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Original article

# A miniaturized method for HPLC-MS/MS identification of wine markers in figured pottery

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ABSTRACT

The study of Organic Residue Analysis (ORA) in archaeological pottery aims to detect traces of organic materials, such as food and beverages, that were once contained in the vessels. Chemical analysis of organic residues can confirm hypotheses made by archaeologists regarding some important aspects of ancient daily life, from ancient diet to rituals. If non-invasive analytical techniques, such as spectroscopies, could provide issues with overinterpretation of the data, chromatographic techniques combined with various detectors may be more suitable for separating and identifying the different components within a complex matrix, like archaeological pottery. This research aims to develop a new rapid, reproducible and efficient method for the identification of organic acids as wine markers in archaeological figured vessels through HPLC-MS/MS. The procedure included a derivatization step and an extraction step, both designed based on green analytical chemistry principles. It employed ultrasound-assisted liquid extraction and dispersive liquid-liquid microextraction (dLLME). dLLME allowed to remove compounds that induce signal suppression, thereby minimizing the matrix effect. Except for tartaric acid, which had a recovery around 20 %, the other analytes had recoveries that ranged from 40 % to 60 %, while LODs were comprised between 0.01 and 0.05 ng $mL^{-1}$. This method was applied to examine the potential presence of wine in figural pottery, validating the method on historical samples in the frame of the wide-scope project Imag-ORA.



## 1. Introduction and research aim

### 1.1. ORA and archaeological pottery

Organic Residue Analysis (ORA) of archaeological pottery focuses on identifying traces of organic substances, such as food and beverage, that were originally contained within the vessels.

According to their use, ancient pots may be classified in different groups: vessels for the preservation and storage of foods, containers such as amphorae for transport of liquids and dry elements, tools for cooking and preparing food [1,2], and vases of different shapes used for the consumption of substances (dry and liquids) during banquets and rituals and other daily life occasions. If the shape of these vessels and their decoration allow to draw hypothesis on their use and possible contents, chemical analysis of organic residues can confirm these assumptions, shedding light on some important aspects of ancient daily life.

Organic traces of substances once contained in the vessels can be preserved both as visible residues and as invisible traces adsorbed within the porous ceramic surface of the pots [3,4]. Pioneering studies in the chemical analysis of substances contained in archaeological vessels emerged in the 1970s [5], and since then, chemical investigations of ancient vessel contents have proven invaluable in providing a wealth of information about past civilizations and various aspects of daily life, including diet, medical practices, and ritual customs. Recently interdisciplinary approaches have been adopted to identify organic residues preserved in archaeological pottery. Projects such as MAGI (Funerary offerings of biological products in Celtic, Etruscan, Italic and Phenico-Punic worlds [6]) PERHAMO (Antic Perfumes and scented-oils: Chemical

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and paleogenetic analyses of organic contents from ancient western Mediterranean Sea [7]), GEPRiCo (Gallic, Etruscan, Phocaeans Rites of Commensality [8]), LAIT'AGES (Archéologie des produits laitiers en Bretagne protohistorique (âge des métaux) et gallo-romaine [9]) have made significant contributions to providing new data on ancient daily life, concerning diet, medical practices, and ritual habits [10–13].

Among the primary challenges encountered in applying analytical techniques to determine ancient contents are the identification of markers associated with various substances, the selection of appropriate analytical methods, and the nature of the materials analyzed.

In order to reach reasonable conclusions about the content of the ancient vases, it is essential to establish the molecular markers associated with specific substances. However, the choice of suitable molecular markers may vary among different studies, resulting in a confusing situation for chemists who develop new analytical procedures and archaeologists who need specific information [14]. The main drawbacks in identifying these markers are related to the extraction step and the possible alterations, due to anthropic treatments, chemical degradation and transformation by bacterial activity.

With regard to the analytical techniques employed, recognizing the paramount importance of archaeological objects, non-invasive analytical techniques have become invaluable tools for investigating organic residues [5], providing general information or a specific fingerprint on the nature of organic compounds [15]. Notably, a key advantage of this approach lies in its ability to identify both inorganic and organic molecular species, irrespective of the shape or size of the archaeological sample [16]. However, while Fourier-transform infrared (FTIR) and Raman spectroscopies offer the advantages of minimal sample preparation and the ability to collect spectra from various items [17,18], these techniques have limitations in definitively identifying organic residues and over-interpretation of spectroscopic data for organic residues can sometimes occur [19]. In such cases, chromatographic techniques coupled with different detection methods may provide a valuable alternative. Chromatography, in fact, excels at separating the diverse components within a complex matrix like archaeological pottery, facilitating their subsequent identification.

If the analysis of organic residues in perfume containers, cooking pottery, and transport vessels has yielded significant insights into ancient practices, the application of such methodologies to figured pottery (black-figure, red-figure, and polychrome), extensively utilized in ritual and banquet contexts across the Greek and Mediterranean worlds, has been less explored (Project "Containing Commodities: Determining Organic Residues in Greek Painted Pottery" [20]). The finer clay composition, shorter contact periods with contents, and post-excavation storage conditions, often involving extended storage and restoration, make the identification and analysis of organic residues on these artifacts particularly challenging.

Within the framework of the Imag-ORA project, which takes an interdisciplinary approach to exploring the uses and functions of Attic and Etruscan-Italic figured pottery in pre-Roman Italy, this study investigates the potential for extracting wine markers from figured vases. The project presents a minimally invasive HPLC-MS/MS protocol specifically designed for analyzing figuredh pottery yet possessing the potential for broader application to a wider range of archaeological ceramic vessels.

### *1.2. Detecting wine markers in ancient pottery*

Organic residue analysis (ORA) of archaeological pottery primarily focuses on the identification of residues derived from foodstuffs and other organic materials that were once contained within the vessels. Common targets for ORA in archaeological ceramics include wine, cheese, oil, honey, cereals, and beeswax [21–23]. Notably, perfumes also feature prominently among the investigated substances [12]. Wine, in particular, held immense significance as a beverage throughout the ancient Mediterranean, Mesopotamia and the Near East. It served as an offering in funerary rituals of ancient Egypt and played a central role in near eastern societies and in social ceremonies like the symposium and the banquet across Greece, pre-Roman Italy, and the Roman world [24,25]. Classical literary sources such as Homer's Odyssey (Book IX) [26]and Cato's De Agricultura (160 BCE) [27] further emphasize the centrality of wine as a social lubricant in daily life for ancient civilizations.

Tartaric acid has been established as the primary chemical marker for identifying wine residues in ancient pottery. While its polar nature may limit its long-term preservation within archaeological ceramics, this very characteristic also allows it to form strong interactions with the Brønsted or Lewis acid sites on ceramic surfaces, or to form hydrogen bonds with silicates. These interactions can enhance its detectability [14]. In addition to tartaric acid, several other analytes are of interest for detecting wine residues in ancient pottery. These include fumaric, maleic, succinic, malic, and syringic acids. However, syringic acid presents a challenge due to its ubiquity in various plant materials, including olives, spices, pumpkin, honey, and grapes [28]. Syringic acid has been proposed as a marker for red wine specifically, due to red wine's richness in malvidin, which forms stable compounds with phenolic components, acetaldehyde, and pyruvic acid during aging. Notably, Guasch-Jane et al. [29] identified syringic acid released from anthocyanin-derived pigments through analysis of Egyptian vases using alkaline fusion with potassium hydroxide (KOH) [24]. According to their study [29], the presence of syringic acid indicates that the wine contained in those vases was a quality of red wine.

