30 min at 3500 rpm (Thermo Scientific, Heraeus Biofuge primo centrifuge)

2.2.4. Samples extraction procedure

The consequent supernatants of the prepared samples have been extracted with solid-phase extraction, using the Bond Elut™ Certify cartridges 130 mg (Agilent) as reported in Table 3 (Di Candia et al., 2022; Giordano et al., 2023a).

Then, the eluates were prepared for the analytical investigation as reported in Table 4 (Di Candia et al., 2022; Giordano et al., 2023a).

The instrumental conditions of the Thermo Scientific™ TSQ Fortis™ II Triple-Quadrupole Mass Spectrometer are reported in Tables 5 and 6.

2.2.5. Qualitative and quantitative investigation

The molecules under investigation were identified following international standard guidelines for forensic toxicology (American Academy of Forensic Science, 2019), and all identification criteria were met (signal-to-noise ratio above 3, parent ion, characteristic fragmentation, retention time and mass spectral ion ratio) (Di Candia et al., 2022; Giordano et al., 2023a). The chromatographic spectra and mass spectral ion ratio of each molecule are reported in Figs. 2–7.

The quantitative analyses were performed with the preparation of a linear calibration model for each molecule under investigation. The calibration curves were developed starting from working solutions of the analytes of interest and spiking blank mummified brain tissue with increasing concentration. The coefficient of determination (r^2) for each linear calibration model was calculated $\geq$0.99 for each molecule with a coefficient of variation (%CV) $\leq$ 10.

However, the quantification on brain tissues with such a long post-mortem interval (about 400 years) cannot be compared to the concentration that can be obtained in fresh biological samples since the specimens underwent extensive decomposition and taphonomic effects. Nonetheless, what cannot be quantified is the exact quantity of the substances in circulation at death because decomposition may concentrate or dilute components of brain, depending on too many factors – but the presence of the analyte, when detected, is certain. Due to the state of preservation of the samples, quantification was performed for completeness and should not be considered representative of the actual concentrations present at the time of death of the subjects.

The chromatographic spectra of each molecule are reported below for completeness (Figs. 2–4).

3. Results

Toxicological analyses revealed the presence of substances of toxicological interest in two out of the nine preserved brain tissues examined, as reported in Table 2. Different active components of the *Erythroxylum* spp. were detected, namely cocaine, benzoylecgonine (inactive metabolite of cocaine) and hygrine, as reported in Table 7 and Figs. 5–10. Of these two individuals, one was a male of 30–45 years with pathological signs of tertiary syphilis (C2-B2), whereas the other was the only preserved brain tissue for which the cranium of origin could not be reassociated during the archaeological excavation (B9).

Table 3
Solid-phase extraction procedure.

EXTRACTION PROCEDURE STEPS	SOLVENTS
CARTRIDGES CONDITIONING	−2 mL MeOH, 2 mL of pH 6 phosphate buffer
SAMPLE LOADING	−8 mL of solution
CARTRIDGES WASHING	−6 mL of H_2O, 3 mL 1 M acetic acid, desiccation for 5 min and 6 mL of MeOH, and drying of the cartridges
CARTRIDGES ELUTION	−2 mL of DCM/IPA/NH_4OH (78:20:2, v:v)

Table 4
Procedure for the analytical investigation.

STEPS FOR ANALYTICAL INVESTIGATION	
STEPS	- Eluates dried in a vacuum rotary evaporator (Thermo Scientific, Savant SpeedVac Concentrator) - Restored with 100 µL of MeOH −2 µL of final solution analyzed with Thermo Scientific™ TSQ Fortis™ II Triple-Quadrupole Mass Spectrometer

Table 5
Gradient of the solvent utilized for the analyses.

Time (min)	Solvent A % (H2O at 01% of formic acid)	Solvent B % (MeOH)
0.00	95	5
1.00	95	5
4.00	5	95
6.00	5	95
6.10	95	5
10.00	95	5

Table 6
Instrumental conditions of the Thermo Scientific™ TSQ Fortis™ II Triple-Quadrupole Mass Spectrometer.

Instrumental conditions	
Capillary and vaporization temperature	330 °C and 280 °C
Electrospray tension	3.5 kV with positive mode
Positive ion spray voltage	3,500V
Sheath gas, aux gax, sweep gas	45 Arb, 20 Arb, 10 Arb
Q1 and Q3 resolution	0.4 FWHM, 0.7 FWHM
CID gas	1.5 mTorr
Resolution power of FS	70.000 FWHM
Mass range	50–650 m/z
Acquisition mode	Inclusion list
Data Independent Acquisition mode	MS2 spectrum, Positive mode at 35.000 FMHM
Automatic gain control (AGC)	5×10^{-4}
Maximum injection time	100 ms
Quadrupole filtered precursors ions	Isolation range of 2 m/z
LOD and LLOQ	S/N > 3 and S/N > 10

4. Discussion

4.1. Possible external contamination

The above-mentioned remains were found inside chamber O of the Ca' Granda crypt. The chamber was accessed exclusively via a ceiling opening, with the manhole having been sealed until the very first opening. To perform their initial site inspection, the archaeologists accessed the chamber, equipped with personal protective equipment, in accordance with established safety protocols. After preliminary inspection, the first archaeological excavation campaign begun. The samples designated for further investigation were collected by archaeologists wearing personal protective equipment and gloves, under the supervision of toxicologists. The crania, along with their preserved brain tissue, were stored in sterilized, sealed jars and kept inside the Ca' Granda crypt to preserve the sepulcher chamber's environmental conditions until analysis. For each investigation (toxicological and histological analyses), well-preserved brain tissue samples were extracted using a

