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Protocol Comparison for Organic Residue Analyses from Waterproofing Materials and Shards of Roman Archaeological Amphorae

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Abstract: With the aim of addressing the impact of extractive protocols in molecular characterization of ceramic content, sixteen archaeological shards and waterproofing coatings of Roman amphorae were studied to compare the extractive capacities of protocols prevalently mentioned in wine amphorae analysis. A microwave-assisted protocol is developed in order to esterify grape-derivative markers from archaeological pitch and shard. Gas chromatography-mass spectrometry is used to highlight the great capacities of a two-step protocol that combines organic extraction with BF_3-etherate complex butylation applied on archaeological shards. Instead, simultaneous alkaline fusion and direct-resin acid-catalyzed butylation are favored for the characterization of waterproofing material. The identification of tartaric acid, together with succinic, fumaric pyruvic and syringic acids provide valuable insights on the archaeological grape-derivative content, possibly wine. Diterpenic markers highlighted *Pinus* pitch and wood tar, originally used to waterproof the amphorae. Since markers are reliable tools in organic residue analyses, protocols exhibiting high extractive capacities are favored to avoid false conclusions drawn through the absence of markers.

Keywords: organic residue analyses; biomolecular archaeology; tartaric acid; microwave-assisted butylation; gas chromatography-mass spectrometry; ceramic content; wine

1. Introduction

In the early 1990s, Evershed introduced the archaeological biomarker concept to trace back the original use of potteries. Focusing on either the carbon structures or the pattern distributions, molecules act as chemical fingerprints [1]. They can reveal information regarding the initial composition, natural ageing, anthropic degradation as well as contamination. This way, organic residue analyses became an established field of research; fundamental to address the archaeological content by rending microremains identifiable [2]. For instance, the earliest consumption of wine could be dated back to the Neolithic in the South Caucasus through molecular markers [3].

Although pioneering studies laid the foundation using Feigl spots, infrared spectroscopy or HPLC to identify tartaric acid [4–6], the emergence of chromatographic tools offered great substitutes to traditional methods to prevent from false-positives [7–9]. As

rightly criticized in literature, neither molecular identification through sole retention or migration time, nor the precision of UV detection are reliable [10,11]. Enhancing the specificity and sensitivity required for wine markers identification as well as providing structural information, gas chromatography coupled with mass spectrometry (GC-MS) became frequently employed [12–15]. Although recent techniques on the cutting edge of technology showed great analytical advancements, equipment costs remain highly limiting. Even though tandem MS mode or selected ion monitoring mode in liquid or gas chromatography coupled with MS were published to grandly improve the limit of detection to specifically target the presence of archaeological wines [3,16–19], their usage remains rare since it implies ultra-advanced equipment. GC-MS consequently appears as a good compromise to turn routine analyses into efficient molecular markers searches, decisive for their identification. This way, fermented grape-beverage has been evidenced in archaeological ceramic jars and amphorae through the presence of tartaric acid [6,20]. Although the molecule is not exclusively produced by grapes, its concentration remains higher than in other exotic plant sources such as tamarind, yellow plums [18] or pomegranates [21]. Moreover, it better survives upon archaeological time compared to other grape acids [9]. For this reason, it is usually considered as a grape biomarker when supported by archaeological contexts and/or archaeobotanical grape evidence [3,11,12,22]. Although synthesized by numerous plants and fungi, pyruvic, fumaric and malic acids are additionally produced during the fermentation process [23]. Syringic acid highlights red wine since it arises from malvidin, the pigment responsible for dark grape coloration [16,18].

Beyond the presence of markers, two concepts need however to be separated: the extraction and the analytical detection. While analytical developments focused on the former, the latter remains of a first interest. Indeed, robust protocols must be employed to increase the chance of extracting tartaric acid and other grape beverage markers from the ceramic matrix. Since the biomarker concept relies on the presence/absence of characteristic features, it becomes necessary to avoid false-negative induced by extraction strength flaw [24]. Among the protocols most mentioned to promote the breaking of the intramolecular hydrogen bonding between tartrate salts and the silicate-rich ceramic, either acid or alkaline conditions are favored [9,12,25]. Alkaline treatment is followed by acidification to make tartaric acid more soluble in ethyl acetate before extraction [13,26]. Differently, the acido-catalyzed butylation of the unsolved material after polar extraction greatly evidenced grape derivatives in Neolithic, Etruscan or even Early Medieval Islamic jars [12,27,28]. The esterification of organic markers aimed at enhancing their solubility into the extractive solvent.