Gas chromatography coupled to mass spectrometry (GC–MS) is considered the gold standard technique for the detection of molecular markers associated with wine [30]. In 1978 Condamin and Formenti determined tartaric acid using Thin-layer Chromatography (TLC) and GC [5]; subsequent studies, applied transmission and diffuse-reflectance infrared spectroscopy (DRIFTS) followed by HPLC-UV and/or Feigl spot tests to identify tartaric acid [31,32]. More recently, Guasch-Jané et al. [29] used liquid chromatography-tandem mass spectrometry (LC-MS/MS). In addition, increased confidence in identification can be achieved by high resolution mass spectrometry (HRMS) as shown by McGovern et al. [33]. On the other hand, the effectiveness of the analysis relies on the extraction protocols used for the isolation of analytes. Ultrasound bath is the most used technique for the extraction of molecular markers from archaeological ceramics [34,35]. Other techniques, such as microwave-assisted extraction (MAE) [36] and pressurized liquid extraction (PLE) [37] have recently been exploited.

This study aimed to develop a novel, efficient, reliable, and rapid method for identifying wine markers in ancient pottery, particularly figured vases, using HPLC-MS/MS analysis. The extraction step was developed according to the characteristic of figured vases and the principles of green analytical chemistry and involved ultrasound-assisted liquid extraction and dispersive liquid–liquid microextraction (dLLME) for enrichment and clean-up. This pretreatment technique was first developed in 2006 for environmental science [38] and has since been successfully applied in analytical chemistry fields, including forensic, food, and clinical analysis [39–41]. Compared to other enrichment strategies such as immunomagnetic bead enrichment (IMBs) and solid phase microextraction (SPME), dLLME is most cost-effective and provides a greater contact surface between the analyte and the extracting solvent, facilitating the transfer of the analyte into the organic phase. The method was validated and applied to analyse molecular mark-

ers of wine preserved in figured vases used for the consumption of wine discovered in Falerii Veteres (now Civita Castellana, Viterbo, Italy) (see below § 4. Real samples).

## 2. Materials and methods

### 2.1. Standards and reagents

Analytical standards for L-(+)-Tartaric acid (≥99.5 %), Malic acid (≥99.0 %), Maleic acid(≥99.0 %), Succinic acid (≥99.0 %), Syringic acid (≥95 %), Fumaric acid (≥99.0 %) and Succinic acid-2,2,3,3-$d_4$ (≥98 %) were purchased from Merck Life Science S.r.l. (Milan, Italy) as a crystalline powder. Water, methanol, acetonitrile, chloroform, ethyl acetate, 2-propanol, pyridine, all LC-MS grade, were supplied by Merck Life Science S.r.l. (Milan, Italy) Italy). Formic acid (HCOOH) and hydrochloric acid (HCl) were provided by VWR Chemicals (Milan, Italy). 1-Ethyl-3-(3′-dimethylaminopropyl) carbodiimide hydrochloride (EDC), 3-Nitrophenylhydrazine hydrochloride (3-NPH), potassium hydroxide (KOH), sodium chloride (NaCl), sodium dihydrogen phosphate ($NaH_2PO_4$), disodium hydrogen phosphate ($Na_2HPO_4$) and ammonium acetate were purchased from Merck Life Science S.r.l. (Milan, Italy). A buffer solution 100 mM of $NaH_2PO_4$ /$Na_2HPO_4$ pH 8, a solution of KOH 1 M and a solution of HCl 6 M were prepared in water and were stored at 10 °C.

Stock solutions of the single analytes were prepared in methanol at 1 mg $mL^{-1}$. A working solution, containing the six analytes at 10 μg $ml^{-1}$ and 500 ng $ml^{-1,}$ was obtained in water : acetonitrile (50:50) by appropriate dilution of the stock solutions and was stored at −20 °C. A working solution of succinic acid-d4, which was used as internal standard at 10 μg $ml^{-1}$, was prepared in water : acetonitrile (50:50) (ISWS). 40 mM 3-NPH solution and 24 mM EDC – 6 % pyridine solution were freshly prepared in $H_2O$: Isopropanol (30:70).

### 2.2. Sample collection

Different samples from figured vessels from the ancient Ager Faliscus (Table S1), the actual Civita Castellana's territory, near Viterbo in central Italy, were collected in minimally invasive procedures, considering the historical, artistic, and cultural value of the vessels. Utilizing sterile nitrile gloves, 35 mg aliquots of the archaeological ceramic material were collected by scraping the vessel's interior surface with a disposable scalpel. Each sample was collected in a separate, pre-labeled Eppendorf tube to prevent cross-contamination and then sealed for transport to the laboratory at Sapienza University of Rome for subsequent analysis. All samples were processed individually.

### 2.3. Sample preparation

#### 2.3.1. Extraction

The extraction of wine molecular markers from samples was performed by Ultrasound-Assisted Extraction (UAE), according to the method described by Pecci et al. [34], with some modifications. An aliquot of 35 mg of each powdered samples was spiked with 10 μL of internal standard (IS) solution (succinic acid-2,2,3,3-$d_4$, 10 μg $mL^{-1}$) and 200 μL of KOH 1 M was added. The Eppendorf tubes were vortex-shaken for 30 s and then immersed for 90 min in an ultrasonic bath thermostated at 70 °C. After cooling, the extract was centrifuged at 12,000 g for 10 min to promote the sedimentation of solid residues; the supernatant was recovered into a 15 mL conical tube and acidified with 30 μL of HCl 6 M to neutralize the base. Then, 35 μL of 100 mM buffer solution of $NaH_2PO_4$ /$Na_2HPO_4$ (pH 8) was added to obtain a slightly alkaline solution.

#### 2.3.2. Derivatization

Analyte derivatization for LC–MS/MS analysis was carried out according to the methods described by Han et al. [42,43], Liebisch et al. [44] and D'Addario et al. [45] with some modifications. The extract obtained in the first step was sequentially mixed with 150 μL of 40 mM 3-NPH solution and 150 μL of 24 mM EDC – 6 % pyridine solution. After vortex-shaking the mixture for 30 s the derivatization was carried out at 45 °C for 30 min by magnetic stirring. The reaction was quenched with 200 μL $H_2O$ + 0.1 % HCOOH. Subsequently, each sample was centrifuged at 10,000 g for 10 min, to precipitate possible ceramic particulates left in solution after the first centrifugation and the supernatant was collected into a conical tube.

#### 2.3.3. Clean-up

For each sample, the derivatized extract, obtained as previously described, was mixed with 0.2 g of NaCl and 1.2 mL of UHPLC-grade water. Once homogenized, 100 μL of 2-propanol, as dispersing solvent, and 100 μL of chloroform, as extraction solvent, were quickly injected into the solution by means of a 250 μL Hamilton syringe. To facilitate the homogenization of the mixture and promote the extraction of the analytes from aqueous phase, the obtained sample was vortex-mixed for 30 s, sonicated at 25 °C for 10 min, and then, centrifuged at 2600 g for 10 min at 4 °C to induce the chloroform sedimentation at the bottom of the tube. The latter was collected and dried under a gentle $N_2$ stream. Samples were resuspended in 100 μL of methanol. 3 μL was injected into the HPLC-MS/MS system for analysis.