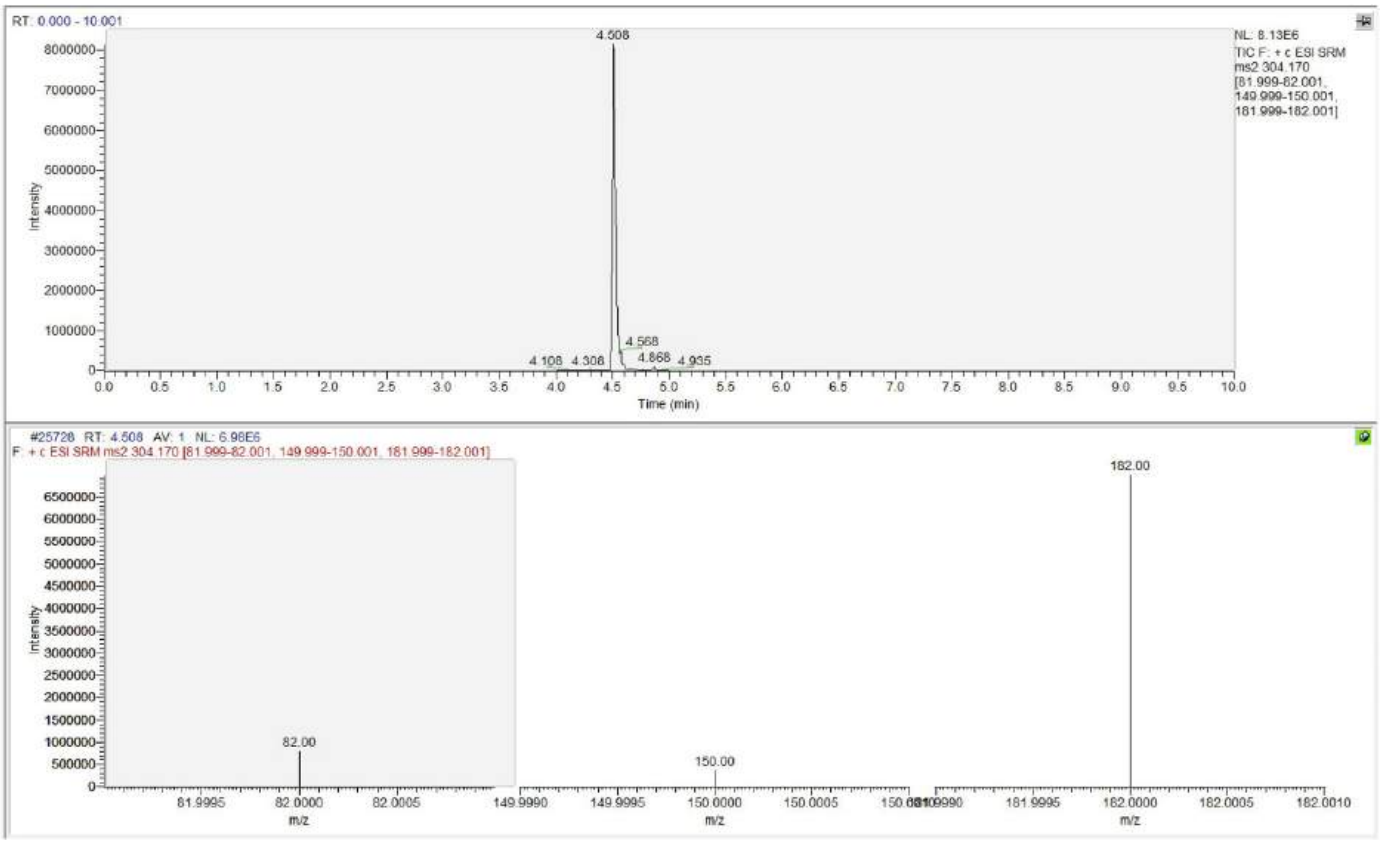

Fig. 2. Chromatographic spectrum and fragmentation of cocaine.

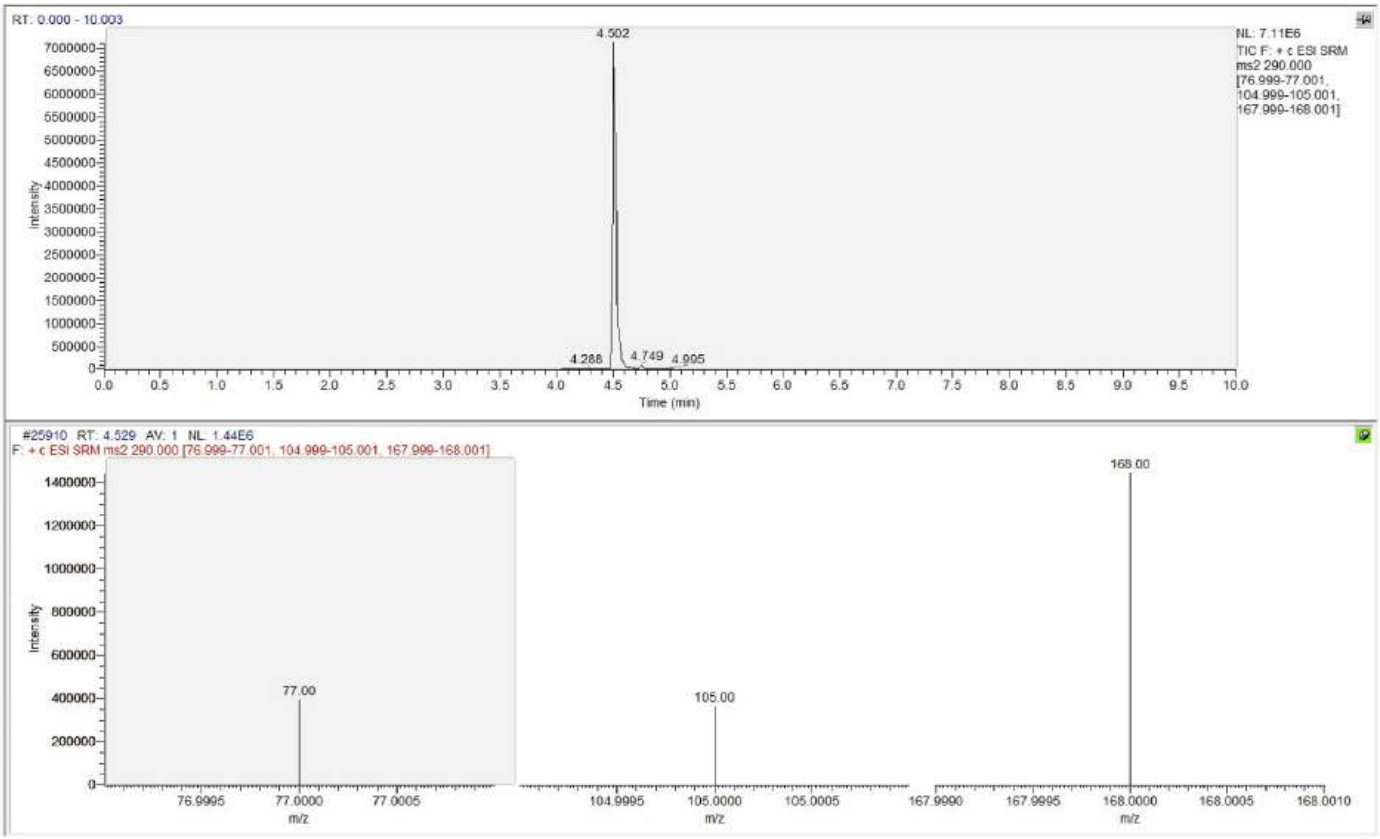

Fig. 3. Chromatographic spectrum and fragmentation of benzoylecgonine.

