Chromatography and DNA: New Perspectives

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1. Introduction

Since ancient times clay was used for the production of pottery. The use of ceramic vessels is prior to the Neolithic Revolution with the beginning of farming and the adoption of sedentary life traditionally seen as the main reason for the construction and use of ceramic food storage containers.

Archaeologists traditionally rely on textual sources or use typological and macroscopical analysis of clays as their primary instrument on the study of ancient ceramic vessels. In fact, one of the most interesting problems related to those pots is the knowledge of the products transported by different shapes and typologies, or from distinct production centres. The conventional approach is based on the analysis of the vessels typology and on the visual inspection of ceramics. However, this methodology is highly subjective and prone to errors (e. g. similar vases used to accommodate different matrices or reused vases) and therefore needs to be reinforced with scientific evidences, as physical, mineralogical and chemical analysis. Amongst the array of analytical techniques available, X-ray diffraction (XRD), Scanning Electron Microscope with Energy Dispersive X-ray Spectroscopy (SEM-EDS), Raman spectroscopy or Fourier Transform Infrared Spectroscopy (FTIR) are the most popular. The main goal of these methods is to recognize patterns in the chemical compositions of ceramics and to relate them with those from clays of geographically restricted locations. The close acquaintance between the pottery production centres and the production areas facilitates both the identification of the products traded and the commercial routes used.

This is not an immediate operation nor a simple task to perform, as ceramic vessels are not typically produced from raw clay straight out of the ground. In fact, raw clays are often refined, removing plant roots and small rocks, together with sand and silt. Tempering materials are often added, making the chemical composition of ceramics different from raw clay. It is also common the mixture of different clays to better adjust the ceramic properties to their function (e. g. small containers in contrast with big containers as dolia). The use of distinct firing temperatures and/or different kiln atmospheres (e. g. oxidation or reduction firing) can also modify their crystallographic composition, making only possible to exclude potential sources and assign probable locations with "comfortable" statistical probabilities. An additional problem is the possibility of post-depositional alterations. In those cases, the comparison between the ceramics composition and the debris from the suspected manufacturing sites is not enough to achieve a conclusion.

The discussion already presented demonstrates that knowing the chemical composition of clays, despite extremely useful on provenance studies, is not enough to unquestioningly establish the function of ancient ceramic containers – in other words, to define what the vessels content was. In fact, the vessels purpose can only be demonstrated by analysing the organic remains preserved in ceramics, where the chromatographic techniques coupled with mass spectrometry and the DNA analysis play primordial roles in this task.

2. Clay Properties

Clay grains easily absorb and loose water, swelling or shrinking if it's gaining or losing liquids (Baeshad 1955). Ceramics are obtained by moulding, drying and firing clay in a kiln, being their properties directly related with the clay characteristics. They have a laminar structure and are disposed by layers, therefore with large surface areas and superficial electrical charges which attract water and ionic species, promoting the circulation of charged species from the outside to the internal structure of ceramics (Fig. 1). Together with water, soluble organic species

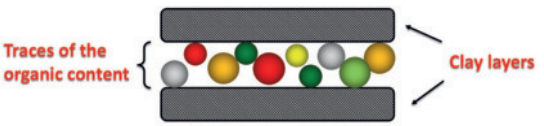

Fig. 1 Organic remains preserved between two ceramic clay layers.

also penetrate inside the ceramics structure, becoming immobilized between clay layers and therefore apart from the contact with oxygen and other oxidizing species. This fact limits the degradation processes and preserves the organic compounds. The use of extraction procedures allows the recovery of the organic remains adsorbed on the surface or trapped and preserved in between clay layers, allowing the identification of chemical compounds characteristic of different organic matrices (Fig. 1).

3. Organic Residue Analysis by Chromatographic Techniques

Natural organic matrices as wine, honey, olive oil, ointments, animal fats or waterproofing agents consist in hundreds of different chemicals, making the identification of the entire range of constituents an ultra-laborious task. Exposure to sunlight, oxygen, microorganisms and water can produce chemical reactions that affect the composition of archaeological materials (Malainey 2011). Additionally, post-depositional phenomena as the thermal and oxidative degradation, contaminations by lixiviation (Fig. 2), post-excavation adulterations or even a reuse of an empty vase previously filled with a different organic content, make a successful chromatographic analysis a difficult task to accomplish. The identification of natural substances and their degradation products is a complicated job as organic materials are more subject to degradation than inorganic ones.