### 2.4. HPLC-MS/MS analysis

Liquid chromatography was performed on an ExionLC AC System from SCIEX (Framingham, MA, United States), equipped with an RS auto sampler. An API 2000 mass spectrometer from AB-Sciex (Toronto, ON, Canada), equipped with an electrospray ionization source (ESI) which can operate in both positive and negative ionization, was used for the mass spectrometric detection. The analytes were separated with a reversed phase Kinetex PFP (100 mm x 2.1 mm ID) column, packed with 2.6 μm core shell particles from Phenomenex (Torrance, CA, USA); the column oven was set at 40 °C. Mobile phases were water (A) and Methanol (B), both acidified with 0.01 % formic acid; flow rate was 0.3 mL $min^{-1}$. The elution gradient was structured as follows: the elution started with 30 % of phase B and was increased to 55 % in 1 min, then isocratic step up to 55 % to 59 % in 2.5 min, finally increased from 59 % to 100 % in 0.5 min and held in these conditions for 1 min. In the following 0.5 min phase A was brought, to the initial conditions, and maintained for 2 min. All the analytes were detected in negative ionization with a Ionspray voltage (IS) of −4500 eV, curtain gas (CUR) set at 20 psi, collision gas set at 6, source temperature set at 350 °C (TEM), with Ion Source Gas 1 (GS1) and Gas 2 (GS2) individually set at 45 psi and 65 psi. All source instrument parameters for the monitored analytes were tuned by injecting standard solutions at a concentration of 100 ng $mL^{-1}$ at 7 μL $min^{-1}$ by a syringe pump. MS/MS analyses were carried out in Multiple Reaction Monitoring (MRM) acquisition mode. Peak areas were detected trough AB-Sciex package Analyst 1.4. The selected transitions, together with the main LC–MS/MS parameters, are reported in Table 1.

## 3. Results and discussion

### 3.1. Optimization of HPLC-MS/MS

Six molecules were initially chosen as targets for analysis, representing the most established molecular markers of wine: tar-

**Table 1**
Quantifier ions are highlighted in bold (Rt: retention time; Q1: precursor ion mass; DP: declustering potential; EP: entrance potential; Q3: product ion mass; CE: collision energy; CXP: cell exit potential.

| Analytes | Q1 (*m/z*) | DP (eV) | EP (eV) | Q3 (*m/z*) | CE (eV) | CXP (eV) |
|---|---|---|---|---|---|---|
| Fumaric acid (biderivatized) | 385.0 | −10 | −3 | 122.0 | −26 | −17 |
| | | | | **232.1** | −27 | −24 |
| Malic acid (biderivatized) | 403.0 | −10 | −10 | **207.8** | −29 | −20 |
| | | | | 137.0 | −48 | −13 |
| Maleic acid (monoderivatized) | 250.0 | −10 | −2 | **206.0** | −21 | −23 |
| | | | | 121.9 | −25 | −12 |
| Succinic acid (biderivatized) | 387.0 | −60 | −8 | **233.8** | −25 | −5 |
| | | | | 137.0 | −43 | −5 |
| Syringic acid (monoderivatized) | 332.0 | −125 | −2 | **166.2** | −31 | −15 |
| | | | | 137.0 | −42 | −15 |
| Tartaric acid (biderivatized) | 419.0 | −10 | −2 | **208.0** | −31 | −21 |
| | | | | 137.0 | −43 | −16 |

taric acid, fumaric acid, syringic acid, malic acid, maleic acid, and succinic acid. We initially attempted to leverage separation methods reported in the literature [23] which employed apolar chromatographic columns utilizing C18 stationary phase. However, this approach proved unsuitable due to the predominantly polar nature of the targeted analytes. Despite employing gradient elution and exploring combinations of various solvents, adequate separation and detection of these analytes remained elusive under the tested conditions [24]. To improve the chromatographic retention and the mass spectrometric detection, a hydrophilic interaction column (SEQUANT® ZIC®-PHILIC 150 mm x 2.1 mm, 5 µm, polymeric beads; Merck KGaA, Darmstadt, Germany) was subsequently tested, using different combinations of mobile phases; the best results were obtained with $H_2O$ + ammonium acetate 5 mm pH 8.5 and acetonitrile as organic phase. However, it must be pointed out that in all the tested conditions, only syringic acid, succinic acid, fumaric acid and maleic acid could be detected and for the last two analytes a single MRM transition was detected, hindering an unequivocal identification of the analytes. Moreover, succinic acid and syringic acid had chromatographic peaks with low signal and irregular shape. To solve these problems, we decided to derivatize the molecular markers, with the aim of improving their chromatographic retention, the ionization efficiency and the fragmentation behaviour (Fig. 1). Generally, the carbonyl group is involved in the reaction, with a final loss of a water molecule. We selected the method described by Han et al. [42,43], followed with some modifications by Liebisch et al. [44] and D'Addario [45]. Carbonyl compounds can react with hydrazine in aqueous solution to form hydrazones using carbodiimides, such as EDC as the condensation reagent, and pyridine as a catalyst. EDC activates the carbonyl group, so it is more available to the nucleophilic attack of the nitrogen lone pair. The derivatization followed a mechanism of nucleophilic addition/elimination and was conducted in slightly alkaline conditions. In the present study, we used a nitro-phenylhydrazine (3-NPH), obtaining, as product, 3-nitrophenylhydrazone. The reaction is in two steps: in the first there is the nucleophilic attack of 3-NPH on carbonyl carbon and in the second the loss of a water molecule. A standard solution containing 10 µg $mL^{-1}$ of each analyte in water: acetonitrile (50:50) was used to find suitable reaction conditions for the derivatization reaction. 40 µL of standard solution, 4 µL ISWS, 20 µL of 3-NPH 200 mM and 20 µL of EDC-HCl 120 mM were transferred in an Eppendorf tube. After stirring for 30 min at 45 °C, the reaction was quenched with 200 µl of aqueous solution containing 0.1 % formic acid. It was interesting that, for all dicarboxylic acids, except maleic acid, the reaction involved both terminal carboxylic sites. (table S2). Therefore, through this process, a biderivatized product was obtained. It was hypothesized that for maleic acid only the monoderivatized product was observed because of the steric encumbrance given by its structure with the two carboxyl groups on the same side. Later, further experiments were carried out to identify the optimal concentration of reagents. By employing 3-NPH at 200 mM, problems of signal suppression were found for the monoderivatized analytes. To improve the signal intensity and the chromatographic peak and to reduce ion suppression different concentrations of 3-NPH and EDC were tested, dividing by 2, 5, 10, 20 and 25 their initial concentration. These investigations led to the identification of optimal concentrations: 40 mM of 3-NPH and 24 mM of EDC-6 % of pyridine. The reduction of derivatization reagents concentration led to improved signal of maleic acid but reduced signal intensity of the biderivatized products. Despite the reduction of concentration of 3-NPH and EDC, the problem of signal suppression of syringic acid and maleic acid persisted. Consequently, these analytes were excluded from further analyses in this study (Fig. 2).

### 3.2. Extraction

For molecular markers extraction from ceramic, a method published in the literature was initially considered, with some modifications [34]. This adaptation aimed to minimize the amount of ceramic sample utilized, prioritizing the preservation of the vase's integrity. In the literature, the quantity of ancient sample utilized for analysis with hyphenated techniques typically ranges from 100 mg to 1 g [30,34]. Significantly, this novel method utilizes a mere 35 mg of powdered archaeological sample. This reduction in sample quantity also translates to a substantial decrease in the required extraction solvent volume. Compared to previously reported methods employing several milliliters of solvent [29,34] the presented analytical approach necessitates only hundreds of microliters. This innovation minimizes both the time and cost associated with the extraction step.