The primary aim of this paper is to describe an adapted microwave-assisted protocol for the acid-catalyzed extraction in order to ensure easy-going and accelerated approach for grape-derivative detection, applicable on both organic and inorganic artefacts. Using GC-MS, we additionally provide a comparative study of the extractive capacities offered by alkaline, acidic and polar extractions with the specific aim of proposing a robust and cost-effective methodology directly applicable on archaeological artefacts (waterproofing material and shard). In this study, three protocols were conducted depending on the nature of the samples. The first one consisted of a basic extraction applied on both pitch and shard. The second one encompassed a two-step protocol with consecutive lipid extraction, including microwave-assisted optimization for the butylation. This protocol was tested on pitch and shard. The last protocol corresponded to a variant of the second one, with the application of the two-step extraction handled separately (i.e., not consecutively but in two different ways) and both steps directly conducted on the waterproofing matter. This study was carried on sixteen Roman amphorae coming from two different maritime archaeological sites. A total of ten shards and eight resinous coatings were investigated in order to promote a great representativeness of the comparison and prevent from any bias that would arise from local or material specificities. A glossary of all of the identified compounds was presented at the end of the article.

2. Materials

2.1. Archaeological Samples

This comparative study integrated 16 archaeological amphorae coming from two different contexts. A total of 11 amphorae were excavated from the shipwreck of Planier 3 (France) and 5 came from the ancient anchorage of San Felice Circeo (Italy). The amphorae were selected to increase the variety between objects in order to make the protocol comparison the more likely to be applied on any further artefacts. Thus, the objects present a strong variability in terms of ceramic pastes, provenance and marine archaeological context, visual presence of coating and conservative conditions (Table 1). Although they all belong to the Roman period, they came from maritime context that can be interpreted differently. The amphorae from Planier 3 were all excavated from the same shipwreck, sunk in 49 BC near the Planier's island (43°11′54″ N; 5°13′48″ E) close to the Marseille coasts (France). Coming from the region of Brindes, the cargo might have stopped first in the Sinus Tarentinus region to load Lamboglia 2 amphorae and then at Pozzuoli before heading towards the Narbonnaise. Excavation campaigns conducted from 1968 to 1975 revealed the important distribution of Dressel 1B, Lamboglia 2, ovoid and Brindisium amphorae in the cargo [29]. Along with the tremendous quantity of amphorae, Planier 3 exhibits the more important evidence of archaeological pouzzolane stoppers. Different mineral pigments were also reported in the cargo, such as realgar, white lead and the precious Egyptian blue of which the production was settled in Pozzuoli [30]. Such discoveries outlined the luxurious assumptions of traded products transported on the ship.

Table 1. Archaeological amphorae investigated.

Amphora	Archaeological Site	Typology	Coating	Shard	Grape Derivatives	Pinaceae Products
1014	Planier 3	Dressel 1	X	X	Fermented	Wood tar
749	Planier 3	Lamboglia 2	X	X	Fermented	Wood tar
6570a	Planier 3	Dressel 1	X		Fermented	Wood tar
SFC1	San Felice Circeo	Dressel 1	X		Fermented	Wood tar
SFC2	San Felice Circeo	Dressel 1	X		Fermented	Wood tar
SFC3	San Felice Circeo	Mañà C2	X		Fermented	Wood tar
SFC4	San Felice Circeo	Greek-Italian	X		Fermented	Wood tar
SFC5	San Felice Circeo	Lamboglia 2	X		Fermented	Wood tar
6904	Planier 3	Chios amphora		X	Fermented	Pitch
6828a	Planier 3	Lamboglia 2		X	Fermented	Wood tar
6828a	Planier 3	Lamboglia 2		X	Fermented	Wood tar
6565	Planier 3	Lamboglia 2		X	Fermented	Wood tar
6793	Planier 3	Lamboglia 2		X	Fermented	Wood tar
6545	Planier 3	Dressel 5		X	Fermented	Wood tar
6566	Planier 3	Lamboglia 2		X	Fermented	Wood tar
6828c	Planier 3	Lamboglia 2		X	Fermented	Wood tar

