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The challenge of extracting proteins from potteries

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***Abstract* – Proteomic approaches based on mass spectrometry (MS) have been used successfully in the recent past for the molecular characterisation of several proteins-containing art works and archaeological objects. However, there are still relatively few examples of the successful recovery and identification of proteins from archaeological pottery. This is mostly because ceramics often contain a much lower amount of proteinaceous material, that is also highly contaminated and degraded, thus making proteomic analyses quite challenging. In the attempt to address this issue, we herein report efforts in developing new methods for the detection and characterisation of protein residues deposited in pottery, with results in the analysis of archaeological samples from Pani Loriga as case studies.**

I. INTRODUCTION

Paleoproteomics, the study of ancient proteins, is a rapidly growing field that leverages the longevity and diversity of proteins to explore fundamental questions about the past. Technological gains over the past 20 years have allowed increasing opportunities to understand better preservation, degradation and recovery of the rich bio-archive of ancient proteins found in works of art and archaeological records [1].

In particular, in the last few years, proteomic analysis of adsorbed residues has emerged as critical strategy to identify the foodstuffs processed in ancient ceramic vessels [2]. The most abundant objects recovered in archaeological sites, ceramic artefacts, were largely employed for cooking and storing materials. As a result, traces of proteins and other food-deriving organic materials can occur in porous ceramic vessels as preserved surfaces or adsorbed residues [2]. Proteomic analysis of such protein residues can yield important information about past cultural and technological activities, giving us an insight into social life of the specific population [2].

However, recovery and detection of proteins from archaeological ceramics remains to date somewhat limited at an early stage of development, potentially undercutting the analytical power of proteomic analyses as a means to understand ancient food processing habits [3].

Indeed, technical and methodological challenges in the study of protein residues in pottery are still huge. These problems range from (i) the small amount of protein samples allowed for analyses and their frequent dispersion in a heterogeneous matrix; (ii) to the intrinsic contamination problems that originate from environmental exposure; (iii) to the modifications that occurred during ageing and deterioration due to physical and microbial agents [4]; (iv) to the effect of humics due to interactions between soil and clay [3].

In particular, protein extraction process represents a key bottleneck in archaeological research, especially owing to the strong attraction forces between proteins and the ceramic matrix, which, although aiding their preservation, makes their extraction difficult [3]. Altogether, these aspects make the analysis of protein residues in pottery extremely challenging.

For such reasons, many efforts are oriented towards the development of novel analytical procedures for recovery and identification of proteins from ceramics. These protocols have to cope with very complex, contaminated or damaged proteinaceous materials.

To this purpose, a variety of reagents and protocols have been explored in recent years for dislodging proteins from ceramics, but there are still few examples of successful protein recovery and detection [5].

In this context, deep eutectic solvents (DESs) have been

recently proposed as promising media to extract proteins from ceramic [3]. These innovative solvents were introduced as an alternative to ionic liquids and form a relatively new area of research, with the first report of a DESs liquid published only this century. DESs are a mixture of Lewis or Bronsted acids and bases, which form a eutectic mixture with a melting point much lower than the melting points of the constituent compounds [3].
Although there are various comprehensive reviews of the applications of DESs in protein extraction from different vegetal and animal matrices [6], DESs have not yet been fully exploited in the field of cultural heritage.

A first reported use of a DES for the extraction of ceramic-bound proteins has been proposed only last year by Chowdhury M. P. et al. [3]. The authors developed a novel DES-based extraction protocol to recover proteins from ceramics. It involves protein recovery by an ultrasonication step using a 2:1 urea-guanidine hydrochloride (GuHCl) DES as extraction medium, followed by the ultrafiltration of the extract and the subsequent preparation of the sample for LC-MS/MS analysis. This novel DES-based extraction procedure was applied to a wide set of both artificially prepared and historical ceramic samples. In addition, as a control procedure, the same samples were subject to a traditional extraction protocol that replaces a 6 M GuHCl aqueous solution to the newly developed DES.

The results obtained by Chowdhury M. P. et al. [3] have clearly demonstrated that the DES-based extraction strategy results in greater protein recovery from ceramics compared to the traditional GuHCl-based extraction technique. These encouraging results open up new perspectives for the use of DESs for the extraction of proteins from pottery residues.

In the framework of this project, we aim to develop novel experimental procedures for improved recovery and detection of proteins in pottery residues. The feasibility of a DES-based extraction strategy will be therefore explored as a promising alternative for the traditional protein extraction protocols.