Preliminary experiments were carried out to define the protocol for the extraction of the analytes from ceramic. To this purpose, initially, 1 mL of KOH 1 M containing the analytes at a concentration of 250 ng $mL^{-1}$ was transferred in a 15 mL conical tube and then immersed for 90 min in an ultrasonic bath at 70 °C; this step was necessary to evaluate the stability of the analytes at this temperature. Then, 140 µl of HCl 6 M were added; the addition of HCl was important to obtain a pH value lower than the pKa of the analytes, to improve the extraction rate in the following dispersive liquid liquid extraction step. The results obtained showed that the analytes were stable in the extraction conditions used. Subsequently, the volume of solutions was further reduced to 200 µL of KOH 1 M and 30 µL HCl 6 M. Considering that the derivatization occurs in slightly alkaline conditions 35 µL of 100 mM buffer solution of $NaH_2PO_4/Na_2HPO_4$ pH 8 was also added.

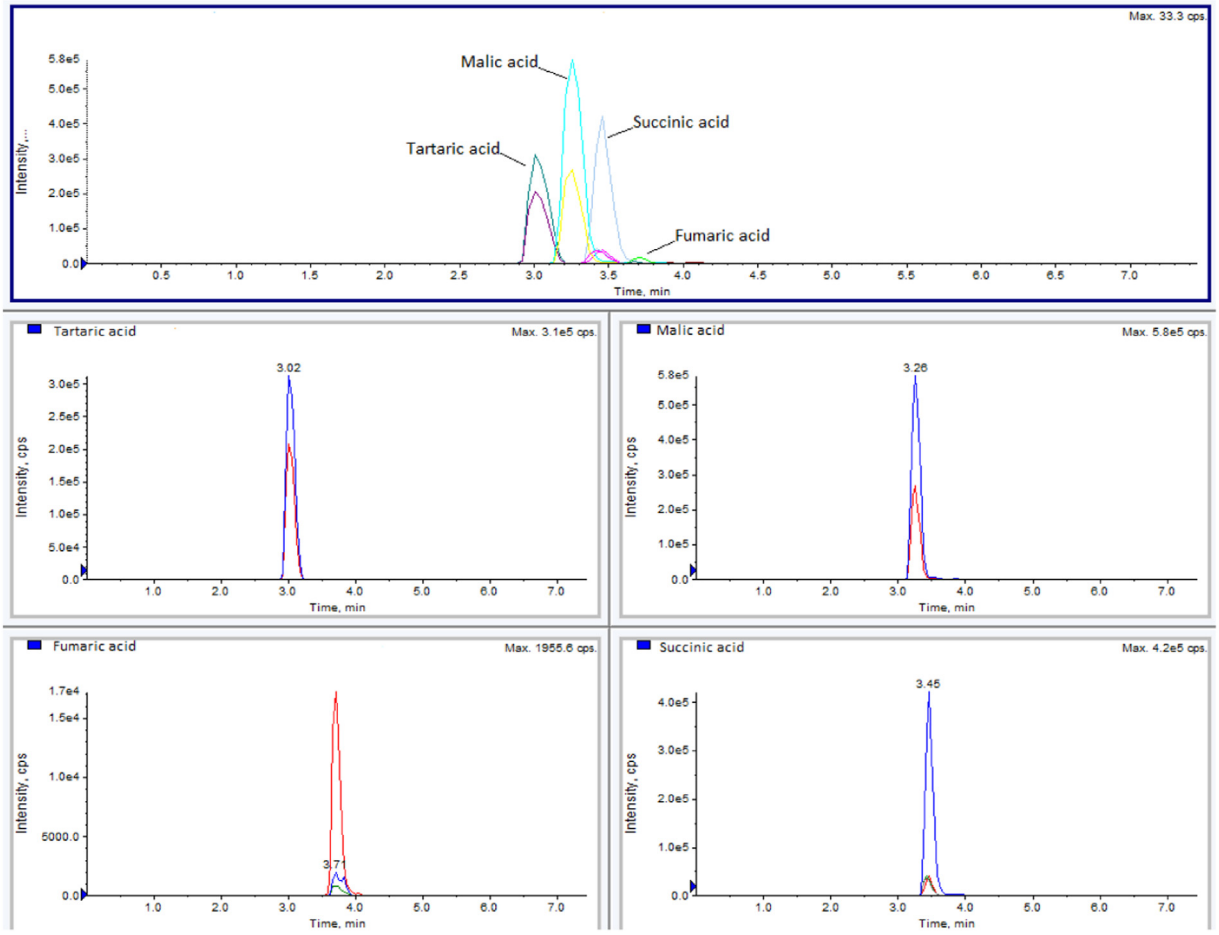


**Fig. 1.** Chromatogram post derivatization.

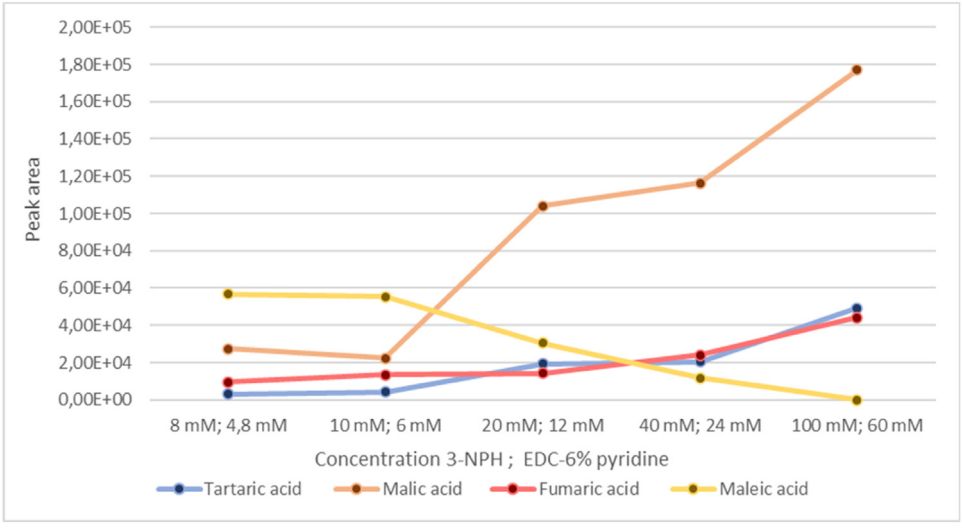


**Fig. 2.** Influence of the concentrations of 3-NPH and EDC in the reaction mixture on peak areas of the derivatized analytes ($n = 3$ for each time point).

### 3.3. dLLME

Archaeological ceramic is a complex matrix and it is therefore necessary to remove interfering components before analysis. Keeping in mind that a significant enrichment factor would be beneficial because of the very low concentrations expected, dLLME emerges as a good candidate for the clean-up of ceramic extracts [37]. This study presents the first application of a miniaturized clean-up step specifically designed for the analysis of wine markers in archaeological vessels. The effectiveness of this approach has been experimentally demonstrated. This liquid–liquid microextraction technique exploits ternary solvent mixtures to achieve effective extraction of compounds, with decreased amount of solvent and time. It consists in the quick injection of a disperser solvent