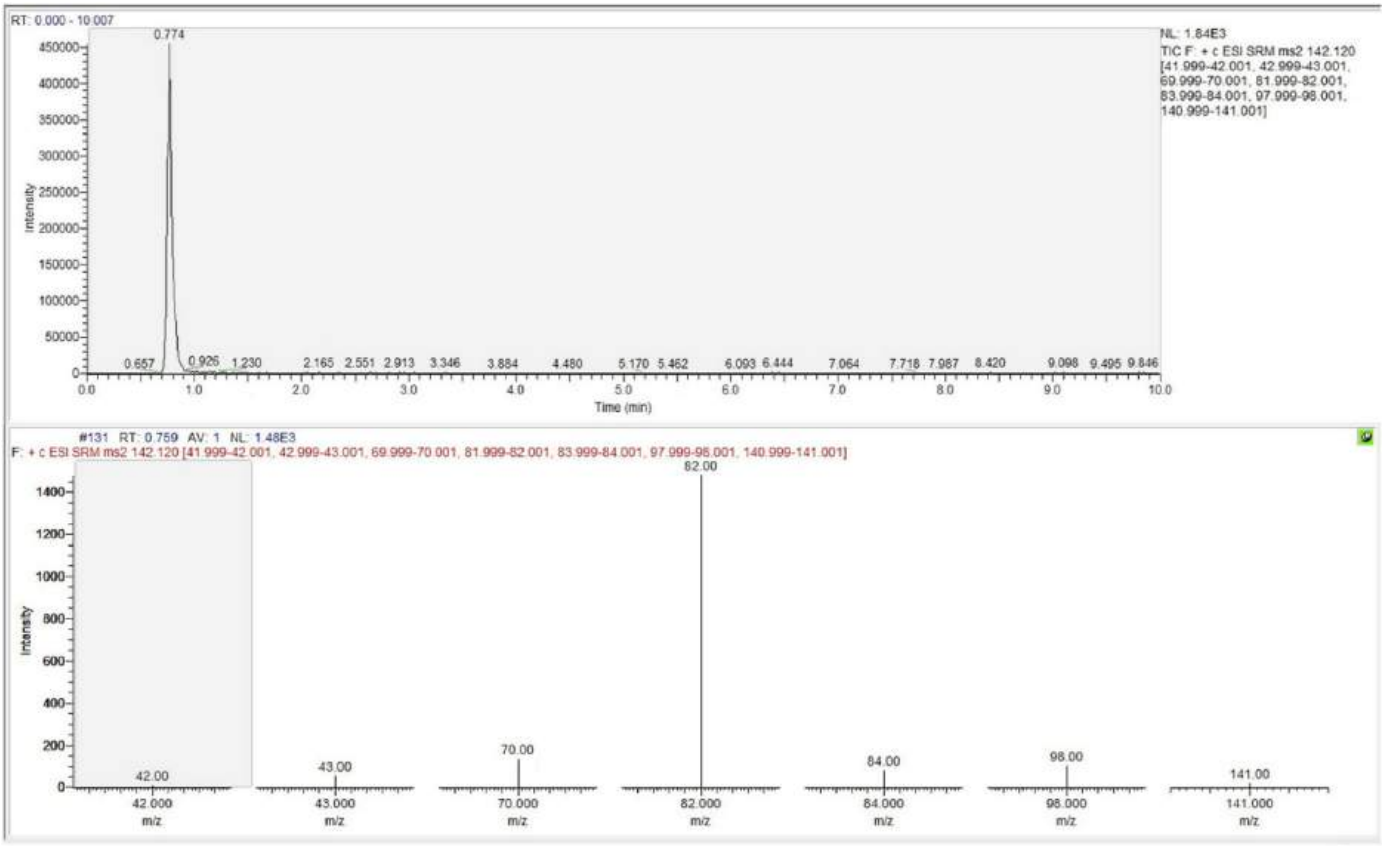

Fig. 4. Chromatographic spectrum and fragmentation of hygrine.