Five amphorae were excavated in the ancient anchorage of San Felice Circeo (41°13′49.0″ N; 13°06′30.1″ E) located 90 km SE of Roma. They were uncovered due to the winter storm of 2018, along with an important scattering of archaeological records located few hundred meters from the coast at a depth of 5 to 7 m under the sea level. The Soprintendenza (the local Office of the Italian Ministry of Culture) supervises ongoing excavation campaigns. The time scale established by the archaeological finds ranges from the Republican period through the Late Roman period to the post-medieval period. The diversity of the excavated ceramics, in terms of morphology and the time period they belong to provided valuable insights of ancient anchorage (Delpino and Melandri, unpublished paper) although the hypothesis of a shipwreck cannot be excluded.

The Late Greco-Italian/Dressel 1 typologies refer to 150 to 10 BC. They originate from south-central Italy, from Campania to Etruria, as attested by important kiln finds along the coastal area [31]. These amphorae were widely used to trade wine in the Mediterranean Basin, from Central Europe to Spain via Gaul. Lamboglia 2 amphora originate from the

Adriatic coast [32,33]. They have largely contributed to the trade of wine in the western Mediterranean [34]. Chios amphora were surely meant for wine trading since the island was prized for the great quality of wine they produced [35]. Maña C2 amphora arose from the Punic tradition amphorae, originating from North African coasts. Although oil and fish preserves are frequently mentioned in such typology, the amphora was included in the study [36]. Dressel 5, also referenced as Rhodian amphorae, usually contained wine from the Aegean coast [37]. The present study focuses on 8 samples from waterproofing coatings and 10 samples of ceramic shards (see Table 1). Sample Nos. 749 and 1014 were analyzed with both the coating and the ceramic clay.

2.2. Solvents and Reagents

All of the organic solvents were of analytical grade. Methanol (MeOH), dichloromethane (DCM), diethyl ether (DEE), ethyl acetate and KOH were purchased by Merck (Darmstadt, Germany). Hexane and N,*O*-Bis(trimethylsilyl)trifluoroacetamide/trimethylchlorosilane (BSTFA/TMCS) and commercial standard molecules such as maleic, succinic, fumaric, malic, pyruvic, tartaric, syringic and dehydroabietic (DHA) acids were supplied from Sigma-Aldrich (Darmstadt, Germany). Anhydrous butanol and cyclohexane were purchased by Acros Organics (Illkirch France) and BF_3 diethyl etherate from Alfa Aesar (Kandel, Germany). The fresh colophony resin standard was purchased by Kremer Pigmente GmbH & Co. KG (Aichstetten, Germany).

3. Methods

3.1. Optimization of the Acid-Catalyzed Esterification: Microwave-Assisted-Butylation

The protocol aiming at butylating wine acid markers was adapted from Garnier and Valamoti [12]. It was firstly developed for tartaric acid before being extended to other standard molecules of maleic, succinic, fumaric, pyruvic, malic, syringic acids. A total of 5 mg of commercial standard were treated with a mixture of boron trifluoride, butan-1-ol and cyclohexane (1:2:4 $v/v/v$) in a sealed vial placed in CEM Discover® LabMate microwave synthesizer (MW) (CEM Corp., Orsay, France). The instrument was used in single-mode (50 Hz; 300 W maximum output power). An infrared sensor positioned below the circular vessel continuously measure the routine temperature. The self-adaptive circular waveguide technique allows the circular cavity to automatically be adjusted in order to optimize the energy provided for the reaction [38]. Continuous pressure measurements permit on-the-fly changes for power control to maintain a maximal temperature, set at 80 °C with the CEM Synergy™ software (software version 0.9, Kamp-Lintfort, Germany). Different reaction times were tested (single dynamic cycle of 5 min, 10 min and three successive dynamic cycles of 5 min each) to evaluate the heating run necessary for the butylation with high stirring speed. The butylation advancement was followed by thin-layer chromatography with cyclohexane:ethyl acetate (1:1 v/v). After the butylation was completed, the solution was neutralized with a saturated solution of sodium carbonate. The esterified compounds were extracted two times with DEE. The combined organic fractions were washed twice with Milli-Q water and dried with anhydrous sodium sulphate before filtration on a PTFE cartridge (0.45 µm).

