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Review Article

# Is it possible to identify ancient wine production using biomolecular approaches?

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**ABSTRACT**
Chemical analysis of archaeological artefacts is used with increasing regularity to understand how wine was produced, traded, and consumed in the past and to shed light on its antiquity. Based both on an extensive review of the published literature and on new analyses, here we critically evaluate the diverse range of methodological approaches that have been used for wine identification. Overall, we conclude that currently none of the proposed chemical 'biomarkers' for wine provide unequivocal evidence. Nevertheless, valid interpretations may be offered if systematically supported by additional contextual data, such as archaeobotanical evidence. We found the extraction and detection method to be particularly crucial for successful identification. We urge the use of controls and quantification to rule out false positives. DNA sequencing offers potential for identifying wine and provides much higher taxonomic resolution, but work is needed to determine the limits of DNA survival on artefacts.

## 1. Introduction

The identification of archaeological artefacts associated with the production and consumption of alcoholic beverages has had a long and chequered history, not least due to the lack of consensus regarding methods of analysis amongst the scientific community (Stern et al. 2008; McGovern and Hall 2016). This is particularly true for wine, an important commodity in the Mediterranean attested to by the earliest written records and pictorial evidence from this region (e.g. Hamilakis 1996). Whilst these sources are of course immensely useful, they say little about the antiquity of wine processing itself, its economic importance through time or the pervasiveness of wine consumption within and beyond the Mediterranean region. To answer these questions, it has long been recognised that chemical analysis of artefacts associated with wine, such as pottery vessels, transport amphorae, vats, and presses offers a potential answer. Unlike botanical remains of the grape, artefacts potentially offer rich contextual detail regarding the process of viticulture, the circumstances of wine consumption, the scale of its production and direction of trade.

Most of what we know of prehistoric wine consumption comes from organic residue analysis of ceramic containers. Recent chemical analysis of potsherds from Georgia (McGovern et al. 2017) claims to have pushed the antiquity of wine production back to the Early Neolithic period (around 6000–5800 BC), some 1000 years earlier than its previous earliest identification in a vessel from the Zagros Mountains (Iran, 5400–5000 BC; McGovern et al. 1996). Wine was also claimed to be identified in vessels from Northern Greece (around 4300 BC; Garnier and Valamoti 2016), Egypt (from 3000 BC; Guasch-Jané et al. 2004, 2006a, 2006b), Northern Italy (1500–1300 BC; Pecci et al. 2017), and in Southern France (around 500 BC; McGovern, Luley, et al., 2013), providing important insights into early establishment of the tradition throughout the Mediterranean. Analysis of Egyptian vessels has highlighted the role of wine in many aspects of political and religious life (Guasch-Jané et al. 2006a, 2006b, 2004; McGovern 1997). At Bronze Age palatial sites of the Eastern Mediterranean, residue analysis claims to directly demonstrate the importance of wine in social feasting to maintain political and social relations (Koh, Yasur-Landau, and Cline 2014; Koh and Betancourt 2010; McGovern et al. 2008). Wine seems also to have been adopted by populations living in areas where grapevines do not grow naturally, such as Scandinavia (c. 1500 BC to the first century AD; McGovern, Hall, and Mirzoian 2013).

Detecting wine in ancient containers is, in particular, a way to study the Mediterranean economy, by

identifying pressing and storage structures for wine, and distinguishing them from those for olive oil production (Pecci, Cau Ontiveros, and Garnier 2013, Pecci, Giorgi, Salvini, et al. 2013). Historical sources from the Classical period described the following amphorae as specific wine containers and several studies tried to check this hypothesis: amphorae from Corfu and Mende (Greece, fifth to third century B.C; Foley et al. 2012), Roman Dressel amphorae (first century BC to first century AD; Arobba et al. 2014; Condamin and Formenti 1978; Formenti, Hesnard, and Tchernia 1978; Garnier et al. 2003; Pecci et al. 2017), Late Roman (LR, fourth century AD; Pecci, Salvini, and Cantini 2010), and Keay amphorae (fourth to fifth century BC; Woodworth et al. 2015). More recently, the wine was claimed to be identified in an Early Celtic princely site (seventh to fifth centuries BC), which demonstrates trade between the Mediterranean and the Celtic spheres (Rageot et al. 2019).

The identification of wine in ancient vessels has been attempted using a considerable number of analytical methods, both for extraction and detection. Furthermore, the interpretation of the molecular markers varies considerably from one author to another, resulting in a confusing situation for both chemists who attempt to carry out new analyses and archaeologists who need reliable information. To clarify this situation, in this paper, we strive to give a critical review of the chemical approaches that have been used to identify the wine in the archaeological record. We provide new empirical data resulting from testing the efficiency of these approaches and propose new guidelines for the interpretation of such datasets.

## 2. Tartaric acid: a reliable biomarker for wine in archaeological samples?

In addition to water and ethanol, wine is composed of hundreds of other molecules present in much lower amounts and responsible for the aroma, taste, and colour of the wine. Tartaric acid ($TH_2$) (Figure 1(a)) is the
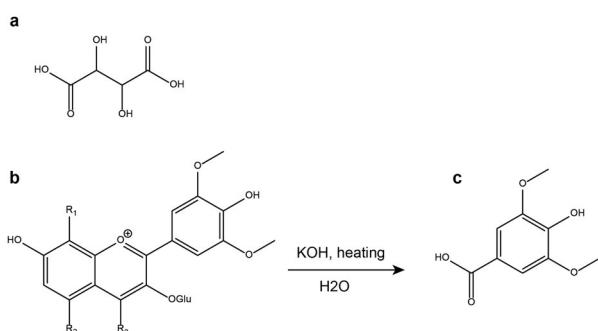