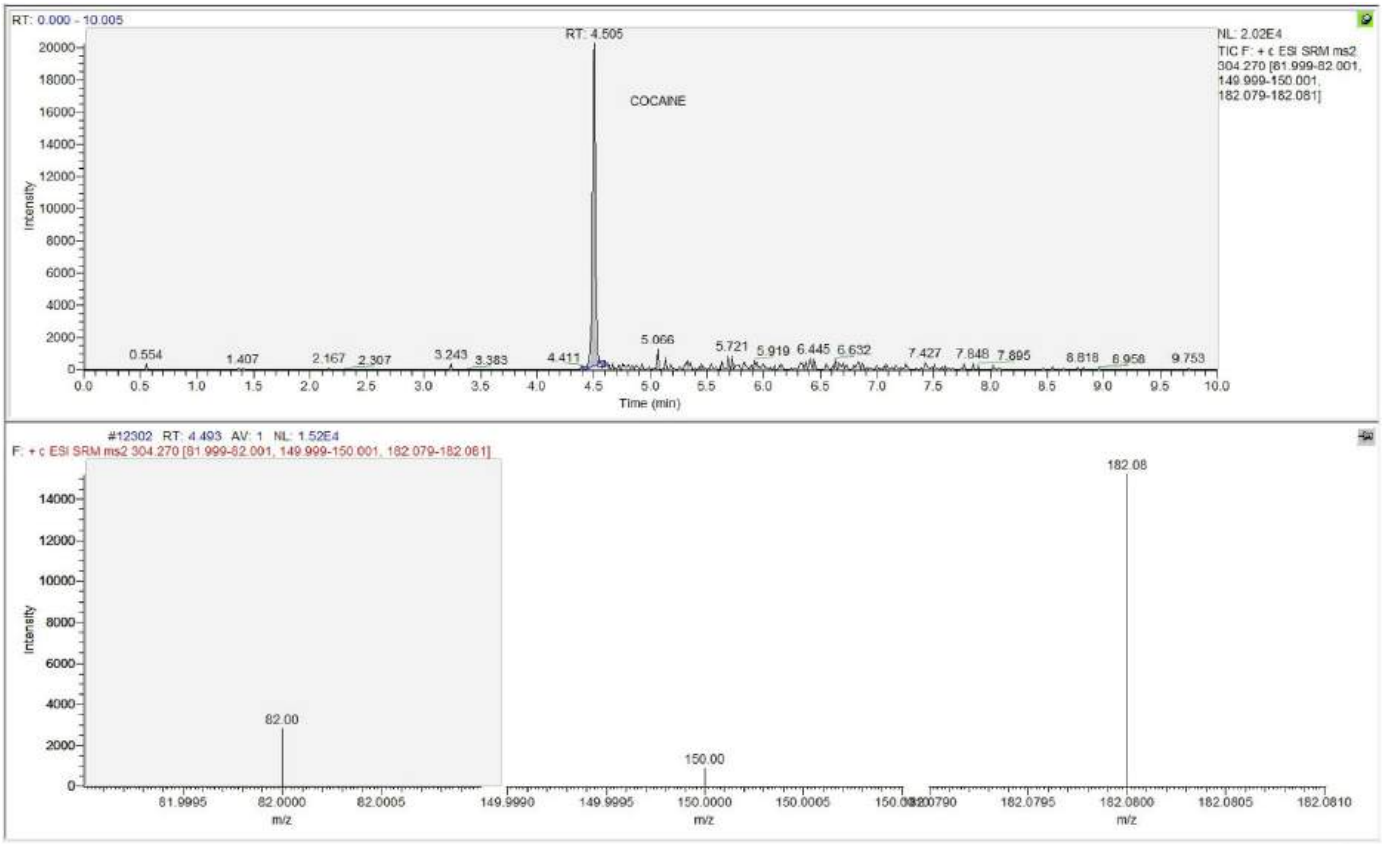

Fig. 5. Chromatographic spectrum (top) and mass spectral ion ratio (bottom) of cocaine detected in B2.

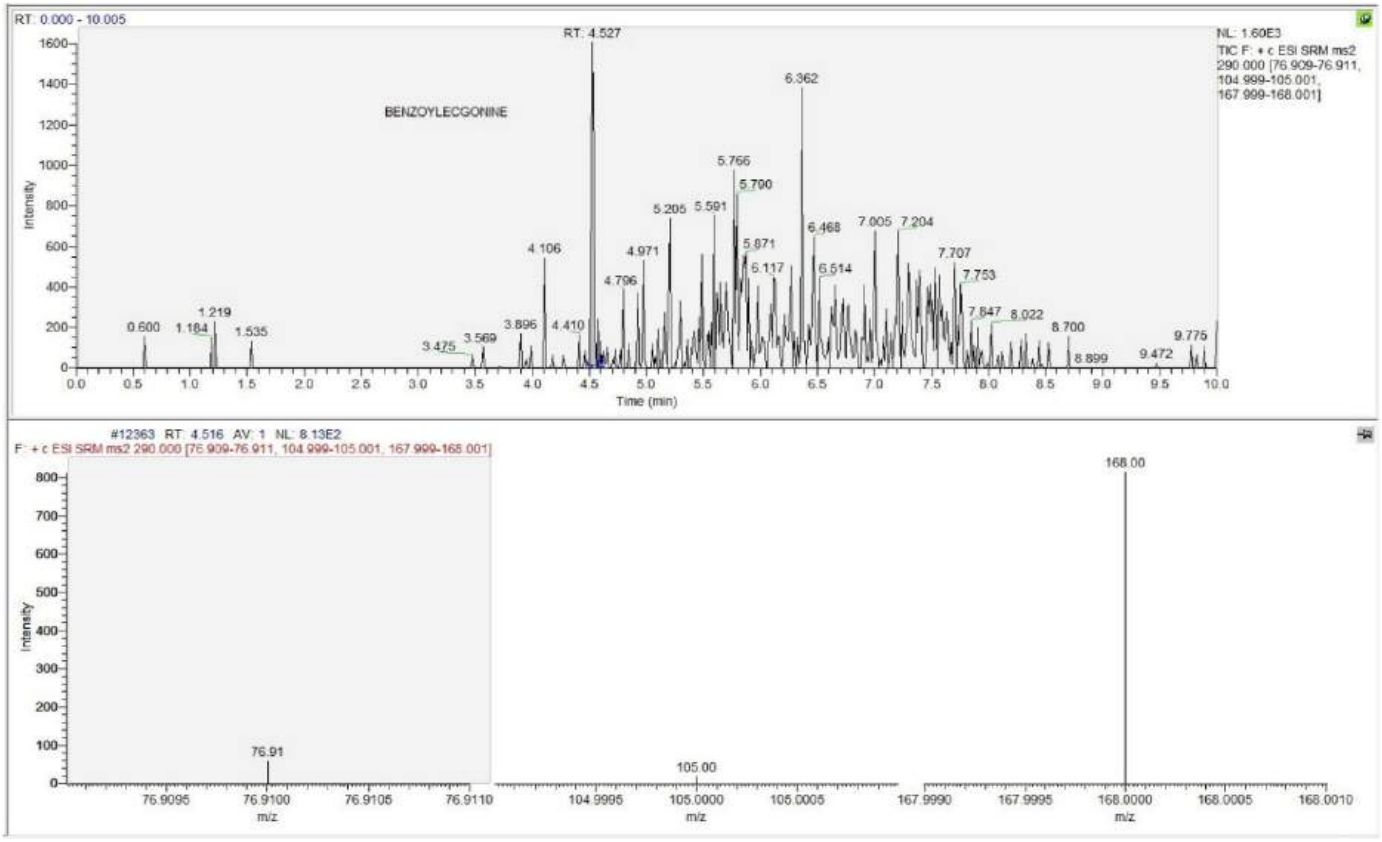

Fig. 6. Chromatographic spectrum (top) and mass spectral ion ratio (bottom) of benzoylecgonine detected in B2.