3.2. Analytical Procedures for Inorganic Shards

For the comparative analyses of the archaeological shards, two protocols were applied: a basic extraction adapted from Pecci et al. [9] and a two-step lipid extraction including the MW-assisted optimization for the butylation. To reduce the risk of external contamination, a thin layer was initially removed from the inner surface of the shard before sampling the drilled ceramic over 1–2 mm in depth.

On the one hand, 100 mg of crushed shard were extracted three times with KOH (1 M; 2 mL) using an ultrasound probe (VCX 130 Vibra-Cell Sonics, Sonics and materials, Newtown, CT, USA) for 3 min. After centrifugation, the successive extracts were combined

and acidified up to pH 2. The organic phase was extracted 3 times with ethyl acetate (3 mL), filtered on a PTFE cartridge (0.45 µm) and evaporated to dryness.

On the other hand, 100 mg of crushed shard were extracted 3 times with DCM:MeOH (1:1 v/v) with the ultrasound probe for 3 min. After centrifugation, the organic supernatant of was filtered with PTFE (0.45 µm) and evaporated to dryness. This first step of the lipid extraction was recorded as 1LE. The remaining powder after the organic extraction was treated for the MW-assisted butylation with a mixture of BF_3 etherate complex, butan-1-ol and cyclohexane (1:2:4 $v/v/v$) for 3 times 5 min. After neutralization with a saturated sodium carbonate solution, the organic fractions were extracted with DEE and washed twice with H_2O before drying over sodium sulphate. The corresponding extract was labelled 2LE-MW. After filtration on a PTFE cartridge (0.45 µm), both extracts (1LE and 2LE-MW) were individually evaporated under a gentle N_2 stream.

3.3. Analytical Procedures for Organic Coatings

For the comparative analyses of the archaeological coatings, three protocols were tested. The first one was the basic extraction already reported (see Section 3.2). The second was the adapted two-step lipid extraction with MW optimization for the butylation as previously described (see Section 3.2). The only difference was the starting amount of 50 mg of organic coating versus 100 mg of shard. The third protocol consisted of the same two-step lipid extraction, with each step independently conducted on the coating: the organic extraction with DCM:MeOH (1LE) and the extraction after butylation (R-2LE-MW) were hence handled separately. A total of 10 mg of coating material were necessary for the direct-pitch esterification. Butylation was performed as previously described for the MW parameter and heating runs.

3.4. GC-MS Analyses

GC-MS analyses were carried out on a Thermo Scientific™ Focus system equipped with an Al 3000 autosampler and an ITQ™ 700 Series Ion Trap Mass Spectrometer (ThermoFisher Scientific, Illkirch-Graffenstaden, France). A ThermoGOLD™ TG-5MS fused silica capillary column (5% diphenyl; 95% dimethyl polysiloxane) of 30 m length × 0.25 mm i.d. × 0.25 µm thickness ensured the separation of the mixture carried with helium at a constant flow rate of 1 mL min^{-1}. A total of 1 µL solution was injected in splitless mode at 250 °C. Transfer line, ion trap and manifold temperatures were respectively 300 °C, 200 °C and 50 °C. Mass spectra were recorded in electron impact mode with an electron ionization energy of 70 eV, with ionization time of 25,000 µs. Scan are recorded in the range of 40–650 m/z. The oven temperature stayed isothermal for 2 min at 50 °C, increased at 8 °C/min to 140 °C held for 2 min before heating to 160 °C at 2.5 °C/min and finally 330 °C at 15 °C/min and held for 3 min.