**Figure 1.** Structures of the main molecules reported in the literature to identify the wine in archaeological vessels: tartaric acid (a) and syringic acid (c) formed from the alkaline treatment of malvidin-3-glucoside polymers (b). Adapted from Guasch-Jané et al. (2004).

principal organic acid in grapes, together with malic and citric acids (Hale 1962; Ribéreau-Gayon et al. 2006, 4). In wine, tartaric acid mainly occurs in the form of dissociated tartrate salts ($TH^-$ and $T^{2-}$ ions): potassium bitartrate, potassium tartrate, calcium tartrate, potassium calcium tartrate, and calcium tartromalate, with quantities generally between 1 and 5 g/L (Ribéreau-Gayon et al. 2006, 21–22; Singleton 1996).

The polarity of tartaric acid and its salts makes it highly soluble in water and thus unlikely to be preserved in archaeological contexts (Barnard et al. 2011; Michel, McGovern, and Badler 1993; Singleton 1996), although tartrates are slightly less soluble in water than the acid. Fortunately, their polarity also offers a means of preservation by permitting binding to the ceramic matrix, through strong interactions between polar groups and Brønsted or Lewis sites of the ceramic paste, or hydrogen bonds with silicates (Garnier and Valamoti 2016; Michel, McGovern, and Badler 1993). Degradation experiments have shown that tartaric acid is better preserved in the ceramic matrix than other small acids (citric, malic, succinic, and fumaric acids) after seven years of burial in natural conditions (Pecci, Giorgi, Salvini, et al. 2013). Because of these properties, tartaric acid has been identified as a potential marker of wine since the inception of organic residue analysis (Condamin and Formenti 1978; Michel, McGovern, and Badler 1993).

### 2.1. How reliable are methods for the extraction and detection of tartaric acid in archaeological samples?

A wide range of approaches has been used to extract and detect tartaric acid from archaeological artefacts (Table 1). For the extraction, the first step must involve breaking chemical bonds between tartaric acid and the ceramic matrix. To do this, alkaline solutions (e.g. NaOH or KOH) have been widely used (Table 1), but acid treatment has also been shown to be effective, probably as it promotes partial dissolution of the ceramic matrix (Correa-Ascencio and Evershed 2014; Garnier and Valamoti 2016). Methods involving only water or methanol extraction are ill suited, as they do not break these bonds. The second step is to solubilise the tartaric acid in the extracting solvent, with a wide range of organic solvents being used (Table 1). Here, we have compared two of the most common extraction methods using modern wine samples and pure authentic tartaric acid, the latter serving as a positive control (Figure 2(a) and Table S2). We have shown that the method described as 'alkaline fusion' and subsequent extraction with ethyl acetate (Guasch-Jané et al. 2004; Pecci, Giorgi, Salvini, et al. 2013) was highly inefficient (less than 0.1% yield for pure tartaric acid standard), due to the low solubility of tartaric acid in ethyl acetate. In our trials, we applied the same protocol but used DCM as the extraction solvent; however,

**Table 1.** Overview of the extraction and detection methods for tartaric acid in archaeological artefacts published in the literature.