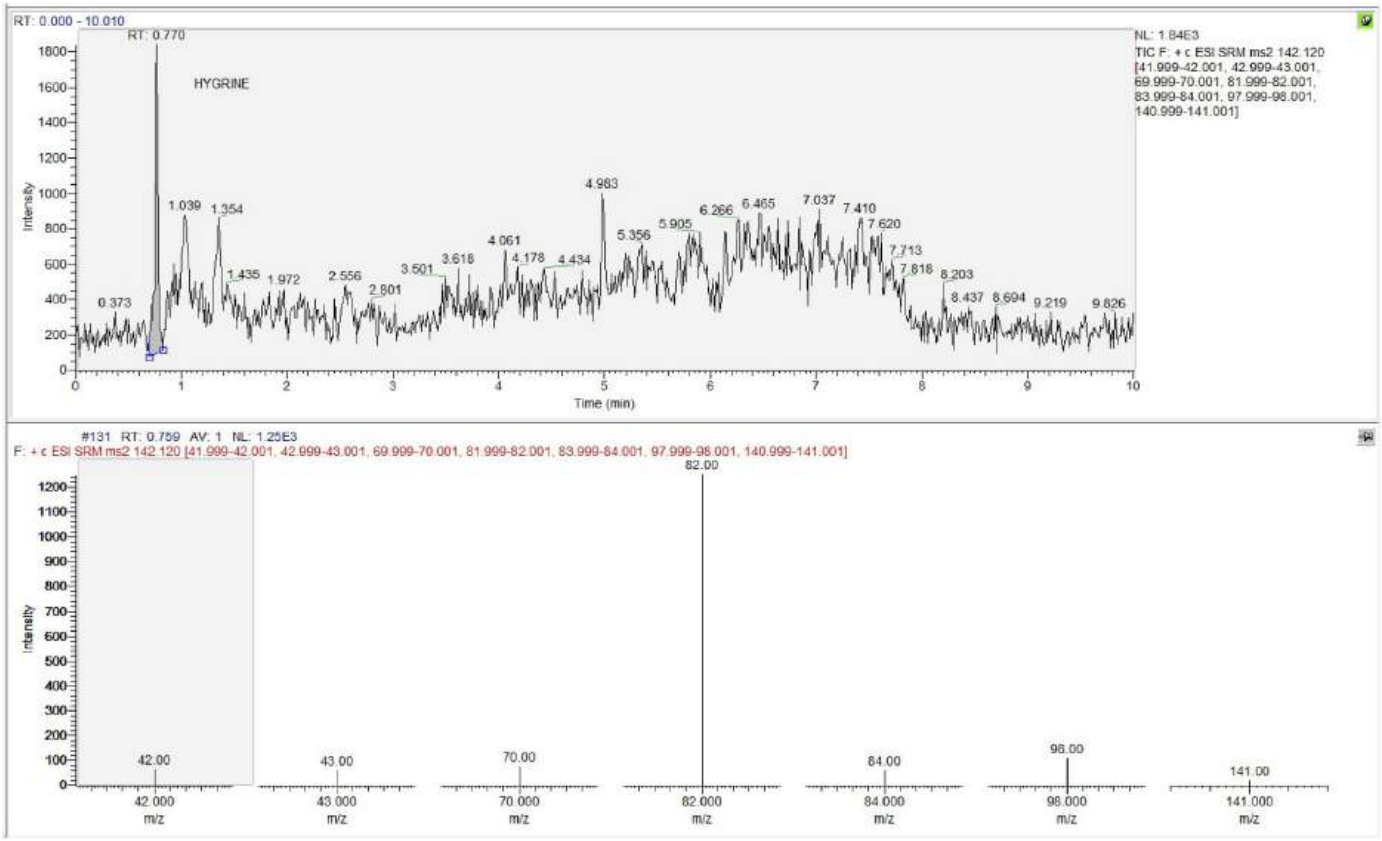

Fig. 7. Chromatographic spectrum (top) and mass spectral ion ratio (bottom) of hygrine detected in B2.

Table 7
Toxicological results of preserved brain samples.

SAMPLE ID	RESULTS	NG/G
B1	/	/
B2	Cocaine	2.2
	Benzoylecgonine	Traces
	Hygrine	Traces
B3	/	/
B4	/	/
B5	/	/
B6	/	/
B7	/	/
B8	/	/
B9	Cocaine	2.98
	Benzoylecgonine	0.21
	Hygrine	Traces

sterilized scalpel. Prior to their arrival at the laboratory, the brains were not removed from the cranium, ensuring their continued protection from external factors. This was accomplished by their placement within the cranium and the use of sterile, sealed jars. Upon discovery during the archaeological excavation, one brain tissue was no longer within the cranium. However, it was immediately placed in a sterilized and sealed jar for transport to the laboratory, where it remained protected until the time of analyses (Giordano et al., 2023a).

The negative results on other tissues and bones surrounding the ones that were positive act as a guarantee of the absence of environmental contamination.

4.2. Confirmation of Erythroxylum spp. Consumption

Cocaine hydrochloride salts may be degraded to benzoylecgonine, and external exposure may contaminate hair samples, incorporating the molecules into this specimen probably due to the porosity of the biological matrix (Hoelzle et al., 2008; Musshoff et al., 2017; Pragst et al., 2010). Nevertheless, the circumstances delineated in this research are distinct from those previously reported in the literature. Indeed, the presence of hygrine, as we will discuss below, confirms that the molecules belonged to coca leaves and not to cocaine hydrochloride salts (Hoelzle et al., 2008; Musshoff et al., 2017; Pragst et al., 2010). Thus, the presence of cocaine and metabolites of coca leaves exclude the possibility of environmental exposure to the plant, as reported in previous articles (Hoelzle et al., 2008; Musshoff et al., 2017; Pragst et al., 2010), which discuss contemporary contamination due to the different formulation of the molecules (cocaine hydrochloride salts). Similarly, because contamination by contact or proximity to coca leaves is not plausible, this excludes the possibility that the cocaine molecule had been degraded to benzoylecgonine, as suggested in the previously cited articles.

In this study, the three molecules detected can be associated to the *Erythroxylum* spp. Specifically, the three molecules were: cocaine, benzoylecgonine, and hygrine. The molecule of cocaine is the major active component present in *Erythroxylum* spp. (Katzung et al., 2012; Zimmerman, 2012).

However, the detection of this molecule in the biological samples under investigation does not confirm the consumption of the plant but rather hypothesizes that the patients of the hospital had been exposed to the plant. To confirm the consumption of the plant, it is necessary to detect at least one metabolite of cocaine. In this specific case, the metabolite in question is benzoylecgonine. Indeed, cocaine is rapidly metabolized by plasma and hepatic cholinesterase enzymes of the organism into benzoylecgonine, its main inactive metabolite (Zimmerman, 2012). Thus, benzoylecgonine is a product of the human metabolism, formed by the hydrolysis of cocaine (Zimmerman, 2012). This implies that the individuals under investigation consumed *Erythroxylum* spp. in order to detect benzoylecgonine in their brain tissue. The