The analysis of a ceramic fragment implies the previous extraction and isolation of the organic compounds from the ceramic matrix (Fig. 3). The collected fragments are normally ground to a fine powder in an agate mortar to homogenize samples and maximize the contact area with organic solvents. The use of extraction procedures allows recovering the organic remains adsorbed or trapped and preserved between clay layers, permitting the isolation and identification of molecular tracers characteristic of different organic matrices. The adopted strategy is the search for compounds that are source specific, in order to get valuable informations with a limited number of compounds identified.

Different extraction methodologies can be used, being Soxhlet apparatus or ultrasound devices the most popular extraction devices. In fact, different authors can adopt diverse strategies to extract and isolate the organic compounds, differing their selection with the characteristics of the samples under analysis, the laboratorial instrumentation and available equipment or even with their personnel experience or preference.

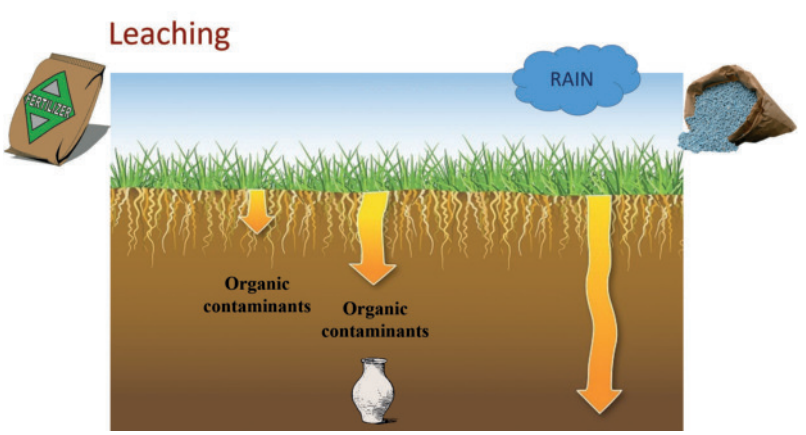

Fig. 2 Post-deposition contamination by leaching.

4. Chamical Tracers

A successful study requires the building up of a profile of the chemical components of the original substance from the ceramic artefact, followed by a comparison to profiles of modern reproductions in order to match the ancient substance to the modern. This procedure is frequently done by searching chemical tracers, e. g. chemical compounds or classes of compounds that are relatively unique to their sources, therefore helping on their identification. A chemical compound must exist in the original organic matrix or being produced by a definite number of sources through well-known degradation mechanisms, as to be considered a tracer. Some compounds are general tracers as they present broad spectrum information as to classify the source of the residue as either animal or plant, while others are specific tracers that provide a relatively unique chemical pattern, presenting solid evidences or clearly demonstrating the residue identification.

The ideal chemical tracers are compounds that react slowly enough as to be chemically preserved, i.e. in an identical form as their primarily source (the organic matrix as wine, olive oil, etc). Other important tracers are those of primary origin and not a sub-product of secondary reactions with unknown mechanisms. The main problem on using the molecular tracer concept is the limited number of tracers known. In fact, the elevated amount of possible organic matrices together with the multiplicity of degradation processes, reuses and post-depositional contaminations, contrasts with the restrict number of chemical tracers known.

One important fact to stress is that the organic tracers absorbed into the ceramic structure tend to be better preserved than those occurring on the surface of an artefact, as they are exposed to oxygen, heat, water, and sunlight, which promotes degradation. Organic residues absorbed into ceramic pores are also less vulnerable to post-depositional contamination than those on the surface.

Performing a successful analysis and interpreting the analytical data is a mental process similar as solving a puzzle (Fig. 4). The main objective is to collect and use a minimum of relevant data and be able to fill in the blanks and reconstruct the initial organic matrix. The permanent study on chemical tracers has significantly enlarged the scope of matrices that can be identified by organic residue analysis. Experimental studies have enriched the known data on the impact of physical and chemical phenomena in the deposition and transformation of organic residues from ceramic matrices, allowing an increment on the number of chemical tracers known. However, it is important to understand that a successful analysis of distinct organic matrices can impose different analytical approaches, with different degrees of complexity and economical investment. This is particularly important when considering the mixture of different commodities, which inevitably complicates the chemical composition of organic residues.