| Type of archaeological sample | Extraction method | Treatment to release sub-product | Detection method | Ref. |
|---|---|---|---|---|
| Original content (liquid part) | Ion-exchange chromatography, acidification, ether extraction | – | GC (and TLC) | Condamin and Formenti (1978) |
| Original content (solid part) | | Alkaline treatment: NaOH, 300℃, 1 h | GC (and TLC) | Condamin and Formenti (1978) |
| Coloured interior deposits | Acetone, boiling | – | DRIFT, Feigl spot test | Michel, McGovern, and Badler (1993) |
| Coloured interior deposits and pottery sherds | EtOH, ultrasonication (2 × 20-min) | – | DRIFT, HPLC-UV, Feigl spot test | McGovern (1997), McGovern et al. (1999, 2004, 2008) |
| Pottery sherds | Water, ultrasonication (80℃, 90 min) | – | HPLC-UV | Zhang et al. (2018) |
| Coloured interior deposits and pottery sherds | 0.1% formic acid in H$_2$O/MeOH (80:20, v/v) | 'Alkaline fusion': KOH pellets (0.2 g in 1 mL H$_2$O) heating (5 min). Acidification, extraction with ethyl acetate | LC/MS/MS (MRM mode, $m/z$ 149 → $m/z$ 87) | Guasch-Jané et al. (2004, 2006a, 2006b) |
| Coloured interior deposits and pottery sherds | 0.1% formic acid in H$_2$O/MeOH (80:20, v/v) | – | LC/MS/MS (MRM mode, $m/z$ 149 → $m/z$ 87) | Stern et al. (2008), McGovern, Mirzoian, and Hall (2009) |
| Pottery sherds | 1–2.8% NH$_4$OH in H$_2$O/MeOH (80:20, v/v) | – | DRIFT, LC/MS/MS (MRM mode) | McGovern, Hall, and Mirzoian (2013), McGovern, Luley, et al. (2013, 2017) |
| Pottery sherds | 0.1% formic acid in H$_2$O/MeOH (80:20, v/v) | – | GC–MS | Salvini, Pecci, and Giorgi (2008), Pecci, Salvini, and Cantini (2010) |
| Archaeological resins found within amphorae | DCM:MeOH (2:1, v/v), ultrasonication | – | GC–MS | Stern et al. (2008) |
| Pottery sherds | MeOH or MeOH/H$_2$O/acetic acid (4.5:4.5:1), 70℃, 1 h | – | GC–MS | Romanus et al. (2009) |
| Pottery sherds | DCM, heating  Ethanol, heating | – | GC–MS | Koh and Betancourt (2010), Koh, Yasur-Landau, and Cline (2014) |
| Pottery sherds, plastered layers | | Alkaline treatment: KOH 1M, 70℃, 1 h 30. Acidification, extraction in ethyl acetate | GC–MS | Pecci, Cau Ontiveros, and Garnier (2013), Pecci, Giorgi, Salvini, et al. (2013), Pecci et al. (2016, 2018), Woodworth et al. (2015) |
| Pottery sherds, plastered layers | CHCl$_3$/MeOH (2:1, v/v) | Alkaline treatment: KOH 1M. Acidification, extraction in CHCl$_3$ | GC–MS | Pecci, Giorgi, Salvini, et al. (2013), Pecci et al. (2016, 2018) |
| Pottery sherds | | DCM:MeOH (1:1, v/v), ultrasonication. Acidic treatment: BF$_3$ in BuOH and cyclohexane (1:2 v/v), 80℃, 2 h. Neutralisation, extraction with DCM | GC–MS | Garnier and Valamoti (2016) |
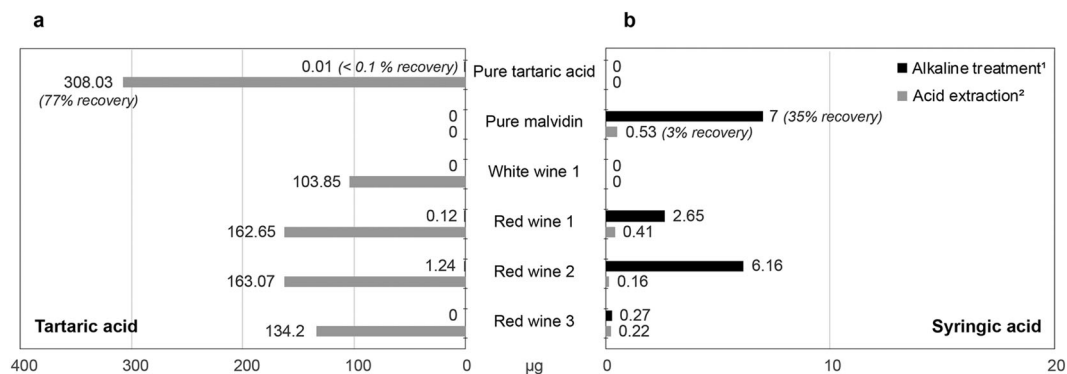
**Figure 2.** Comparative study of two extraction methods for (a) tartaric acid and (b) syringic acid (detection by GC–MS). Methods are taken from [1]Guasch-Jané et al. (2004) and Pecci, Giorgi, Salvini, et al. (2013), [2]Garnier and Valamoti (2016). Details of the materials, methods, and results are available in Supplementary information (Tables S1, S2, and S3).

this approach did not improve extraction yields (data not shown). It is also likely that the alkaline conditions promote the formation of insoluble tartrate salts. In comparison, the protocol involving butylation in acidic conditions (Garnier and Valamoti 2016) yielded much higher amounts of tartaric acid (our trial resulted in 77% yield for pure tartaric acid standard). The reason for this is that butylation significantly increases the solubility of the analyte in organic solvents, such as the DCM used in this method. It is clear then that when considering previous studies, the method of extraction needs to be carefully scrutinised.

Similarly, a wide range of approaches has been used for detecting tartaric acid in extracts from archaeological samples. In a pioneering study, Condamin and Formenti (1978) detected tartaric acid using gas chromatography and thin layer-chromatography (GC and TLC) after two different preparation methods were applied (Table 1). Later studies mentioned the use of transmission and diffuse-reflectance infrared spectroscopy (DRIFTS) as a preliminary test to detect tartaric acid or its calcium salt, followed by 'identification' by liquid chromatography with ultraviolet detection (HPLC-UV) and/or Feigl spot tests (Table 1). More recently, capillary electrophoresis (CE) has been also proposed as a detection method based on experiments on modern wine (Teodor et al. 2014). All these methods, although they have laid the foundations for the study of ancient wine, are not appropriate for the identification of tartaric acid in archaeological samples since they either lack the specificity or sensitivity needed for rigorous identification (Boulton and Heron 2000; Stern et al. 2008). Feigl tests can detect tartaric acid only in concentrations greater than 5–10 µg/mL (Feigl 1960, 390; Stern et al. 2008), and are therefore unsuitable for detecting the levels likely to be present in degraded archaeological samples. In addition, these types of tests can produce false positives in the presence of several other compounds of similar structure (e.g. lactic, glucolic, glucuronic, dihydroxytartaric, citric, and

malic acids; Feigl 1960, 389). This cautious interpretative approach is applied across other analytical applications, such as forensic analysis of narcotics where presumptive tests are used as only part of a set of minimum criteria for positive identification (e.g. United Nations Office on Drugs and Crime 2009). Infrared spectroscopy enables the analysis of bulk samples and identifies only the general type of chemical bonds that occur in the sample. It is therefore also not an appropriate method for the analysis of archaeological artefacts that usually involve complex molecular mixtures, often degraded and potentially contaminated (Boulton and Heron 2000). In particular, absorption bands from other organic products (for example resin or pitch used to seal the surfaces) may hide the tartaric acid bands (Stern et al. 2008). Without mass spectrometry (MS), the use of GC, HPLC or CE relies only on the retention/migration time to identify a compound and therefore lacks specificity. UV detection cannot provide precise enough information about the structure to allow identification of the compound.