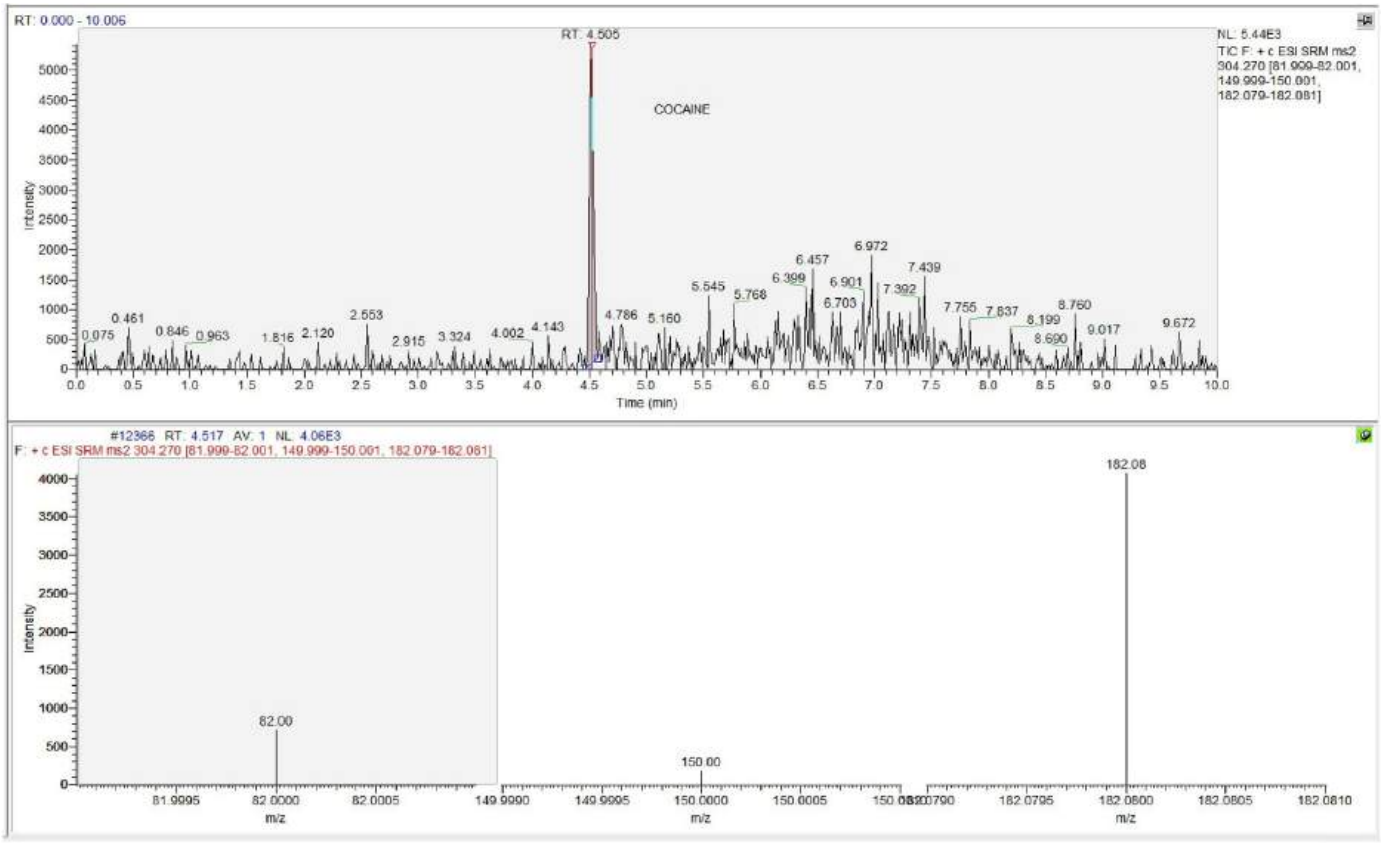

Fig. 8. Chromatographic spectrum (top) and mass spectral ion ratio (bottom) of cocaine detected in B9.

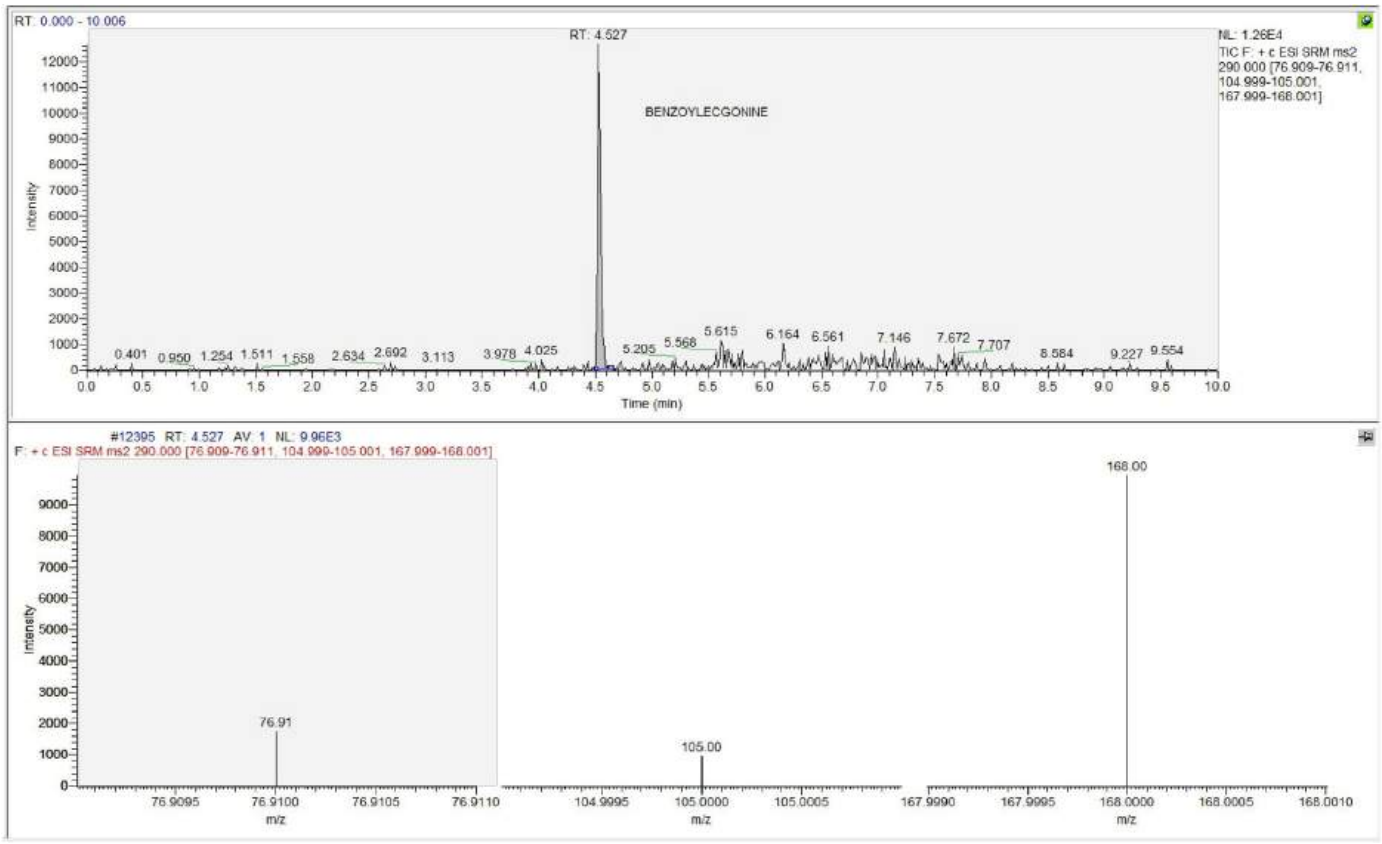

Fig. 9. Chromatographic spectrum (top) and mass spectral ion ratio (bottom) of benzoylecgonine detected in B9.