To this purpose, data collected with the DES-based extraction strategy proposed by Chowdhury M. P. et al. [3] will be shown in comparison to other established extraction protocols as controls. In particular, the 6 M GuHCl-based protocol by Chowdhury M. P. et al. [3] will be used to confirm the control proposed by the authors. Moreover, a 6 M Urea-based extraction protocol that is our standard procedure will be considered as additional internal control [4].

In order to evaluate the best protocol for analyses, preliminary experiments will be performed on test specimens, namely modern ceramic fragments on which known foodstuffs (milk or meat) were artificially deposited. For each test specimen, sampling was carried out by scratching the surface of the fragment where the different substances had been placed, similar to how sampling takes place on archaeological objects.

Once optimised the extraction procedure, the developed protocol will be applied to a set of archaeological samples. In particular, ceramic samples coming from the collection of the archaeological site of Pani Loriga will be analysed as extremely interesting case studies.

Pani Loriga was probably founded at the end of the 7th century BC. The transfer of a community of Eastern traditions to a hill near the modern town of Santadi, in southwestern Sardinia (Italy), can be seen within a specific program of territorial control initiated by the powerful colony of *Sulky*. In this hinterland management system, Pani Loriga occupied a strategic position of primary importance, as the settlement was to link the coastal hinterland and the internal areas of the region, rich in minerals and agro-pastoral products [7]. The settlement maintained its function through the following Punic phase.

Its importance is evidenced by Carthage's strong interest in it, which has been underlined by the recent excavations by CNR. They identified and partially revealed vast living areas built between the end of the 6th and the early 5th century BC in the earliest phase of the North African metropolis's presence on Sardinia. The settlement most likely occupied the entire eastern slope of the hill, from south (Area A) to north (Area B), with a series of aligned and interconnected buildings arranged under the Acropolis (Area C) [8]. On the other hand, the western slope hosted the Phoenician and Punic necropolis. A sacred area, also in operation during the Roman period, was instead located on the extreme eastern edge of the high ground [9].

Fig. 1. Panorama of Pani Loriga hill with the main archaeological evidence (photo by G. Alvito, Terravista; drawing F. Candelato).

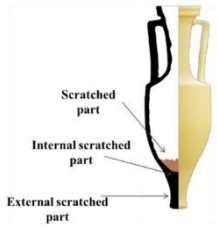
During two excavation campaigns, we collected a significant set of samples (Fig. 2B) from the large building arranged in the north-eastern sector of the hill (Area B).

These samples include (i) scratched parts from different sides of the ceramic vessels and (ii) internal powders recovered near the bottom of the pots and therefore most likely assumed to have been in direct contact with foods.

A graphical representation of how sampling was carried out in ceramic vessels is given in Figure 2A.

Proteomic characterisation, integrated with GC-MS analyses of lipid and saccharide fractions from the same samples will hopefully significantly support historical and archaeological interpretations, revealing new insights into the customs and habits of the Sardinian Phoenician-Punic population.

A



B

Vessel Shape	Sample code
Plates	PLB17. S6N. 1559.10 (RR 300) PL17C US 24 PLB17. S6S.1564.1 PLB17S6N.1576.1 RR308
Cooking trays	PLB17S6N.1524.6 RR277 PLB17S6N.1524.6 BIS
Pots	PLB17S6N.1557.1 PL17C US24
Amphorae	PLB17S6N.1563.41 PLB17S6S.1551.3 RR298 PLB17S6N.1524 RR278 PLB17S6N.1524 RR283 PLB17S6N.1524.1 RR283 PLB17. S6N.1489.1 RR 283PL17C 1A US 12 PLB17S6S.1550.5 RR295 PLB17S6S.1551.1 RR296 PLB17. S6N.1519.15
Lamps	PL17C US 12 PLB17. S6N.1546.14
Basins	PLB17. S6S.1511.1 PLB17. S6N.1546.4
Pitchers	PLB17. S6S.1538.1 PLB17. S6N. 1558.7PLB17. S6N.1519.13 PLB17. S6N.1546.28 PLB17. S6N.1546.23
Cups	PLB17. S6N.1497.1 PLB17. S6N.1546.10 PLB17. S6N. 1539.7

Fig. 2. A. Graphical representation of how sampling was carried out on archaeological potteries. B. List of archaeological samples collected in Pani Loriga, divided according to their shape and function.