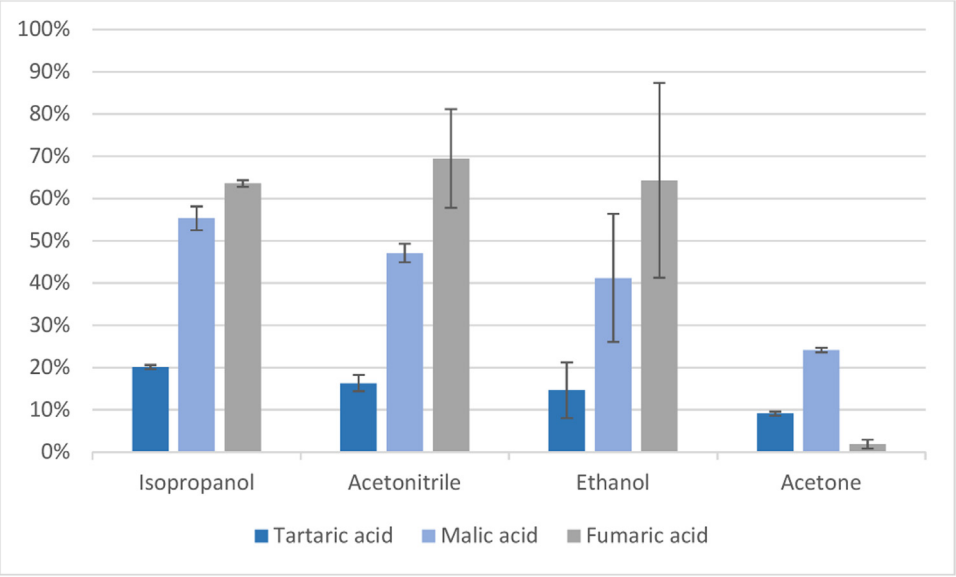


**Fig. 3.** Extractive rates using different dispersing solvents (maintaining chloroform as extracting solvent) ($n = 3$ for each tested condition).

containing an appropriate amount of an extraction solvent into the aqueous sample. The advantages of DLLME are mainly: a) the increase in sensitivity; b) the removal of excess of derivatization reagents before HPLC-MS/MS analysis; c) the high enrichment factor (EF) due to the high phase ratio of the donor (aqueous sample) and the acceptor (extraction solvent). To obtain satisfactory extraction levels in the dLLME step, it was necessary to pay attention to different parameters: dispersing solvent, volumes, pH and ionic strength [46]. In the first case, four different dispersant solvents were initially tried: isopropanol, acetone, ethanol and acetonitrile. Based on the obtained results, isopropanol was chosen, as it allows to slightly enhance the recoveries of tartaric acid and malic acid (Fig. 3).

Moreover, different relative amounts of the extracting and dispersing solvents were explored, namely 200:100, 100:100 µL (extracting : dispersing, v:v) and the latter amount was selected. To improve the transfer in the organic solvent of the analytes, sodium chloride has been added; different amounts (0 g, 0.1 g and 0.2 g) were tested. 0.2 g of NaCl allowed to increase the ionic strength of the aqueous solution, decreasing the solubility of the analytes in water. Therefore, 0.2 g of NaCl was added into the DLLME solutions in the subsequent studies (Fig. 4). Higher amount of salt led to the separation of the dispersing solvent by salting-out effect and resulted in decreasing of recovery values.

### 3.4. Control vase: analysis of blank and mock-up samples

To assess the efficacy and minimize potential biases in the analytical method, a control vase was analyzed prior to examination of the actual archaeological pottery. This control vase consisted of a modern ceramic vessel specifically crafted using techniques that replicate those employed in antiquity. The clay composition of this control vase was sourced locally in the Ager faliscus (Vignale, Falerii Veteres), aiming to mimic the expected chemical composition of the archaeological finds. By analyzing this vase, we sought to eliminate the influence of any pre-existing molecular markers potentially present within the clay body used to manufacture the archaeological pottery locally produced.

A blank sample was first collected from the newly produced control vessel, which had never been in contact with wine. Subsequently, 2 mL of wine was introduced into the vessel. Following a 24-hour drying period to ensure complete evaporation of the wine, the sample was then subjected to accelerated weathering for 72 h within a QUV accelerated weathering tester (Q-LAB EUROPE BOLTON, ENGLAND), that was set at a constant temperature of 65 °C and with UVB radiation at a wavelength of 313 nm. This procedure was aimed to reproduce the damage caused by sunlight, according to specific ASTM G154-23. Our goal was not to simulate the conservation process of the vessels but to subject the analytes into hard conditions of temperature and light radiation and to verify if under these conditions the markers were still identified by HPLC-MS/MS analysis. 35 mg of ceramic powder were then collected and analysed, by scraping the ceramic with a disposable scalpel, from the region that was in contact with the wine.

The chromatograms obtained from the analysis of the blank and the aged mock-up samples are shown in Fig. 7. It should be observed that tartaric acid is only present in the sample put in contact with wine (the mock-up sample). Based on this observation, tartaric acid appears to be a promising molecular marker for identifying wine residues in archaeological pottery. However, further investigation into its specificity is warranted, as this analyte is not only characteristic of grapes but also present in other fruits [47]. In contrast, both malic and fumaric acids were detectable also in the blank ceramic control sample, before the addition of wine. While the intensity of the fumaric acid signal remained relatively unchanged after wine introduction, a noticeable increase was observed for malic acid.

In light of these observations, the sole detection of fumaric and/or malic acid in a sample is insufficient to definitively identify the presence of wine residues. Conversely, the co-occurrence of all three analytes – tartaric, fumaric, and malic acids – strengthens the hypothesis that the vessels were once in contact with wine.

Succinic acid, on the other hand, was also detected in the blank samples (Fig. 5). This finding suggests that either succinic acid itself or an isobaric contaminant might be present in the solvents and reagents used during sample preparation. Consequently, succinic acid cannot be considered a reliable marker for wine residues, despite its frequent mention in the literature.

### 3.5. Evaluation of analytical performance

The analytes that have been selected for validation of the method were: malic acid, tartaric acid and fumaric acid. The proposed method was validated as a qualitative method for the target

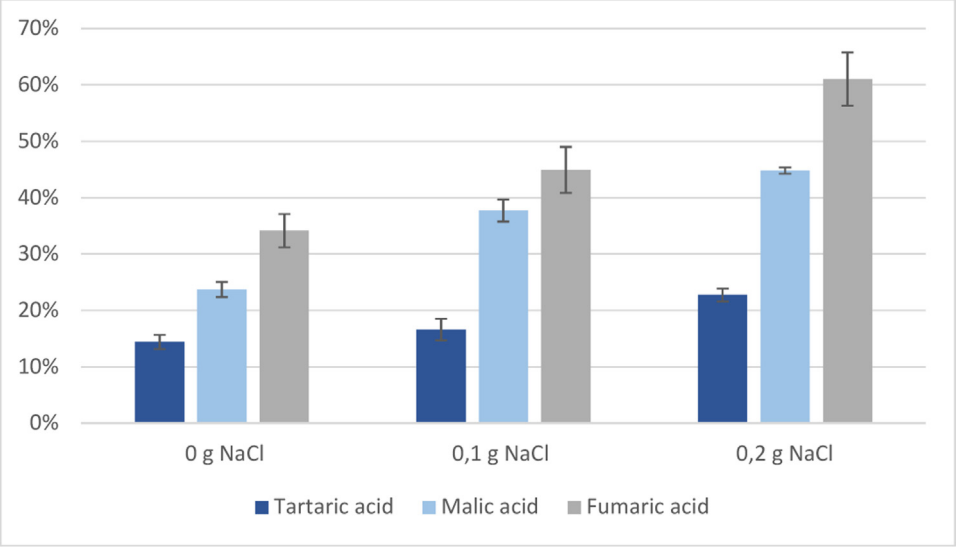


**Fig. 4.** Extractive rates using chloroform: isopropanol (extracting: dispersing) and different amounts of salt. ($n = 3$ for each tested condition).