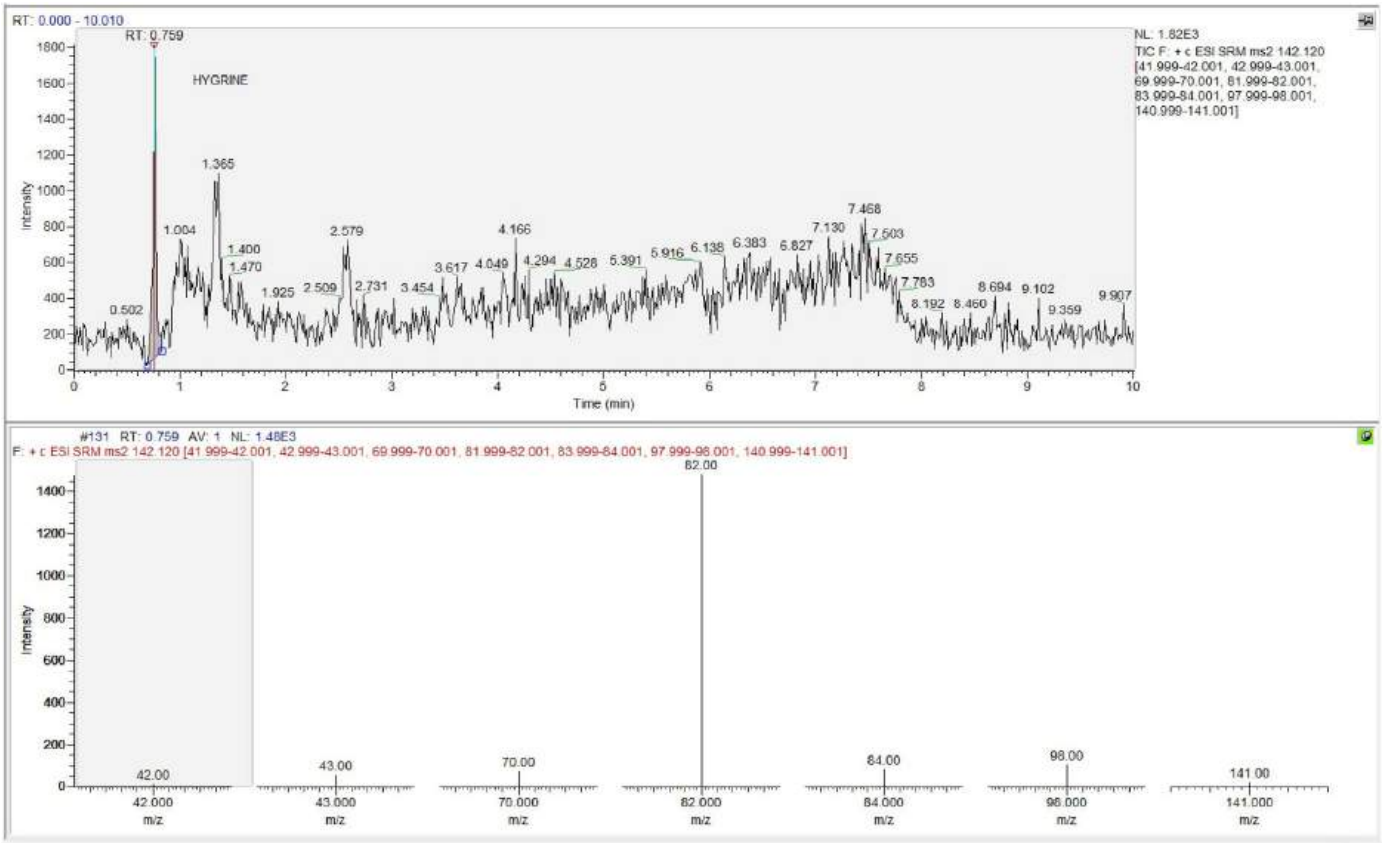

Fig. 10. Chromatographic spectrum (top) and mass spectral ion ratio (bottom) of hygrine detected in B9.

absence of benzoylecgonine would have precluded the hypothesis that the patients were consuming *Erythroxylum* spp. Conversely, it would allow to hypothesize that the patients had been exposed to the plant. Thus, the detection of cocaine and benzoylecgonine were essential to confirm the presence of the plant in Europe, and in Italy in particular, before the 19th century but also to confirm the consumption of the plant by the subjects under investigation.

The presence of cocaine (alkaloid) and benzoylecgonine together indicates that the individuals under investigation consumed the molecule. However, it did not allow us to conclude whether the consumption was in the form of leaves or cocaine hydrochloride salts (Karch, 2017; Rubio et al., 2013, 2015, 2014). Hence, in order to distinguish in which form the drug was taken by the patients, the detection in the human remains of a third molecule was required: hygrine (Rubio et al., 2013, 2015, 2014).

Some studies tried to distinguish coca leaf chewers from cocaine hydrochloride salt abusers by performing toxicological analyses on different biological samples collected from living subjects (Rubio et al., 2013, 2015, 2014). The analyses performed on coca leaf chewers showed molecules of the *Erythroxylum* spp. such as cocaine, ecgonine methyl ester, tropacocaine, cinnamoylcocaine, hygrine and cuscohygrine, whereas those on cocaine hydrochloride salts users did not reveal the presence of hygrine or cuscohygrine, although cocaine and metabolites were detected. These observations were made based on the analyses of urine and hair of coca chewers and cocaine hydrochloride salt abusers: hygrine and cuscohygrine were found in coca chewers but not in cocaine abusers. Since the extraction and purification of cocaine from the coca plant leads to the loss of the alkaloids tropacocaine, cuscohygrine and hygrine present inside the leaves, the authors concluded that the presence of hygrine and cuscohygrine in urine constitutes a discriminating factor to understand whether the subjects consumed cocaine hydrochloride salts or coca leaves.

Therefore, the 3rd molecule detected in the brain tissues of our subjects, hygrine (an alkaloid present in the leaves of *Erythroxylum* spp. only), was essential to determine that the molecules detected in these human remains derived from the chewing of coca leaves or from leaves brewed as a tea, consistent with the historical period (Rubio et al., 2013, 2015, 2014; VanPool, 2019). Therefore, the detection of hygrine excludes that the molecules were derivatives of some external and more recent contamination from anthropic influence.

Moreover, the fact that these molecules were detected in brain tissue samples implies that the *Erythroxylum* spp. intake occurred near the time of death of the individuals (Giordano et al., 2023a). Indeed, the aforementioned matrix is representative of an acute intake of substances, and, as such, can even show a single intake of the plant near the time of death of a subject (Giordano et al., 2021).