Amongst the different known tracers, lipids represent an important group of compounds with archaeological importance as they are water insoluble, have a relatively high stability with increased temperature and therefore minimal decomposition during cooking procedures. Lipids are hydrophobic and are normally low-density particles that inside containers tend to accumulate at the upper part of vessels. For example, olive oil has a lower density than water, forming a separate layer on their surface and therefore accumulating near the containers rim. In case of boiling pots, as vessels are not always filled to capacity and evaporative losses reduce the water levels during boiling, fats should accumulate over the upper third of boiling vessels. Table 1 present examples of chemical tracers and their possible source mechanisms (Fig. 5).

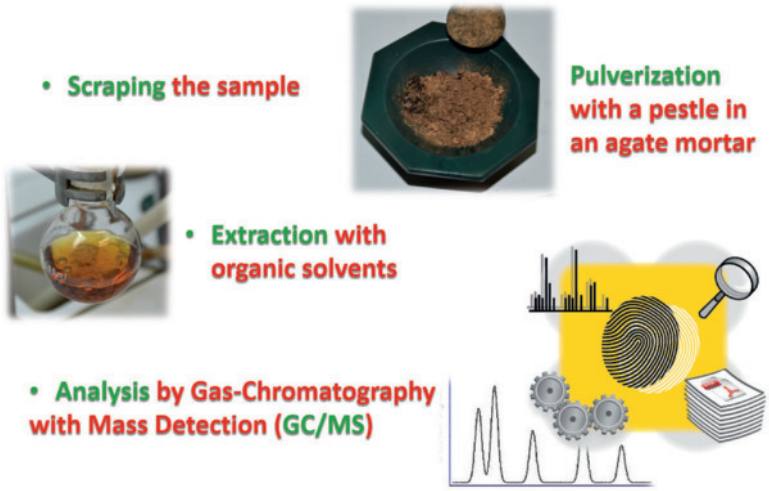

Fig. 3 Generic scheme of a chemical analysis protocol.

5. Best Practice Recommendations to Manipulate Ceramics for Chemical Analysis

To maximize the reliability of data from chemical analysis, actions should be taken to avoid contaminations that could mask the

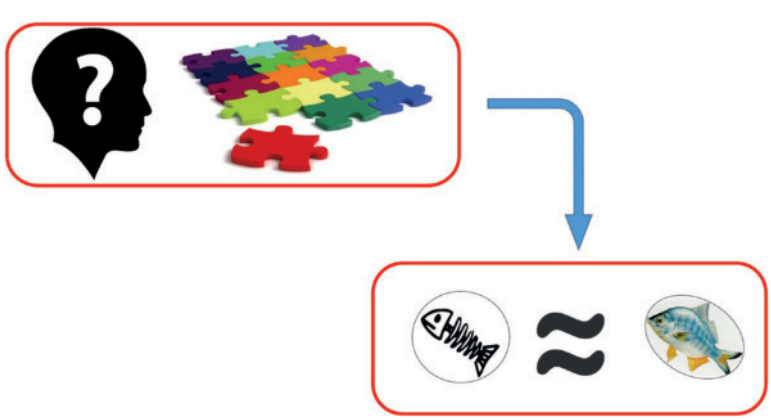

Fig. 4 Mental process for the use of chemical tracers on the analysis of organic residues.

Organic tracers	Source material
Tartaric acid, syringic acid, malic acid	Wine
Malvidin	Red wine
β-sitosterol, stigmasterol, and campesterol	Plant material
Dehydroabietic acid, 7-oxo-dehydroabietic acid, pimaric acid, levopimaric acid	Conifer products
n-alkanes with chains of 23 to 33 carbon atoms and palmitic acid wax esters with chains of 40 to 52 or 54 carbon atoms	Beeswax
ω-(o-alkylphenyl) alkanoic acids with 16 to 20 carbons together with 2 isoprenoid fatty acids	Marine mammal fats
Cholesterol	Animal fats

Fig. 5 Table: Some organic tracers and their sources.

analytical data and conduct to erroneous conclusions.

The introduction of contaminants should be prevented by minimizing sample manipulation that ought to be handled only with clean tools and gloved hands, as improper handling of sherds could result in the introduction of fingerprint oils. Pottery that has been selected for analysis may be air dried but not brushed, washed or overly handled before being wrapped in aluminum foil and not in polyethylene bags, as they introduce industrial plasticizers in samples (Pollard 2007). Washing the sherds with water may lead to the loss of soluble residue components, while the use of detergents will cause some lipid components to dissolve. Visible residues may be lost if a brush is used to clean soil from the sherd. Small fragments of pottery should be preserved without the use of adhesives or consolidants as they mask the organic residues from the vessels content and introduce organic contaminants. Care must be taken to assure that markings are not placed on sherds surfaces that will be used for chemical analysis. Prior to analysis, ceramic surfaces need to be mechanically cleaned to minimize post-depositional contamination.