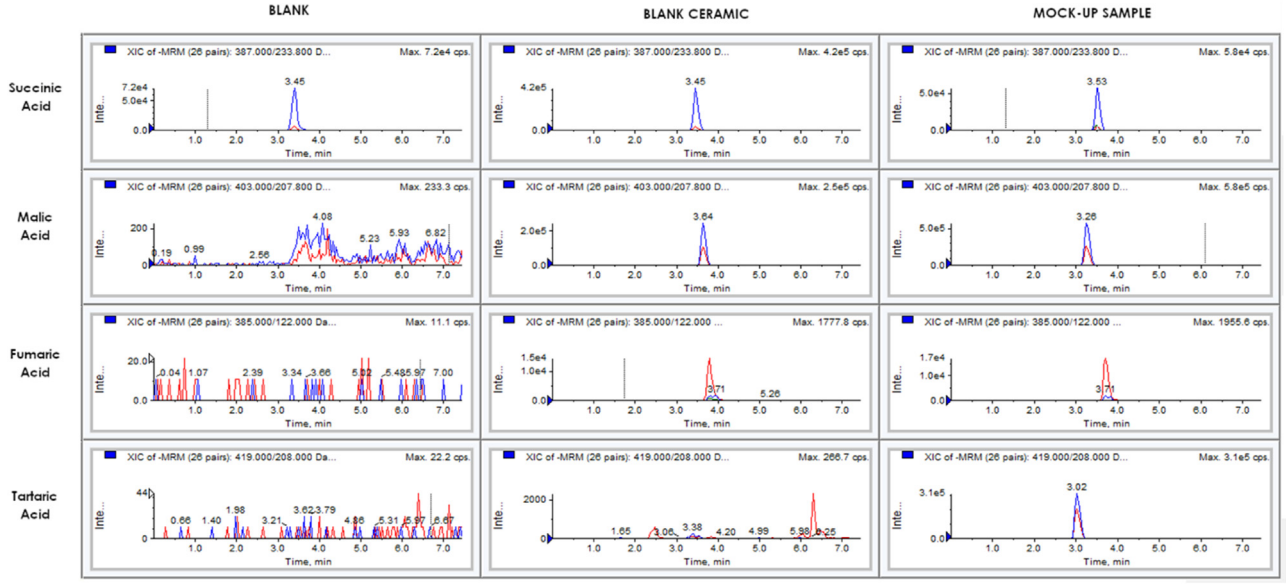


**Fig. 5.** Panel A: Chromatogram of blank sample that showed the presence of succinic acid; Panel B: Chromatogram without addition of wine; Panel C: Chromatogram with addition of wine.

**Table 2**
LOD and precision for tartaric acid, fumaric acid and malic acid. LOD was defined in ng of analyte/ mg of powdered samples ($n = 5$).

| Analytes | LOD (ng mg$^{-1}$) | Precision |
|---|---|---|
| Tartaric acid | 0.05 | 15 % |
| Fumaric acid | 0.01 | 13 % |
| Malic acid | 0.01 | 12 % |

analytes, evaluating the following parameters: LOD, precision and carryover (Table 2). The LOD was calculated as the lowest absolute concentration that provided a signal-to-noise (S/N) ratio >3 for the qualifier ion. For this purpose, decreasing concentrations of the standard mixture were processed according to the described method and analyzed in three different chromatographic runs. Precision was evaluated with independent tests on five quality control samples (QCs). QCs samples were prepared from blank samples spiked with a mixture containing the target analytes at 100 ng mL$^{-1}$. Then, precision was calculated as relative standard deviation (RSD%) and was always found, for all analytes, within ± 15 %. Carryover phenomena were evaluated by the analysis of a blank sample immediately after injection of the highest calibrator and the real sample. Absence of peaks indicated no carryover in the procedure.

## 4. Real samples. Detecting wine markers in figured vases

This study utilizes the previously established analytical protocol to investigate the presence of wine residues within archaeological pottery. A total of 26 vases were selected for analysis, with the primary objective being the development of a minimally invasive extraction method suitable for valuable artifacts, particularly figured pottery.

### *4.1. Provenance and context*

All analyzed vases are preserved in the Museo Nazionale dell' Agro Falisco in Civita Castellana, Italy (Table S1).

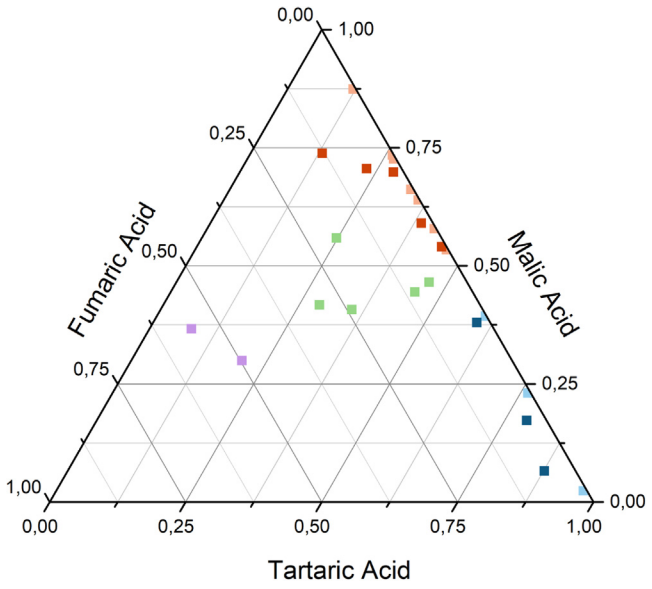


**Fig. 6.** Ternary plot that represents the results of the analyte in the analyzed sample.

These vases were unearthed during the late 19th and early 20th centuries within chamber tombs from various necropolises of the Ager Falisco (Fig. 6), a small cultural enclave in central pre-Roman Italy historically linked to the city of Falerii Veteres (present-day Civita Castellana in the province of Viterbo).

Archaeological evidence suggests commercial links between the Ager Falisco and Athens for the import of figured pottery as early as the 6th century BC. This trade relationship fostered the development of local workshops by the beginning of the 4th century BC, leading to the production of red-figure and superimposed color wares. Eventually, these locally produced ceramics supplanted imported vessels in common use [48].

The current study encompasses both Attic imports of the late 6th-5th centuries BC (black-figure and red-figure vases) and locally produced vases from the 4th century BC (red-figure, superimposed color, and black-glazed vases).

### *4.2. Selection criteria, functional considerations: and sampling methodology*

The study focused on whole, closed-shape vessels to facilitate micro-sampling on inconspicuous areas of the inner surface. This approach aimed to preserve the historical and artistic integrity of the artifacts. A significant portion of the selected vessels likely served for wine consumption during symposia and banquets. Notably, the majority are stamnoi, a frequently encountered shape within the Ager Falisco. While some vessels possessed an interior glaze, others are unglazed. Importantly, none of the chosen vases exhibited visible residues on their inner surfaces.

Micro-samples were obtained by scraping various regions of the bottom and inner surface with a sterile metal scalpel. Sampling was performed at two different stages (January 2022 and January 2023).

### *4.3. Addressing potential biases*

To minimize contamination from modern restoration materials, the analysis primarily focused on whole, unrestored vases. Subsequently, vases reassembled from fragments were also included to compare the results. During the sampling process, meticulous attention was paid to avoid areas near the joins, thus minimizing potential interference from restoration adhesives. The consistency observed between results from whole and reassembled vessels confirmed the method's applicability to restored materials. To further validate the method's efficacy, control samples were obtained from vessels not associated with symposia or wine consumption (samples 6171 and 1556).