To improve the sensitivity and the selectivity in the detection of tartaric acid, Guasch-Jané and co-authors (2004) proposed using liquid chromatography/mass spectrometry in tandem mass spectrometry mode (LC/MS/MS; Table 1). This method is highly reliable because it is based on the retention time, molecular mass, and on the MS fragmentation pattern. Furthermore, triple quadrupole instruments enable the use of multiple reaction monitoring (MRM) mode to improve detection, with a limit of detection (LOD) at 0.01 µg/mL (Guasch-Jané et al. 2004; Stern et al. 2008). To date, this method remains the best option, but GC–MS is also suitable for detecting tartaric acid, albeit with higher LODs (Table 1).

## 2.2. Interpretive issues: is tartaric acid a reliable biomarker of wine?

Given suitable methods for the extraction and detection of tartaric acid, as discussed above, a number of
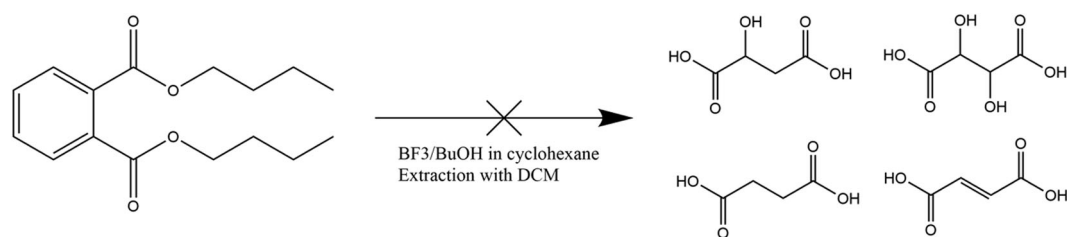
**Figure 3.** Absence of formation of 'wine markers' following application to phthalates of the acidic extraction method.

interpretative challenges still need to be considered before the wine can confidently be identified. First, as with many of the small organic acids, tartaric acid occurs in a large range of plants (e.g. Stafford 1959). Fortunately, in many edible fruits, tartaric acid is present at much lower concentrations (generally less than 100 mg/L; Singleton 1996) than in grapes. Consequently, such fruits are unlikely to release enough tartaric acid to be detected in pottery, especially after degradation. Some plants do have high levels of tartaric acid; these include tamarind (Rao and Mathew 2012; El-Siddig et al. 2006), the fruit of the Chinese hawthorn tree (McGovern et al. 2004), and some cultivars of pomegranate (Hasnaoui et al. 2011; Melgarejo, Salazar, and Artés 2000; Poyrazoğlu, Gökmen, and Artık 2002). Note, however, the concentration of tartaric acid in many modern edible plant species, especially wild plants, has not been measured. Secondly, unlike lipids which are generally targeted in organic residue analysis due to their relative insolubility in the burial matrix (Heron, Evershed, and Goad 1991), tartaric acid is highly susceptible to migration from the surrounding sediment to the artefact studied (Barnard et al. 2011). Therefore appropriate controls, such as the exterior surface of the sherd or the surrounding soil, should always be used when looking for such compounds (Koh and Betancourt 2010; McGovern et al. 2017, McGovern, Luley, et al., 2013). Phthalates derived from plastics used to conserve and store artefacts offer another potential source of tartaric acid contamination given the structure of the precursor molecules (Figure 3). Since phthalates are frequently identified in potsherds stored in plastic bags, it was important to verify that acid treatment of these compounds did not lead to the release of tartaric acid. Using the acid extraction method (Figure 3, details of materials and methods in Supplementary information), testing of a pure phthalate standard and plastic bags did not produce tartaric acid, although other sources should also be considered (glue, consolidants, etc.) in the future.

Due to its lack of specificity, the identification of grape juice/wine through the presence of tartaric acid needs to be carefully considered and supported by information on the archaeological context. Firstly, knowledge of the burial environment is important. The presence of tartaric acid in arid archaeological contexts such as Egypt (Guasch-Jané et al. 2006a, 2006b,

2004; McGovern, Mirzoian, and Hall 2009) would seem to be justified, but in other more humid contexts, tartaric acid is more likely to have been leached from the artefact (Singleton 1996).