II. PRELIMINARY RESULTS

Preliminary characterisation studies have been conducted on historical samples coming from the archaeological site of Pani Loriga. In particular, early GC-MS analyses allowed for the classification of the samples based on their lipid and sugar compositions.

On the basis of the GC-MS profiling some of the samples were selected and investigated for their protein component. These samples were subject to a standard 6M Urea-based protein extraction protocol and analysed by LC-MS/MS as reported in ref. [4].

As successful example, we report the case of sample PLB17.S6N.1519.13, a pitcher on which proteomic analysis enabled to identify several bovine serum proteins (Fig. 3A). These results were consistent with the hypothesis proposed on the basis of previous GC-MS analyses (Fig. 3B). The simultaneous identification of animal fatty acids, animal-type steroid hormones and bovine serum proteins suggests that the vessel might have been used to contain beef or beef broth.

A. Results of the LC-MS/MS analysis on sample PLB17.S6N.1519.13

Protein	Mascot score	Protein sequence coverance (%)	No. of Peptides
Serum albumin OS=Bos taurus	5097	31	15
Apolipoprotein A-II OS=Bos taurus	145	55	3
Fibrinogen alpha chain OS=Bos taurus	52	4	2
Apolipoprotein C-III OS=Bos taurus	37	35	2

B. Results of the GC-MS analyses on sample PLB17.S6N.1519.13

Identified compounds	Chemical category
Rhodoxanthin	Organic pigment
Acetic acid	Carboxylic acid
Glycerol	Polyalcohol
Palmitic acid C16	Fatty acid
Stearic acid C18	Fatty acid
Oleic acid C18:1	Fatty acid
Crocetane	Hydrocarbon
Pregnane-3,20-dione	Steroid hormone
Methyl-androstane-2,3-diol-1,17-dione	Steroid hormone

Fig. 3. Preliminary results obtained for sample PLB17.S6N.1519.13 from Pani Loriga's collection. A. List of the proteins identified by the LC-MS-MS analyses. B. List of the organic molecules identified by GC-MS. The extraction of organic molecules and their analysis by GC-MS were performed according to ref. [10].

However, only in very few cases successful protein recovery and identification were obtained, prompting for the search of further extraction strategies. This emphasises how critical it is to design novel experimental strategies dedicated to a sensible and reliable diagnosis in the field of cultural heritage.

III. CONCLUSIONS AND NEXT STEPS

The analysis of lipids (fats, oils and waxes) adsorbed within archaeological pottery has revolutionized the study of past diets and culinary practices. However, this technique can lack taxonomic and tissue specificity and is often unable to disentangle signatures resulting from the mixing of different food products [11].

The analysis of ancient proteins offers an alternative approach for identifying foodstuffs adsorbed onto ceramics with improved tissue and taxonomic resolution. However, challenges in the study of ancient protein residues in pottery are still huge, with protein extraction process being a key bottleneck [3].

In this work, we report our efforts in developing new methods for the detection and characterisation of protein residues deposited in archaeological potteries.

In particular, we attempt to design novel analytical procedures for protein recovery and identification, that can handle highly complex, contaminated or degraded proteinaceous materials.

The feasibility of a DES-based extraction strategy will be investigated as a promising alternative for the traditional protein extraction protocols. The application of this analytical procedure to case studies from Pani Loriga

will provide a strong validation of the proposed strategy.

We expect the novel protocol set-up in this work will provide new insights into the proteomic analysis of pottery-adsorbed residues, a still extremely challenging issue in the panorama of cultural heritage diagnosis.

Future efforts will be aimed to further improve experimental and bioinformatic strategies. Special attention will be given to the design of innovative procedure for protein validation and authentication as well as to the characterization of protein degradation/ageing.

The obtained results will provide keys to deciphering ancient ceramics materials, revealing new insights into ancient peoples' uses, customs and habits that preceded us.

IV. EXPERIMENTAL SECTION

The sample preparation procedure is divided in three major steps, e.g., protein extraction, enzymatic hydrolysis and sample analysis/database searching for protein identification.

A. Protein extraction from modern and archaeological samples

Proteins from about 5 mg of ceramic powder will be extracted in this study using the following three experimental procedures.