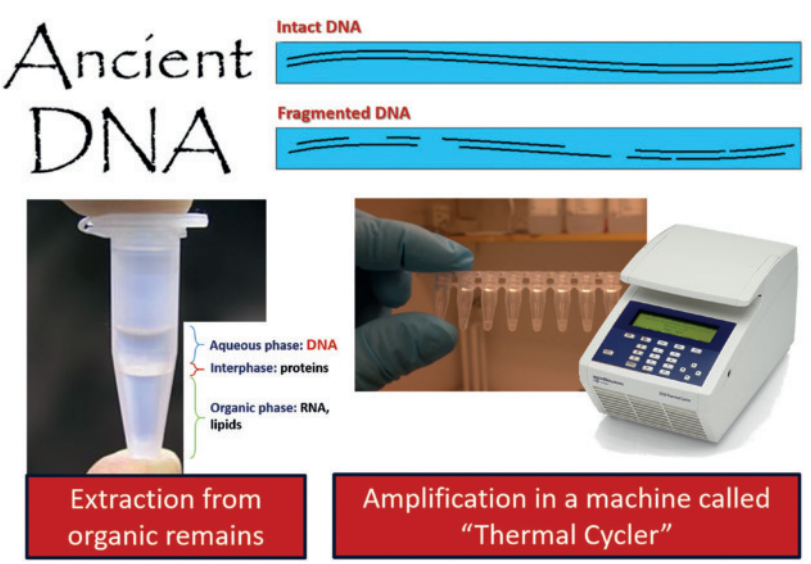

Fig. 6 Basic scheme of the extraction and amplification of ancient DNA fragments.

6. DNA Analysis

A successful identification of chemical tracers can allow the reconstruction of original organic matrices and consequently to discover the function of ancient ceramic containers. However, despite extremely important, this data could be insufficient when more concrete informations are needed, as to distinguish different fish species used on the preparation of a fish-based product, as garum, or other fish derivative, or on the individual DNA identification from ancient human remains. In fact, the lack of more source specific tracers does not enable chemists to infer on the fish species neither to differentiate DNA residues from distinct individuals, posing this question a different analytical strategy.

Despite the changes on the chemical composition of the original organic matrix due to degradation effects that can occur prior to, or after the deposition, it is sometimes possible to isolate traces of short and damaged DNA fragments which have to be reconstructed to allow their identification. During the 1980's the advances on biomolecular biology and genetics allowed recover and analysis of DNA from ancient specimens, as an Egyptian mummy (Pääbo 1985; Hall 2008). However, DNA is a very fragile molecule, putting its reconstruction technical challenges by their high degradation level and possible contaminations with modern biomolecules. In fact, all natural molecules suffer deterioration after the death of the living organisms, producing tiny quantities of ancient biomolecules that are very difficult to detect. The contamination with modern DNA molecules difficult even more the detection process and could lead to inaccurate results. This is an important issue, forcing the researchers to carry out their experiments in ultraclean laboratories and to adopt rigorous procedures to avoid contaminations by modern DNA.

The amount of ancient DNA sequences in an extract is relatively small to be directly analysed without their previous replication by PCR (Polymerase Chain Reaction). In that case, primers (short pieces of RNA or DNA that serve as a starting point for DNA synthesis) are chosen and added to the sample, allowing the selective amplification of particular bits of the genetic code and dramatically increasing their concentration in the sample. This automated process uses an equipment called thermocycler (Fig. 6) that mimics under controlled laboratory conditions the natural DNA replication process that occurs in cells, producing in a test tube millions of copies of a DNA sequence even with a very small initial amount of DNA.

The replicated extracts are analysed by agarose gel electrophoresis (Fig. 7) to confirm the existence of genetic material clearly visible in the form of bands. This method uses differences in the electrical charge of molecules in a mixture to separate them. As DNA molecules have negative charges, they migrate towards the positive pole when positioned in an electric field, depending their velocity on their charge-to-mass ratios, so similar DNA molecules migrate at about the same speed. Additionally, the agarose gel has a network of pores that have to be crossed by the DNA fragments. The length of the DNA molecules also limits their velocity as the smaller DNA molecules can migrate faster through the gel. In that case, molecules are separated according to their length, originating different bands containing identical DNA fragments (Fig. 8).