Second, most studies typically report analysis of fewer than 15 vessels, usually where there is a prior belief that wine was a potential commodity, either from the context (e.g. Mediterranean amphorae) or associated botanical remains. Samples from regions or periods where wine is less likely to have been available are rarely analysed for the presence of tartaric acid. Here, there is a danger of circular reasoning leading to false-positive conclusions. To examine this last point, we tested a range of Russian prehistoric potsherds from the site of Zamostje 2 ($n = 6$, Table S4) located ca. 110 km north of Moscow (Bondetti et al., in press), using the acidic extraction and GC–MS approach (Garnier and Valamoti 2016 method). Although these vessels are similar in date to those from the Caucasus (sixth millennium BC) where early viniculture has been identified (McGovern et al. 2017), they are from a hunter-gatherer site in a region not suitable for wine cultivation and with no archaeological evidence for contact with regions implicated in early wine cultivation. Instead, previous lipid residue analysis of these samples has shown the presence of fish and plant biomarkers (Bondetti et al., in press) as well as carbonised remains of plants, such as *Viburnum* spp., present in charred surface deposits (foodcrusts). Surprisingly, tartaric acid was identified in all of these samples, but in variable quantities (Figure 4). Foodcrusts containing plant macro remains were particularly enriched in the compound (>5 µg/g of foodcrust). The results clearly point to a false positive for wine identification, most likely explained by the presence of tartaric acid in *Viburnum* fruits (Ersoy, Ercisli, and Gundogdu 2017; Özrenk et al. 2011). These results show that in the absence of other botanical or historical data, the presence of tartaric acid in significant quantities in archaeological samples cannot be taken as definitive evidence of the presence of wine, but only of fruits. Extensive testing of potential sources of tartaric acid must be carried out taking into account the historical context and environmental setting before a robust interpretation can be made. The presence of other acids (e.g. cinnamic, malic, citric, ascorbic, aconitic, and gluconic acids) cannot be used as corroborative
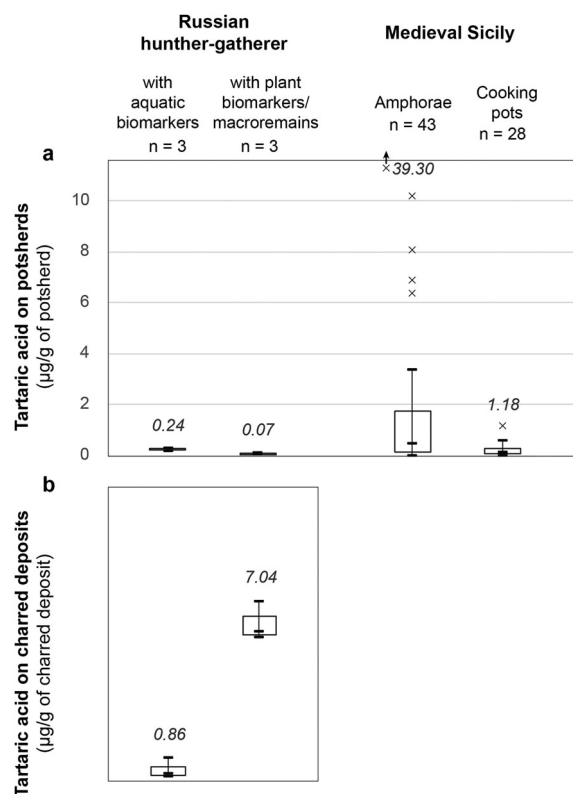
**Figure 4.** Box plot of the quantities of tartaric acid extracted from (a) sherds and (b) charred surface deposits from different archaeological contexts. ˣ indicates outliers (below or above 1.5 times the interquartile range). The maximum concentration (µg/g) is indicated in italics. Details of the materials and methods are available in Supplementary information (Table S4).

evidence for ancient wine because these compounds occur in a very wide range of plants (e.g. Table 2).

Comparative analyses of amphorae and cooking pots from early Medieval Sicily (Table S4) show that tartaric acid was identified in both sets of samples, although in different concentrations (Figure 4). The presence of tartaric acid was unexpected in cooking pots as these predominantly contained lipids from animal products. This result shows that when detected at low concentration, tartaric acid could be the result of contamination leading to the false-positive identification of wine/fruit. We suggest that a minimum limit of detection should be considered for more robust

interpretations of the presence of fruit. From our results, we propose a limit of >1 µg per g of ceramic, but ideally, this should be assessed with reference to suitable controls from the same contexts, such as sediments and forms that are unlikely to have come into contact with wine or grape juice (e.g. handles, ceramic tiles, exterior bases).

We also suggest that the extraction of tartaric acid should be undertaken routinely. Increasing the number of samples analysed would help verify the robustness of the method. For example, at the Iron Age site of Vix Mont-Lassois in Eastern France, tartaric acid was found to be only associated with specific vessel forms (Greek fine wares) despite the fact that a much larger assemblage was analysed, thereby increasing the confidence of the interpretation (Rageot et al. 2019). The acid extraction method is also very effective in extracting polar compounds strongly bound to the ceramic matrix so, if applied more widely, this approach could greatly enhance the detection of plant products in general (especially fruits) in the future.

## 3. Red or white: can wine pigments be extracted from archaeological vessels?

Wines are characterised by a large diversity of phenolic compounds: phenolic acids and derivatives and polyphenols (flavonoids, anthocyanins, and tannins) mainly derived from grapes, but also possibly from storage containers (e.g. oak barrels; Ribéreau-Gayon et al. 2006, 142–152). Ageing plays an important role in phenolic composition as polyphenols undergo a wide range of reactions, mostly polymerisations, which are still not fully characterised (Ribéreau-Gayon et al. 2006, 141). Malvidin (usually occurring as its glycoside, malvidin-3-glucoside, Figure 1(b)), is the main anthocyanin in red wine and is largely responsible for its red colour (Ribéreau-Gayon et al. 2006, 146). Directly after fermentation, 100s of mg/L of free malvidin are detected in wine, but this concentration decreases rapidly during ageing of wine, due to polymerisation processes (Ribéreau-Gayon et al. 2006, 147).

The polymerisation process improves the stability of the compounds and favours their preservation over

**Table 2.** Comparative study of modern fruit beverages extracted using the protocol of Garnier and Valamoti (2016).

| Modern reference | Molecular compounds (Bu derivatives) recovered from ceramic sherd (µg/g) | | | | |
| --- | --- | --- | --- | --- | --- |
| | Succinic acid | Fumaric acid | Malic acid | Tartaric acid | Syringic acid |
| White grape juice | – | 1.44 | 1.42 | 7.98 | – |
| Red grape juice | – | 2.26 | 1.97 | 2.73 | 0.01 |
| White wine | 0.44 | 59.84 | 38.64 | 55.88 | – |
| Red wine | 0.42 | 1.69 | 31.32 | 209.10 | 11.16 |
| Apple juice | 0.04 | 2.80 | 2.64 | – | – |
| Apple cider | 0.05 | 0.78 | 4.00 | – | – |
| Cherry juice | – | 0.40 | 0.33 | 0.02 | – |
| Pear juice | – | 0.91 | 0.84 | – | – |
| Hawthorn fruit (pressed) | 0.24 | 3.16 | 66.51 | – | – |
| Blueberry juice | – | – | 0.06 | – | – |
| Cranberry juice | – | 4.49 | 22.62 | – | – |