To explore the feasibility of a DES-based strategy as an alternative to the traditional extraction methods, a urea−GuHCl DES will be used for protein extraction, as reported in ref. [3]. According to ref. [12], a 2:1 urea-GuHCl DES will be prepared for this strategy by mixing the compounds and heating them at ∼70−75 °C until a homogeneous clear liquid is obtained. 1 mL of the obtained DES will be added to ground ceramics and the mixture will be incubated in an ultrasonic bath for 4 h at 65 °C with ultrasonication. The resultant liquid slurry recovered by centrifugation will be diluted with 1 mL of distilled water and ultrafiltered using 3 kDa molecular weight cutoff filters. The retentate will be then redissolved into 50 mM ammonium bicarbonate (Ambic) for enzymatic hydrolysis.

To also reproduce the control conditions reported in ref. [3], a GuHCl solution (6 M) will be used to extract the proteins at 65°C for 4 h with ultrasonication. Also in this case, the supernatant liquid will be recovered by centrifugation, and ultrafiltered using 3 kDa ultrafilters.

Lastly, a traditional urea-based extraction protocol will be performed as additional internal control, as reported in ref. [4]. Briefly, 30 μL of a solution of 6 M Urea will be added to ground ceramics and incubated for 10 min at room temperature, followed by sonication for 20 min. Urea will be then 6-fold diluted with water. Lastly, enzymatic digestion will be carried out as reported in the following subsection.

B. Enzymatic hydrolysis

In all cases, proteins will be then reduced, alkylated and digested with trypsin as reported in ref. [3].

Briefly, the mixture will be reduced by addition of 21 μL of 100 mM dithiothreitol (DTT; 45 min at room temperature) and then alkylated with 42 μL of 100 mM iodoacetamide (IAM; 45 min at room temperature in the dark), and the alkylation will be subsequently quenched by the addition of 21 μL of 100 mM DTT. Next, trypsin (Sigma Aldrich) will be added to a final concentration of 10 ng/μL to samples as directly suspended in 50 μL of Ambic 10 mM. After incubation at 37 °C for 16-18 h, the resultant tryptic peptides will be desalted and concentrated on in-house-made C18 extraction stage tips, as described by Cappellini et al. [13].

C. Sample Analysis and Database Search

Peptides were eluted with 20 μL of 50% acetonitrile and 0.1% formic acid in Milli-Q water and analysed by LC-MS/MS as reported in ref. [14]. Samples will be analysed on LTQ Orbitrap XL Hybrid Ion Trap-Orbitrap MS System (Thermo Scientific, Bremen, Germany) on a C18 capillary reverse-phase column (200 mm, 75 μm, 5 μm) at 250 nL/min flow rate, using a step gradient of eluent B (0.2% formic acid, 95% acetonitrile LC-MS grade) in eluent A (0.2% formic acid in 2% acetonitrile) from 5 to 50% over 80 min and to 80% over 5 min. Mass spectrometric analyses were performed using data-dependent acquisition (DDA) mode over the 400 to 1800 m/z range, at a resolution of 60 000, and the automatic gain control (AGC) target was set to 1 × 106, followed by acquisition in MS/MS of the five most abundant ions. For the MS/MS scans, the resolution was set to 15 000, the AGC target was set to 1×105, the precursor isolation width was 2 Da, and the maximum injection time was set to 500 ms. The CID normalized collision energy was 35%; AGC target was set to 1 × 105. Data were acquired by Xcalibur software (Thermo Fisher Scientific).

The acquired MS/MS spectra will be transformed in Mascot Generic files (.mgf) format and routinely used to query the SwissProt database. A licensed version of Mascot software (www.matrixscience.com) version 2.4.0. will be used. Standard parameters in the searches will be trypsin as the enzyme; 3, as allowed number of missed cleavages; 10 ppm MS tolerance and 0.6 Da MS/MS tolerance; peptide charge from +2 to +3. Carbamidomethylation of cysteines will be inserted as the sole fixed chemical modification, while possible oxidation of methionines, formation of pyroglutamic acid from glutamine residues at the N-terminal position of peptides, deamidation at asparagines and glutamines, and hydroxylation on lysine and proline will be considered as variable modifications. To simplify species assignment and recover more focused results, ultimate searches will be carried out using a homemade database that collects all sequences most likely to be detected in adsorbed protein residues. Only proteins presenting two or more peptides will be considered as positively identified. Individual ion score threshold provided by Mascot software to evaluate

the quality of matches in MS/MS data will be applied and the protein with scores lower that this will be rejected [13].

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