After evaporation, all of the extracts were derivatized with BSTFA (200 µL, 70 °C, 30 min) before injection in splitless mode in GC-MS in 200 µL of hexane:DCM (1:1 v/v).

Data treatment was performed on Xcalibur™ software (software version 4.3, Thermo Scientific, Waltham, MA, USA). Peak identification was achieved by comparison of retention time and mass spectra with molecular commercial standards and from the NIST MS Search 2.0 database (last access in May 2021).

3.5. Radar Plot Construction

The building of the radar plots (Figures 1 and 2) relies on the presence/absence of 22 targeted molecules, that account for the characterization of the grape derivatives content and the resinous coating. Among them, 7 organic acids referred to grape composition (tartaric and syringic acids), fermentation (maleic, succinic, pyruvic, fumaric, malic acids) and 15 diterpenic derivatives indicative of the nature of the coating (dehydroabietic acid DHA, dehydroabietic methyl ester DHAM and retene) and its ageing (3-hydroxy-, 7-hyrdoxy-, 15-hydroxy-; 7,15-dihydroxy-, 7-oxo- and 15-hydroxy-7-oxo-DHA and DHAM-derivatives).

We notably emphasized on highly significant markers, indispensable for grape derivative and coating identification (i.e., tartaric acid, retene and DHAM compounds).

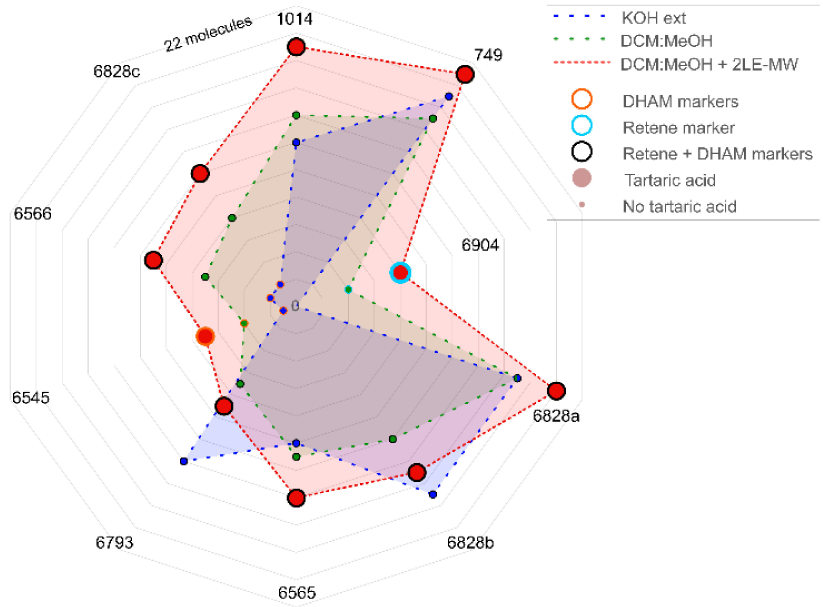

Figure 1. Radar plot of the shards.