### *4.4. Results*

Interestingly, all the analyzed samples tested positive for tartaric acid. Among them, some also contained malic acid, while fumaric acid was detected in only a few samples. The results, summarized in Fig. 6, were expressed as normalized peak area of the analyte in the analyzed sample in a ternary plot, in order to represent the different relative content of the three species and the related plausibility of presence of wine in the vessels. The samples highlighted in green should correspond to the vessels that likely


**Fig. 7.** Two of the vases analised. A: Attic red-figure stamnos, Villa Giulia Painter, 470–450 BCE (Civita Castellana, Museo Archeologico dell'Agro Falisco, 983. From Falerii Veteres, Celle necropolis, Tomb *67, XCII*); B: Faliscan red-figure stamnos, Nazzano Group, 380–370 BCE circa (Civita Castellana, Museo Archeologico dell'Agro Falisco, 6208. From Corchiano, Ager faliscus, *Il Sepolcreto del Vallone, Tomb 15*) Photos A. Pola, courtesy of the Polo Museale del Lazio- Civita Castellana (VT), Museo Archeologico dell'Agro Falisco e Forte San Gallo).

contained wine in antiquity: the content of all the three acids is over their limit of detection and their relative amount is over 50 %, in some cases remarkably comparable (the two green points at the centre of the plot). The purple points, instead, represent the vessels with the lowest probability of contact with the wine: the relative content of fumaric acid is highly over the other two acids, and, with reference to its ubiquity, no hypothesis about the presence of wine can be done. Finally, blue/light blue and red/light red sets of points can be considered as intermediate situations. The blue points represent the samples where the relative amount of tartaric acid is over 50 %. On the opposite, the red points present a relative amount of tartaric acid between 10 % and 50 %, but with a high content of malic acid (over 50 %). In the two groups, moreover, red and blue points present a content of fumaric acid over the LOD, while the light red and the light blue points correspond to samples where this acid was under the LOD. Comparing these two sets of data, with reference to the general higher concentration of malic acid for wine containing vessels observed in the study on the mock-up, some possible hypotheses could be formulated. For the blue-marked data, the content of tartaric acid is generally high, so it is possible to hypothesize the presence in antiquity of products deriving from fruit fermentation, even if the content of malic acid is not very high and, for the three light blue samples, the detection of fumaric acid did not occur. For the red-marked vessels, both tartaric and malic acid can be considered present, but the higher relative amount of malic acid could make the hypothesis more ambiguous and should address to further investigations.

Fumaric acid was found, in low amount, only in five samples. In samples 1207, 6208, 8341, 983 high amounts of tartaric acid and malic acid were found simultaneously. These data suggest that these vessels have been in contact with wine for long time. In samples 1062 and 6451, there were high values of all three analytes object of our study. This leads to consider almost certain the presence, in the past, of wine in the sample 1062. The highest amount of fumaric acid was detected in sample 6451. However, this vase according to excavation records was used as a cinerary urn, and it still contains fragments of burnt bones. Therefore, in this case, it cannot be excluded that the source of fumaric acid could be different from that of wine.

Samples 6171 and 1556 contained traces of soil impregnated with oily substances. For these reasons and according to their shape (askos) these vessels are believed to had been used as containers for perfumed oils. The results obtained, can confirm that in the past these two vases have not been used as containers for wine.

## 5. Conclusions

In this work, an efficient and versatile extraction technique has been developed for identifying molecular markers of wine in archaeological pottery, specifically in figured vases, materials rarely investigated through ORA. The development of this method is included in a comprehensive project on the uses and function of figured pottery in preroman Italy (Imag-ORA), and it allows for the first time a better assessing of the actual use of figured vases. While designed specifically for figured pottery, this method demonstrates applicability to a broader range of archaeological vessel types, facilitating a substantial reduction in the sample size required for analysis compared to previously employed methods.

The implementation of a miniaturized extraction and clean-up method pursued a dual purpose. First, it aimed to reduce the amount of sample analyzed, thereby prioritizing the preservation of the archaeological object's integrity. Secondly, from a green analytical chemistry perspective, this approach aimed to enhance the enrichment factor. In complex matrices like archaeological ceramics, the target biomarkers are frequently present in minute quantities, necessitating enrichment for successful detection.

When working with HPLC-MS/MS analysis, the main issues are related to detecting the analytes of interest. Chemical derivatization was shown to be pivotal in improving their detectability, allowing optimal performance in terms of recovery, chromatographic retention, and high selectivity and sensitivity. From the analysis of archaeological samples, it was shown that the developed method allowed corroboration of the presence of wine in some of the analyzed samples, supporting the hypothesis suggested by the archaeological data and proving the possibility of applying ORA to figured vases. Also, the method proved to be applicable both to entire and restored vases, even those preserved for long time in museum storerooms.

This novel method offers several advantages, including a reduction in the required sample size, high recovery rates, and minimal consumption of organic solvents. Notably, the significant decrease in sample amount to 35 mg, compared to the 100–250 mg typically required by methods reported in the recent literature, allows for the greatest possible preservation of the analyzed archaeological materials [10]. Furthermore, the developed procedure is readily applicable in laboratory settings due to its ability to process multiple samples simultaneously. Additionally, the reduced amount of extraction solvent translates to faster analysis times and lower overall costs.

Future research endeavors will focus on identifying more specific molecular markers for various substances once contained within figured pottery. Building upon the success of the current green analytical method with its minimal extraction requirements, the analyzed vases are now undergoing further investigation to detect markers of honey, waxes, and animal and vegetable fats. A critical aspect of future project development involves refining the analytical method through modifications to the sampling procedure. This optimization aims to minimize the invasiveness of sample collection, ultimately resulting in a less destructive technique that prioritizes the paramount preservation of the vases' integrity. With reference to achieved results and to a further optimization, new perspective of the research involve: (1) an experimental design study, conducted to determine the optimal conditions for improving the recovery of the target analytes, with reference also to the combination of their concentration; (2) a focus on minor markers of the wine matrix.

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## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.culher.2024.11.010.