Note: Details of the materials and methods are available in Supplementary information (Table S1).

time in archaeological samples (Guasch-Jané et al. 2004; Singleton 1996). However, it also makes them difficult to extract from archaeological samples (Singleton 1996). To overcome this issue, the presence of tannins has been "detected" through spot/presumptive tests (Table 3; Petit-Domínguez, García-Giménez, and Rucandio 2003; Romanus et al. 2009) to measure the phenol content. However, such methods are not appropriate (Boulton and Heron 2000; Stern et al. 2008) as they are susceptible to false positives in the presence of monomeric phenolic compounds, such as ascorbic acid, gallic acid, vanillin (Singleton and Rossi 1965) that occur widely in the plant kingdom.

An alternative analysis for polyphenol relies on chemical cleavage and analysis of the resulting products. Garnier and co-authors (2003; Table 3) used a thermally assisted hydrolysis and methylation-gas chromatography–mass spectrometry method (THM–GC–MS), after *in-situ* treatment with tetramethylammonium hydroxide (TMAH). Pyrolytic markers released by this method, however, are difficult to trace to specific polyphenols and the pyrolysis approach can cause unwanted side reactions leading to analytical artefacts. Alkaline degradation (Table 3) was used as early as the 1970s (Condamin and Formenti 1978; Formenti, Hesnard, and Tchernia 1978) to break the polyphenol polymers and release various unspecific small acids. More recently, this method has been used to characterise syringic acid (Table 3), a small acid released from malvidin and malvidin polymers by a two-step cleavage of the C ring (Figure 1(c); Guasch-Jané et al. 2004; Singleton 1996). Similarly, an acid-catalysed reaction has been proposed for releasing syringic acid from malvidin polymers, using boron trifluoride solution in butanol and cyclohexane (Table 3; Garnier and Valamoti 2016). As with tartaric acid, the best methods for detecting syringic acid are GC–MS or LC/MS/MS in MRM mode (Table 3).

Syringic acid itself is not a biomarker for wine as it is found widely in natural products (e.g. in swiss chard, pumpkin, honey; Trifunovic et al. 2015; Rezig et al. 2012; Pyrzynska and Biesaga 2009), has been found in soils (Mukherjee et al. 2006) and can be released on the degradation of lignin, for example during wood combustion or tar production (Simoneit et al. 1993). Natural degradation of cork stoppers has also been suggested to be an alternative source of syringic acid in archaeological artefacts (Woodworth et al. 2015). Therefore, syringic acid can only be considered as a biomarker of (red) wine, if it can be shown to directly derive from malvidin or malvidin polymer precursors. The extraction method is therefore crucial as it is imperative that free syringic acid is removed from the sample before the treatment that cleaves malvidin. Methods to extract free syringic acid including solid phase extraction (SPE) and washing with DCM/MeOH have been applied to date (Table 3; Barnard

et al. 2011; Garnier and Valamoti 2016). However, there has been no assessment of the risk of false positives related to free syringic acid tightly bound to the artefact surface or released by molecules other than malvidin (e.g. lignins) when using these methods.

The effective release of syringic acid from malvidin using different approaches has also not been systematically assessed. Here we have examined the efficiency of acid and base treatment for releasing syringic acid from purified authentic malvidin, and red and white wines (Figure 2 and Table S3). Interestingly, the alkaline conditions favoured by some researchers (Guasch-Jané et al. 2004; Pecci, Giorgi, Salvini, et al. 2013) were shown to be much more effective than the acid protocol (Garnier and Valamoti 2016), the latter barely releasing any syringic acid from the standard or the wine samples analysed (Figure 2(b)).

Overall, the utility of syringic acid as a biomarker for red wine would seem to be limited. Concerns regarding the extraction methods and the specificity of the molecule in its free form need to be carefully considered. Even if appropriate measures and controls are deployed to rule out the presence of free syringic acid, it may still be released from a wide range of other natural substances potentially occurring in archaeological artefacts, including *Pistacia* fruits and leaves (Stern et al. 2008), cereals (Sun et al. 2002), and beer (Nardini and Ghiselli 2004), using acid or alkaline treatment. A final point is that the absence of syringic acid and presence of tartaric acid can never be used as evidence of white wine (Guasch-Jané et al. 2006a), given that the two molecules have different preservation pathways.

## 4. From grape juice and syrup to wine: is it possible to chemically identify ancient fermentation?

As tartaric acid occurs naturally in fresh grapes and other fruits, it cannot be considered as a marker for fermented fruit beverages. The detection of tartaric acid can be related to the presence of fruit juice or fruit syrup that were also consumed in the past (e.g. *defrutum* in Antiquity; Barnard et al. 2011). It has been suggested that malvidin could be a fermentation marker because it is released from the seeds and skin of grapes during the fermentation process (Garnier and Valamoti 2016). However, malvidin release begins at the maceration stages (Ribéreau-Gayon et al. 2006, 191–192), and is present in black grape juice, which excludes its use as a fermentation marker. Succinic, fumaric, pyruvic, citramalic (α-methylmalic), and lactic acids are produced during the fermentation of wine (Ribéreau-Gayon et al. 2006, 6–7) but cannot be used as markers of transformation *per se* because they are synthesised by several plants and fungi that occur naturally in soils (e.g. Bennet-Clark 1933 and Table 2).