4.3. Literature analysis

The present study, to the best of our knowledge, constitutes the first report on the detection of compounds derived from the *Erythroxylum* spp. in European historical and archaeological human remains. Indeed, according to literature, this category of drugs has never been detected in human remains of European origin. Although some evidence of cocaine has been found in ancient Egyptian mummies (Balabanova et al., 1992; Parsche et al., 1993; Parsche and Nerlich, 1995), the validity of these studies has been questioned (Musshoff et al., 2009, 2017) suggesting that positive results for such molecules, not supported by any historical context, should be attributed to external contamination, use of inappropriate techniques, and chemical decomposition. Hence, the only studies reporting the detection of the molecule of cocaine in hair and teeth samples in ancient biological remains come from South America, where the coca plant was endemic and frequently consumed by the population of the Incan Empire (Brown, 2014; Cartmell et al., 1991a, 1991b; Indriati and Buikstra, 2001; Rubio et al., 2013, 2015, 2014; Springfield et al., 1993). Therefore, evidence of *Erythroxylum* spp. in individuals of origins other than South American testify to successful importation to the Old World. Although the presence of cocaine in hair samples was reported on remains of the early 20th century by Musshoff et al. (2017), the same authors stress that their results were congruent with cocaine hydrochloride salt consumption; hence, this is extremely different from what the present authors are saying in this article i.e. that we are witnessing *Erythroxylum* spp. in Europe before the 19th century. Notably, the specimens and the historical context were different from our case: we analyzed mummified brain tissues that reflect the acute consumption of substances and not hair samples that represent a prolonged intake of substances as the papers cited. Moreover, the data presented in literature reported the presence of cocaine (molecule) and metabolites in human remains in which the *Erythroxylum* spp. was endemic (South America), or, alternatively, in Europe after the first synthetization of cocaine hydrochloride salts.

Finally, it is important to compare the toxicological data with the historical context; indeed, an important source of support of the analytical results obtained is the historical evidence. An example can be the results reported by Socha et al. (2022a), who found not only cocaine and benzoylecgonine but also harmaline and harmine in samples collected from South American mummies. The authors suggested that the detection of harmine in the hair of the Nazca mummies can be reassociated to the *Malpighiaceae Banisteria chrysophylla Lam.* However, other authors reported that this plant was native to the eastern most regions of Brazil which is diametrically opposed to the region where the Nazca mummies were found, suggesting that the presence of this plant is unlikely in the mummies under investigation by Socha's group (Greco et al., 2024). Although these papers did not directly address the *Erythroxylum* spp., along with the criticism of Balabanova and Parsche's group (Balabanova et al., 1992; Parsche et al., 1993; Parsche and Nerlich, 1995), they serve as another illustration of the importance of a well-defined historical comparison with archaeotoxicological results.

4.4. Archaeotoxicological considerations

In light of the evidence presented, these archaeotoxicological findings have the potential to shed light on the pharmacological treatments utilized in Milan during the 17th century. Indeed, the two subjects that were positive to *Erythroxylum* spp. compounds could have consumed the plant as a medicinal remedy at the hospital to relieve pain or treat some symptoms or disease (even if it was not in use according to the pharmacopeia in the hospital). Alternatively, the individuals may have ingested the plant shortly before admission into the hospital for medical and/or recreative purposes.

The presence of this plant in European human remains is unprecedented. In fact, the pharmacological archives of the hospital do not report the presence of the *Erythroxylum* spp. until the end of 19th century, which would indicate that the plant did not enter the hospital pharmacy until then (Lemery, 1737; Ospedale Maggiore di Milano - Ca' Granda, 1711). In order to explain these peculiar findings, an alternative hypothesis may be formulated. A synthesis of the historical data presented in section 1.2 of the introduction leads to the conclusion that the effects of the plant, including reduced hunger and thirst, as well as a sense of well-being, were known and controlled by the Spaniards and subsequently diffused to the rest of Europe. Moreover, at the time, the duchy of Milan was under the rule of the Spanish domain; this constitutes a direct connection between the maritime sales and the city of Milan (Crivelli, 2021). It can therefore be argued that the historical testimonies which revealed the arrival in Europe of exotic plants, the direct connection between Milan and the Spaniards (due to their domination of the city), and the sea trades established between the New World and Milan, may explain the presence of *Erythroxylum* spp. before the 19th century in the Italian city. Whether coca leaves were used for recreational purposes, or rather for their reinforcing properties helpful for the population in their hard everyday life, is a topic that requires further debate.

5. Conclusion

In this paper, we present, to the best of our knowledge, the first hard evidence regarding the use of the coca plant in Europe through archaeotoxicological analyses on human remains in the extraordinary context of the Ca' Granda crypt, hence backdating its use in Europe to the 1600's.

Specifically, toxicological analyses performed on preserved brain tissues revealed the presence of the compounds of cocaine, benzoylecgonine, and hygrine in two cases. Hygrine, in particular, indicated that cocaine intake occurred through the chewing of coca leaves. The present study can therefore backdate the arrival of the *Erythroxylum* spp. with respect to what is narrated by historical sources by almost two centuries. This plant was not listed in the pharmacopeia of the Ca' Granda, suggesting that it may not have been administered at the hospital. Coca leaves may therefore have been chewed for their reinforcing properties or for recreational purposes.

This study allows for a better understanding of how the use of cocaine has changed over the centuries in Europe, starting as a recreational or medical substance, evolving as a medicine in the 19th century, and becoming a widespread substance of abuse for its psychoactive properties, as well as the cause of 1/5 of overdose deaths across the world in the 20th century.

Data availability statement

The data supporting the findings of this study are available within the article.

Ethical approval

Approval to conduct this research was issued by the *Sopraintendenza Archeologia, Belle Arti e Paesaggio per la città metropolitana di Milano*, an institution of the Italian Ministry of Cultural Heritage, following the ethical protocol of the agreement itself.

CRediT authorship contribution statement

Gaia Giordano: Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation. **Mirko Mattia:** Supervision. **Lucie Biehler-Gomez:** Supervision. **Michele Boracchi:** Writing – review & editing, Investigation, Formal analysis. **Alessandro Porro:** Supervision, Investigation. **Francesco Sardanelli:** Supervision. **Fabrizio Slavazzi:** Supervision. **Paolo Maria Galimberti:** Supervision. **Domenico Di Candia:** Validation, Supervision, Methodology, Investigation, Data curation, Conceptualization. **Cristina Cattaneo:** Supervision, Project administration.

Declaration of competing interest

The author(s) declare no competing interests.

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