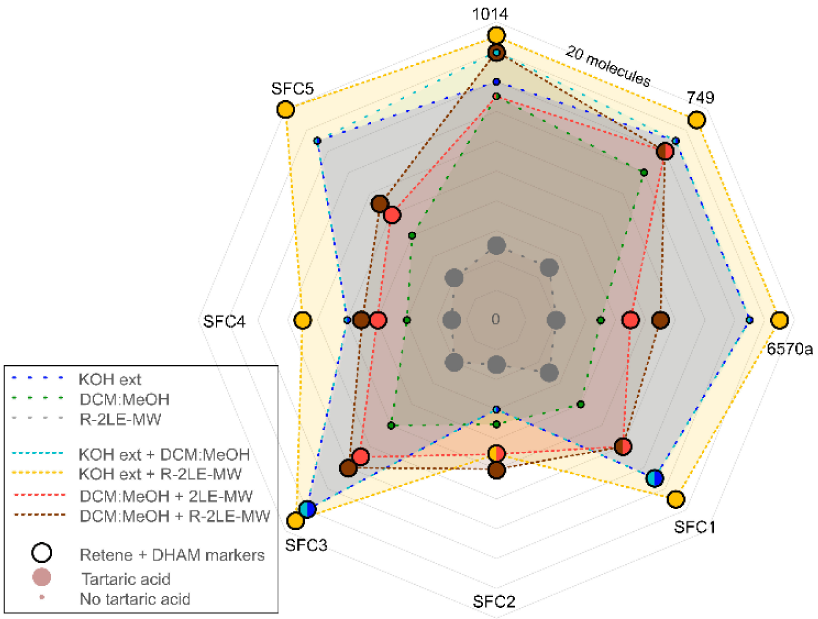

Figure 2. Radar plot of the coating materials.

4. Results and Discussion

4.1. Optimization of the Acid-Catalyzed Butylation

The esterification advancement, followed by thin-layer chromatography, ensured the effective butylation of standard molecules after three dynamic cycles of 5 min each. Butylated compounds were additionally controlled with infrared spectroscopy (FT-IR) and NMR (^{1}H, ^{13}C). GC-MS analyses allowing dibutyl tartrate (DBT) to be characterized with fragment ions at m/z 276, 305 and 391. The same butylation protocol was then applied on commercial standards acids considered as dark grape (i.e., syringic acid) and fermentation markers (i.e., maleic, succinic, pyruvic, fumaric and malic acids) to obtain their retention time and fragmentation patterns.

4.2. Extracting Capacities Comparison on Archaeological Shards

Archaeological artefacts being different from each other, the studied shards cannot be generalized to a set of amphorae. Protocol comparisons and analyses interpretations must respect individual molecular specificities, turning conclusions on the extraction capacities to be singular and object dependent. For this reason, the use of a radar plot was favored to independently outline the number of molecules extracted by each protocol and for each of the ten archaeological shards (Figure 1). The protocol comparison applied on shards encompassed: (i) an alkaline fusion with KOH extraction, (ii) a DCM-MeOH organic extraction and (iii) its coupling with BF_3-catalyzed MW-butylation applied on the dried remaining powder after the organic extraction (2LE-MW extract).

For all the 10 shards, only the BF_3-catalyzed butylation allowed DBT, highlighting the presence of tartaric acid (Figure 1). Partially dissolving the ceramic clay, BF_3, optimized the release of the organic compounds strongly bonded, or even polymerized [25,39]. Increasing the apolar character of the esterified acids, butylation favored their rapid extraction in cyclohexane, hence favoring the butylation of remaining acids by shifting the equilibrium [12]. From there, the extraction from the co-solvent is enhanced with DEE that has a low dielectric constant solvent.

Even though hydroxyl anions arising from the alkaline fusion are supposed to interact with the ceramic matrix to enhance the release of bonded acids [9,13,26], no tartaric acid could be identified with this protocol (Figure 1). By shifting the solubility equilibrium of tartrate salts, KOH should favor the bonding cleavage with the ceramic and leave the marker soluble in the aqueous phase [9,16]. In fact, tartaric acid resists archaeological time thanks to the formation of salts that strongly interact with the clay matrix [6]. Conversion into free tartaric acid would ensure the recovery in the aqueous phase before extraction with ethyl acetate. Extraction issues could originate from the insufficient alkaline robustness or from the poor solubility of tartaric acid in ethyl acetate [11,16]. The hypothesis of tartrate salt that would be formed once the alkaline fusion released tartaric acid from the ceramic cannot be ruled out [11]. It is worth noting the limit of detection involved in every protocols. Starting from pure standard, quantitative analysis comparing the amount of tartaric acid recovered after extraction reported to identify 77% of the acid with butylation while it did not reach 0.1% with alkaline fusion [11]. Additionally, Garnier and Valamoti reported the detection of tartaric acid up to 10 ng/g shard with the acido-catalyzed protocol [12]. In conclusion, neither KOH fusion, nor the organic extraction with DCM-MeOH were suitable for the characterization of grape derivatives.