**Table 3.** Overview of the extraction and detection methods for polyphenols, malvidin and syringic acid in archaeological artefacts published in the literature.

| Target biomarker | Sub-product released after treatment | Type of archaeological sample | Extraction method | Treatment to release sub-product | Detection method | Ref. |
|---|---|---|---|---|---|---|
| Tannins | – | Pottery sherds | Distilled $H_2O$ | – | Folin-Denis reagent | Petit-Domínguez, García-Giménez, and Rucandio (2003) |
| Tannins | – | Pottery sherds | MeOH/$H_2O$/acetic acid (4.5:4.5:1), 1 h, 70°C Drying and dissolution in water | – | Folin-Ciocalteu reagent | Romanus et al. (2009) |
| Tannins (of (+)-Catechin, (−)-Epicatechin, (−)-Epigallocatechin, (−)-Epicatechin-3-O-gallate, (−)-Epigallocatechin-3-O-gallate) | Benzenoid pyrolytic products | Original content (liquid part), archaeological grape seeds | – | Tetramethylammonium hydroxide (TMAH) treatment thermally assisted hydrolysis and methylation-gas chromatography-mass spectrometry (THM–GC–MS) | | Garnier et al. (2003) |
| Tannins | Gallic acid, parahydroxybenzoic acid, and 3,4 dihydroxybenzoic acid | Original content (solid part) | – | Alkaline treatment: NaOH, 300°C, 1 h | GC (and TLE) | Condamin and Formenti (1978) |
| Malvidin | Syringic acid | Coloured interior deposit | 0.1% formic acid in $H_2O$/MeOH (80:20, v/v) | 'Alkaline fusion': KOH pellets (0.2 g in 1 mL $H_2O$) heating (5 min). Acidification, extraction with ethyl acetate | LC/MS/MS (MRM mode, m/z 197 → 182 and m/z 197 → 123) | Guasch-Jané et al. (2004, 2006a, 2006b), Stern et al. (2008) |
| Malvidin | Syringic acid | Pottery sherds, plastered layers | Alkaline treatment: KOH 1 M, 70°C, 1 h 30. Acidification, extraction in ethyl acetate | | GC–MS | Pecci, Cau Ontiveros, and Garnier (2013), Pecci, Giorgi, Salvini, et al. (2013), Pecci et al. (2016, 2018), Woodworth et al. (2015) |
| Malvidin | Syringic acid | Pottery sherds | $H_2O$/MeOH/HCl (150/1.5/0.1, v/v/v, ∼6.7 mM HCl). Extraction on SPE cartridge, elution using ACN/2-propanol/$H_2O$ (25/25/50, v/v/v) | Alkaline treatment: KOH pellets (12 in 3 mL $H_2O$) 50°C, 5 min. Acidification, extraction with ethyl acetate | LC/MS/MS (MRM mode, m/z 197 → 182 and m/z 197 → 123) | Barnard et al. (2011) |
| Malvidin | Syringic acid | Pottery sherds | Pre-treatment: DCM:MeOH (1:1, v/v), ultrasonication (*discarded*). Acidic treatment: boron trifluoride in butanol and cyclohexane (1:2 v/v), 80°C, 2 h. Neutralisation, extraction with DCM | | GC–MS | Garnier and Valamoti (2016) |
| Syringic acid | – | Archaeological resins found within amphorae | DCM:MeOH (2:1, v/v), ultrasonication | – | GC–MS | Stern et al. (2008) |
| Syringic acid | – | Pottery sherds | MeOH or MeOH/$H_2O$/acetic acid (4.5:4.5:1), 70°C, 1 h | – | GC–MS | Romanus et al. (2009) |
| Syringic acid | – | Pottery sherds | DCM, heating EtOH, heating | – | GC–MS | Koh and Betancourt (2010), Koh, Yasur-Landau, and Cline (2014) |

When detected together with tartaric acid, they can, however, be considered as additional compounds possibly originating from a fruit-based fermented beverage. In the absence of tartaric acid, they provide no chemotaxonomic information.

## 5. Can DNA be a more reliable solution?

Genetic testing of archaeological remains represents one of the biggest developments in archaeological science in the past 35 years (Der Sarkissian et al. 2015). While ancient DNA (aDNA) researchers have frequently focused on human skeletal remains (Slatkin and Racimo 2016), the same methodologies have been used to investigate numerous species and substrates, including a few projects that targeted wine-related DNA on archaeological ceramics. As these studies precede the era of "palaeogenomics"— the current approach wherein full genomes can be characterised using high-throughput sequencing – it is important to re-evaluate these findings based on our growing knowledge of DNA survival and degradation (Kistler et al. 2017).

In 2003, Cavalieri et al. (2003) reported the identification of yeast (*Saccharomyces cerevisiae*) DNA from a putative wine jar from the ancient Egyptian tomb U-j of King Scorpion I. As the primary driver of fermentation, *S. cerevisiae* holds a key position in viniculture and exploring its genetic history may lead to new insights into the origins of winemaking. With this in mind, Cavalieri et al. extracted DNA from a yellowish residue in the 5100-year-old jar, which they interpret as lees (dead yeast and fermentation debris). The researchers used the polymerase chain reaction (PCR) to amplify a segment of DNA that encodes ribosomal RNA in fungi, and the residue yielded several DNA sequences between 540 and 840 basepairs (bp) in length. The closest match in a reference database for the longest sequence was *S. cerevisiae* strain 288C, with only four nucleotide differences. The researchers interpret the results to indicate that the ancient Egyptians fermented wine with a *S. cerevisiae* strain which was very closely related to modern yeast.