## References


[1] S. Charters, R.P. Evershed, P.W. Blinkhorn, Quantification and distribution of lipid in archaeological ceramics: implications for sampling potsherds for organic residue analysis and the classification of vessel use*. vol. 35 (1993).
[2] A. Pecci, M.Á. Cau Ontiveros, N. Garnier, Identifying wine and oil production: analysis of residues from Roman and Late Antique plastered vats, J. Archaeol. Sci. 40 (2013) 4491–4498.
[3] M.P. Colombini, G. Giachi, F. Modugno, E. Ribechini, Characterisation of organic residues in pottery vessels of the Roman age from Antinoe (Egypt), Microchem. J. 79 (2005) 83–90.
[4] A. Irto, et al., Lipids in archaeological pottery: a review on their sampling and extraction techniques, Molecules. 27 (2022) Preprint at, doi:10.3390/molecules27113451.
[5] J. Condamin, F. Formenti, Détection du contenu d'amphores antiques (huiles, vin). Etude méthodologique, Revue d'Archéométrie 2 (1978) 43–58.
[6] https://bioarchaeo.hypotheses.org/the-program-magi.
[7] https://bioarchaeo.hypotheses.org/research-programs/perhamo.
[8] https://bioarchaeo.hypotheses.org/research-programs/geprico .
[9] https://laitages.hypotheses.org/le-projet.
[10] D. Frère, Dairy product and wine in funerary rituals: the case of a Hellenistic Etruscan Tomb, J. Hist. Archaeol. Anthropol. Sci. 1 (2017).
[11] Frère, D., Munzi, P. & Pouzadoux, C. Introduction. Manger, Boire, Offrir Pour l'éternité En Gaule et Italie Préromaines . (2021).
[12] Frére, D. & Hugot, L. Les Huiles Parfumées En Méditerranéè Occidentale et En Gaule, (VIII SIÈCLE AV. – VIII SIÈCLE APR. J.-C.). (2012).
[13] Frère D. & Bodiou L. Archéologie et histoire culturelle du lait et du fromage. Âge du Fer, Antiquité et Moyen Âge. in Annales de Bretagne et des Pays de l'Ouest - N° 129-3 (2022).
[14] L. Drieu, et al., Is it possible to identify ancient wine production using biomolecular approaches? Sci. Technol. Archaeol. Res. 6 (2020) 16–29 Preprint at, doi:10.1080/20548923.2020.1738728.
[15] E. Ribechini, F. Modugno, C. Baraldi, P. Baraldi, M.P. Colombini, An integrated analytical approach for characterizing an organic residue from an archaeological glass bottle recovered in Pompeii (Naples, Italy), Talanta 74 (2008) 555–561.
[16] S. Garrigues, M. De la Guardia, Non-invasive analysis of solid samples, TrAC - Trends Anal. Chem. 43 (2013) 161–173 Preprint at, doi:10.1016/j.trac.2012.10.008.
[17] L.M. Shillito, M.J. Almond, K. Wicks, L.J.R. Marshall, W. Matthews, The use of FT-IR as a screening technique for organic residue analysis of archaeological samples, Spectrochim. Acta a Mol. Biomol. Spectrosc. 72 (2009) 120–125.
[18] G.I. Badea, et al., Fourier transform Raman and statistical analysis of thermally altered samples of amber, Appl. Spectrosc. 69 (2015) 1457–1463.
[19] M. Regert, Analytical strategies for discriminating archeological fatty substances from animal origin, Mass Spectrom. Rev. 30 (2011) 177–220.
[20] https://gtr.ukri.org/projects?ref=studentship-2078700.
[21] M.P. Colombini, G. Giachi, F. Modugno, E. Ribechini, Characterisation of organic residues in pottery vessels of the Roman age from Antinoe (Egypt), Microchem. J. 79 (2005) 83–90.
[22] P.E. McGovern, et al., Chemical identification and cultural implications of a mixed fermented beverage from late prehistoric China, Asian Perspect. 44 (2005) 249–275 Preprint at, doi:10.1353/asi.2005.0026.
[23] A. Manhita, S. Martins, M. Gomes da Silva, M. Lopes, C. da, C. Barrocas Dias, Transporting olive oil in Roman times: chromatographic analysis of dressel 20 amphorae from Pax Julia Civitas, Lusitania, Chromatographia 83 (2020) 1055–1064.
[24] A. Amir, The identification of ancient wine through organic residue analysis of ceramic vessels, Atiqot 114 (2024) 179–192.
[25] L. Briggs, et al., There's more to a vessel than meets the eye: organic residue analysis of 'wine' containers from shipwrecks and settlements of ancient Cyprus (4th–1st century bce), Archaeometry 64 (2022) 779–797.
[26] Z. Papakonstantinou, Wine and wine drinking in the Homeric world, Antiq. Class 78 (2009) 1–24.
[27] D. van Limbergen, Wine, Greek and Roman, Oxford Research Encyclopedia of Classics, Oxford University Press, 2016, doi:10.1093/acrefore/9780199381135.013.6888.
[28] C. Srinivasulu, M. Ramgopal, G. Ramanjaneyulu, C.M. Anuradha, C. Suresh Kumar, Syringic acid (SA) – a review of its occurrence, biosynthesis, pharmacological and industrial importance, Biomed. Pharmacother. 108 (2018) 547–557 Preprint at, doi:10.1016/j.biopha.2018.09.069.
[29] M.R. Guasch-Jané, M. Ibern-Gómez, C. Andrés-Lacueva, O. Jáuregui, R.M. Lamuela-Raventós, Liquid chromatography with mass spectrometry in tandem mode applied for the identification of wine markers in residues from ancient Egyptian vessels, Anal. Chem. 76 (2004) 1672–1677.
[30] L. Blanco-Zubiaguirre, et al., Wine markers in archeological potteries: detection by GC-MS at ultratrace levels, Anal. Bioanal. Chem. 411 (2019) 6711–6722.
[31] R.H. Michei, P.E. Mcgovern, V.R. Badier, The first wine & beer chemical detection of ancient fermented beverages.
[32] McGovern, P.E. et al. Fermented beverages of pre-and proto-historic China. www.pnas.orgcgidoi10.1073pnas.0407921102 (2004).
[33] P.E. McGovern, et al., Beginning of viniculture in France, Proc. Natl. Acad. Sci. U.S.A. 110 (2013) 10147–10152.
[34] A. Pecci, G. Giorgi, L. Salvini, M.Á. Cau Ontiveros, Identifying wine markers in ceramics and plasters using gas chromatography-mass spectrometry. Experimental and archaeological materials, J. Archaeol. Sci. 40 (2013) 109–115.
[35] L. Briggs, et al., There's more to a vessel than meets the eye: organic residue analysis of 'wine' containers from shipwrecks and settlements of ancient Cyprus (4th–1st century bce), Archaeometry 64 (2022) 779–797.
[36] J.J. Lucejko, et al., Long-lasting ergot lipids as new biomarkers for assessing the presence of cereals and cereal products in archaeological vessels, Sci. Rep. 8 (2018).
[37] F. Vincenti, et al., Unearthed opium: development of a UHPLC-MS/MS method for the determination of Papaver somniferum alkaloids in Daunian vessels, Front. Chem. 11 (2023).
[38] M. Rezaee, et al., Determination of organic compounds in water using dispersive liquid-liquid microextraction, J. Chromatogr. A 1116 (2006) 1–9.
[39] F. Vincenti, et al., Combination of pressurized liquid extraction with dispersive liquid liquid micro extraction for the determination of sixty drugs of abuse in hair, J. Chromatogr. A (2019) 1605.
[40] P. Viñas, N. Campillo, I. López-García, M. Hernández-Córdoba, Dispersive liquid-liquid microextraction in food analysis. A critical review microextraction techniques, Anal. Bioanal. Chem. 406 (2014) 2067–2099 Preprint at, doi:10.1007/s00216-013-7344-9.
[41] F. Fanti, et al., dLLME-μSPE extraction coupled to HPLC-ESI-MS/MS for the determination of F2$\alpha$-IsoPs in human urine, J. Pharm. Biomed. Anal. 186 (2020).
[42] J. Han, S. Gagnon, T. Eckle, C.H. Borchers, Metabolomic analysis of key central carbon metabolism carboxylic acids as their 3-nitrophenylhydrazones by UPLC/ESI-MS, Electrophoresis 34 (2013) 2891–2900.
[43] J. Han, K. Lin, C. Sequria, J. Yang, C.H. Borchers, Quantitation of low molecular weight sugars by chemical derivatization-liquid chromatography/multiple reaction monitoring/mass spectrometry, Electrophoresis 37 (2016) 1851–1860.
[44] G. Liebisch, et al., Quantification of fecal short chain fatty acids by liquid chromatography tandem mass spectrometry—investigation of pre-analytic stability, Biomolecules 9 (2019).
[45] C. D'Addario, et al., Regulation of oxytocin receptor gene expression in obsessive–compulsive disorder: a possible role for the microbiota-host epigenetic axis, Clin. Epigenetics 14 (2022).
[46] F. Vincenti, et al., Combination of pressurized liquid extraction with dispersive liquid liquid micro extraction for the determination of sixty drugs of abuse in hair, J. Chromatogr. A (2019) 1605.
[47] L. Kırca, S. Kırca, A. Aygün, Organic acid, phenolic compound and antioxidant contents of fresh and dried fruits of pear (Pyrus Communis L.) cultivars, Erwerbs-Obstbau 65 (2023) 677–691.
[48] Angela Pola. La Più Antica Produzione Ceramica Falisca a Figure Rosse. (2024).