The replicated DNA extracts are sequenced (through one of the different sequencing available technologies) to determine the precise order of nucleotides within the DNA fragments, i. e. to determine the order of the four bases adenine, guanine, cytosine, and thymine in a strand of DNA, and the resulting sequences analysed by bioinformatics and compared with data in banks of genomic sequences.

This approach was recently used on the identification of DNA fragments on the remains of fish-based products (Fig. 9) including fish bones and teeth, collected in a doliola from Boca do Rio (Lagos) in a context of the late Roman era to the end of the 4th century or early 5th century (Landi et alii 2015; Oliveira et alii 2015). In fact, the use of extraction techniques allowed the recovery of ancient DNA fragments, being identified several bone fishes and one from a large shark, all from the Portuguese Atlantic coast.

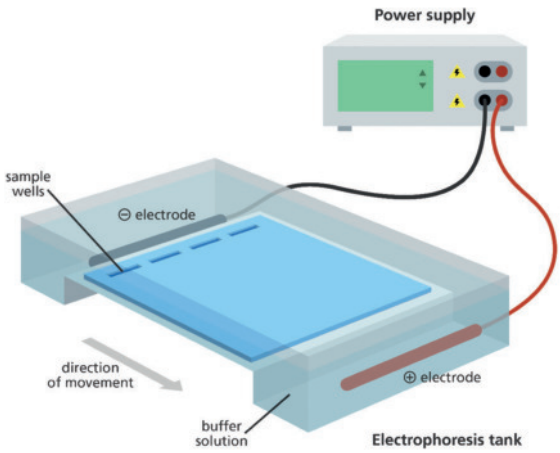

Fig. 7 Scheme of a gel electrophoresis equipment.

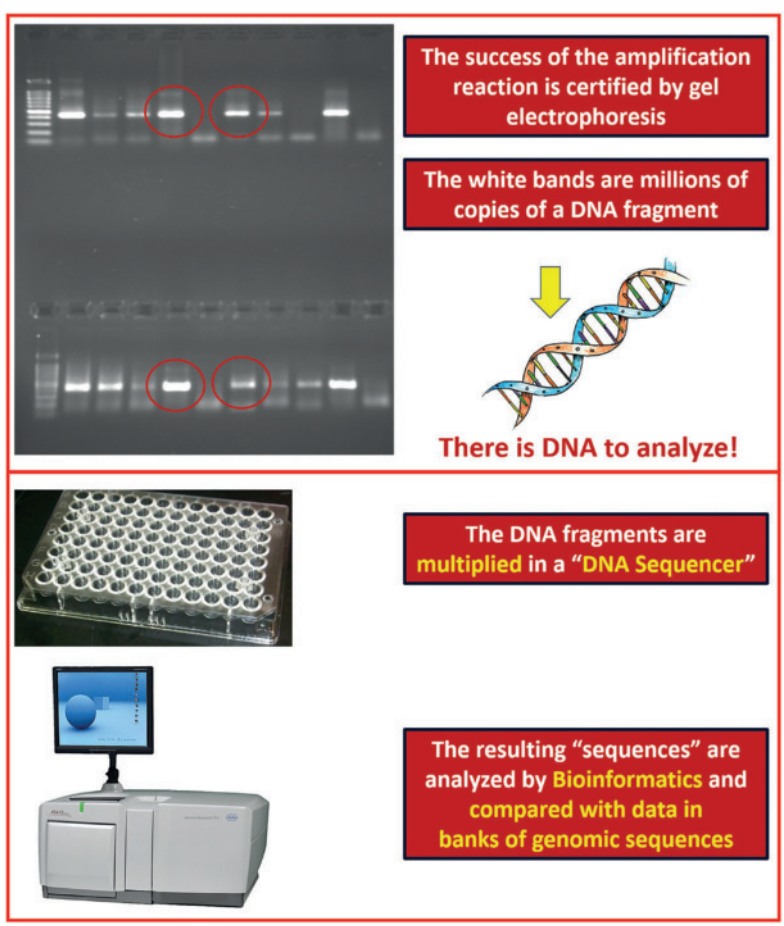

Fig. 8 Detection of DNA fragments by gel electrophoresis. The replicated DNA fragments are multiplied in a DNA sequencer and the results analysed by bioinformatics.

Fig. 9 Fish bones and teeth collected in a *doliola* from Boca do Rio (Lagos), used on the identification of DNA fragments.

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Endnote

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