Aside from the considerable extraction of DBT, esterification also accounted for the extraction of grape acids (Table 2). Maleic acid was only characterized with butylation (m/z 99; 117). Malic acid, although hardly characterized with KOH fusion, was always identified as dibutyl malate (m/z 101; 145; 161; 303). The most important increase of molecules extracted was observed for the amphora No. 6904, where three fermentation acids (over the five considered) could be identified with butylation, hitherto absent with alkaline fusion and DCM:MeOH extraction. Surprisingly, pyruvic acid was directly extracted with traditional solvents in nine shards. Neither KOH nor butylation reached such great extent (Table 2) and the molecule that originates from malolactic fermentation [23] was only identified in two shards with alkaline fusion and never recovered as dibutylcetal. To the contrary, fumaric acid which is considered as marker of alcoholic fermentation [39], was preferentially extracted with alkaline fusion and never identified with butylation (Table 2). Since maleic, succinic, pyruvic, fumaric and malic acids can originate from the fermentation of grapes, they are considered as fermentation markers of wine. However, to compensate for their lack of exclusivity towards grape fermentation only, the greater the number of fermentation markers extracted, the more reliable the fermentation assumption. For this reason, the combination of extractive protocols would allow the number of molecules extracted to be increased. Since only the butylation proved to surely trace tartaric acid, the most fruitful coupling would include it to widen its extractive capacities.

Table 2. Molecules identified in shards. Presence (+) and absence (-) of molecular markers under alkaline fusion (KOH ext.), organic extraction with DCM:MeOH, butylation applied on the remaining fraction (2LE-MW). The number of '+' refers to the number of molecules present. ac.: acid; OH-DHA: hydroxy-DHA (i.e., 3-hydroxy-DHA; 7-hydroxy-DHA and 15-hydroxy-DHA); Oxo-DHA: 7-oxo-DHA; DiOH-DHA: 7,15-dihydroxy-DHA; Oxo-OH-DHA: 7-oxo-15-hydroxy-DHA. DHAM and oxidized derivatives (OH-DHAM, Oxo-DHAM, DiOH-DHAM and Oxo-OH-DHAM) refer to the same skeleton with methyl ester derivatives instead of the carboxylic acid function.

Amphora	Protocol	Maleic ac.	Succinic ac.	Pyruvic ac.	Fumaric ac.	Malic ac.	Tartaric ac.	Syringic ac.	Retene	DHA	OH-DHA	Oxo-DHA	DiOH-DHA	Oxo-OH-DHA	DHAM	OH-DHAM	Oxo-DHAM	DiOH-DHAM	Oxo-OH-DHAM
1014	KOH ext	-	+	-	+	-	-	+	+	+	+++	+	-	-	+	+	-	-	+
	DCM:MeOH	-	+	+	-	-	-	-	+	+	+++	+	+	-	+	++	+	+	-
	2LE-MW	+	+	-	+	+	+	+											
749	KOH ext	-	+	-	+	+	-	+	+	+	+++	+	+	+	+	+++	+	+	+
	DCM:MeOH	-	+	+	-	-	-	-	+	+	+++	+	+	+	+	+++	+	+	+
	2LE-MW	+	+	-	-	+	+	+											
6904	KOH ext	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	DCM:MeOH	-	+	+	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-
	2LE-MW	+	+	-	+	+	+	-											
6828A	KOH ext	-	+	-	+	+	-	+	+	+	+++	+	+	+	+	++	-	+	+
	DCM:MeOH	-	+	+	+	-	-	-	+	+	+++	-	+	+	+	+++	+	+	+
	2LE-MW	-	+	-	-	+	+	+											
6828B	KOH ext	-	+	+	+	+	-	+	+	+	+++	+	+	+	+	+	-	+	+
	DCM:MeOH	-	+	+	-	-	-	+	+	+	++	-	-	-	+	+++	+	-	-
	2LE-MW	+	+	-	-	+	+	+											
6565	KOH ext	-	+	+	-	+	-	+	+	+	-	-	-	-	+	+	+	-	+
	DCM:MeOH	-	+	+	-	-	-	-	+	+	++	-	-	-	+	++	+	-	+
	2LE-MW	-	+	-	-	+	+	+											
6793	KOH ext	-	+	-	-	-	-	+	+	+	+++	-	+	-	+	++	+	+	+
	DCM:MeOH	-	+	+	-	-	-	+	+	+	+	-	-	-	+	-	-	-	-
	2LE-MW	-	+	-	-	+	+	+											
6545	KOH ext	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
	DCM:MeOH	-	+	-	-	-	-	-	-	-	-	-	+	-	+	-	-	+	-
	2LE-MW	-	+	-	-	+	+	+											
6566	KOH ext	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-
	DCM:MeOH	-	-	+	-	-	-	-	+	+	-	-	+	-	+	+	-	+	-
	2LE-MW	-	+	-	-	+	+	+											
6828C	KOH ext	-	-	-	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-
	DCM:MeOH	-	-	+	-	-	-	-	+	+	-	-	+	-	+	+	+	+	-
	2LE-MW	-	+	-	-	+	+	+											