Based on current knowledge of DNA survival, it is rare for archaeological specimens to yield DNA >300 bp, especially from hot environments like Egypt. Using the jar's age and burial location, degradation modelling software (Smith et al. 2003) calculates that all DNA should have been destroyed by depurination ($\lambda = 0.3011$). Thus, it is likely that the amplified yeast DNA was a recent contaminant. Even though the research team followed strict protocols to avoid contamination, trace amounts of modern yeasts could have entered the jar during excavation, storage, or laboratory processing. Recent experiments have also shown that laboratory reagents frequently contain trace amounts of microbial DNA (Salter et al. 2014),

and *S. cerevisiae* contamination has been reported in several instances (e.g. Loeffler et al. 1999). Given that *S. cerevisiae* strain 288C is a common laboratory organism (Mortimer and Johnston 1986) the central conclusion of ancient yeast DNA does not stand up to scrutiny.

In addition to the search for wine-related microbes, two projects have used PCR to target short genetic barcodes (<100 bp) in the chloroplast genome of species likely to have been stored in amphorae. In 2008, Hansson and Foley (2008) tested two 2400-year-old Greek amphorae, finding genetic signatures of olives and oregano in the first vessel and mastic (*Pistacia*) resin in the second. The authors interpret the first amphora as holding olive oil, and the second as a wine container, as the resin is traditionally used as a preservative for wine. In an expanded study of nine amphorae dredged from the sea floor, Foley et al. (2012) again found genetic evidence for olive and *Pistacia*, but also grapevine, thus supporting written accounts that some amphorae were used for transporting wine in antiquity. The researchers also found evidence for many other plant taxa including juniper, pine, legumes (Fabaceae), Zingiberaceae (ginger family), and Juglandaceae (walnut family). Based on these findings, the researchers argue that ancient amphorae were used to transport a much wider assortment of products than is often assumed.

While these researchers followed aDNA methodologies standards for the time, based on our improved knowledge of DNA degradation, it is difficult to surmise how much of the amphora genetic signal originates from ancient contents. Anaerobic underwater environments have been identified as encouraging DNA survival (Kistler et al. 2015), but the amphorae were recovered between one to 20 years before DNA testing and were stored in a museum in Greece (i.e. in the same region where many of the observed species grow today), leading to potential exposure to contaminants. A core concern in these studies is the reliance on PCR, a method that amplifies low levels of DNA and so suffers from being extremely sensitive to trace levels of modern contamination. PCR has been likened to finding a needle in a haystack and it played an important role in the development of aDNA research, but it is also to blame for a series of erroneous studies of fossilised materials (Cooper and Poinar 2000). Hansson, Foley, and their colleagues did an admirable job of testing for modern contamination by trying to amplify relatively long DNA fragments, but it is still plausible that modern DNA was inadvertently amplified and there is no clear way to authenticate the results with other data.

So far, three publications have reported wine-related DNA in amphorae, but each study has shortcomings that lead us to question some findings. In each case, the researchers only used PCR amplification, which

while being an indispensable method in molecular biology experiments, has the unfortunate ability to readily recover trace amounts of modern DNA contamination. Even with many controls, these studies have produced a limited amount of genetic data that could in part (or in whole) originate from recent sources, thus preventing us from confidently inferring the ancient contents of the vessels.

## 6. Conclusions and future work

Through a review of the literature and some validation experiments, we provide the following conclusions related to prospects for identifying wine associated with archaeological artefacts.

- Ideally, the analysis should be undertaken on a substantial number of vessels, including vessels not typically associated with wine, to give confidence in the approach.
- Tartaric acid can be reliably extracted from archaeological artefacts but only using the method of Garnier and Valamoti (2016).
- Interpretation of the presence of tartaric acid has to also consider the wider historical and archaeological context and be supported by archaeobotanical evidence (e.g. Garnier and Valamoti 2016) as it may be derived from other types of fruits.
- Small amounts of tartaric acid are present in soils and have been detected in artefacts that are not typically associated with wine. Therefore, it is recommended that suitable negative controls (soil or other types of archaeological artefacts that should not hold wine) are always analysed in tandem with the archaeological artefact and that quantification is always carried out.
- Syringic acid can be reliably extracted from archaeological artefacts but only using alkaline treatment (Guasch-Jané et al. 2004; Pecci, Giorgi, Salvini, et al. 2013), and after removing free syringic acid.
- It remains doubtful that syringic acid can be reliably traced to malvidin derived from wine.

With the current state of knowledge, it is not possible to identify wine production using only biomolecular approaches. More research into finding new and better molecular proxies for the identification of wine residues is necessary to allow more conclusive interpretations, perhaps with identifications relying on a suite of biomarkers. This can potentially be achieved through a multi-instrumental approach which would increase the range of potentially informative molecules that can be identified.

- A metabolomic approach has been recently tested on wine from the Modern period, with interesting potential (Roullier-Gall et al. 2017), but this should

now be tested on older ceramics of unknown content.
- Polyphenols have real potential as biomarkers because of their resistance to degradation, but their ubiquitous nature and rapid polymerisation are challenging. Proteomics and amino acid preservation in ancient wine could be another possibility that is yet to be explored (Roullier-Gall et al. 2017).
- DNA could offer the potential to identify wine associated with artefacts but requires new approaches deploying high-throughput sequencing and experimental work to determine the limits of survival in different conditions. High-throughput DNA sequencing approaches can provide an unbiased picture of the range of taxa present and reveal tell-tale signs of DNA damage (Llamas et al. 2017).

## Disclosure statement

No potential conflict of interest was reported by the author (s).

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