Although syringic acid is naturally present in many plants and in wood lignin [13,18], malvidin origin has been clearly evidenced [16]. After the reaction of crushed grapes with yeasts present at the fruit surface, the alcohol produced by the spontaneous fermentation starts hydrolyzing malvidin contained in the dark grape skins. To surely state on red grapes, the benefits of a two-step protocol with the first part targeting the free syringic acid are not to be demonstrated anymore [12,26]. Indeed, while the first extraction can include free syringic acid, the second one rather focuses on the acids that are deeply impregnated in the clay matrix. The acid-catalyzed extraction hence promotes the tracing of malvidin origin. Following Table 2, none of the samples revealed the presence of syringic acid as free acid and butyl syringate was identified in almost all the samples (m/z 240, 296, 311, 326), shedding light on the dark-color beverage contained in amphorae. The Chios amphora was the only one that did not show the dark marker, highlighting the white nature of the content.

Coupling organic extraction with esterification extended the overall number of identified compounds from two to ten molecules per sample over the twenty-two molecules targeted (Figure 1). No DHA was extracted for the amphorae Nos. 6904, 6566 and 6828c using alkaline fusion whereas this compound was extracted with DCM:MeOH for all the items (Table 2). Exceptions were nonetheless noticed for amphorae Nos. 6828b and 6793 where organic solvents barely supported the extraction of diterpene derivatives, hence grandly diminishing the number of characterized molecules (Figure 1). Oxidized diterpenes, identified in all the amphorae, provided insights on the ageing of the pitch that occurred through oxygen incorporation into the DHA skeleton to form peroxide intermediates. Hydroxyl and ketone derivatives are produced by peroxide reduction or dehydration, respectively [40].

Almost all the protocols extracted DHAM markers, traducing an archaeological use of Pinaceae wood tar [41] (Figure 1). Indeed, DHAM characterizes wood distillation: DHA is esterified by the methanol contained in the wood during pyrolysis at high temperature. Interestingly, the amphora from Chios (No. 6904) is the unique potsherd only containing *Pinus* pitch. Neither DHAM derivatives, nor triterpenoids were identified yet the island was famous for its mastic resin of *Pistacia lentiscus* L. [42]. The presence of resinated wine such as *retsina* can be assumed since the practice was common in Greece. Aside from antibacterial properties, small amounts of *Pinus* pitch could be added during the alcoholic fermentation to flavor and color wines [43]. No retene was observed for the amphorae Nos. 6566 and 6828c with alkaline fusion whereas it did with DCM:MeOH (Figure 1). Even though no chemical explanation can afford it, the archaeological meaning refers to the heating treatment of the resin [40]. Thermal degradation above 300 °C induces abietane aromatization into retene. Only the amphora No. 6545 (Dressel 5), where retene has never been identified, was coated with a resinous material that had not been produced under a high temperature.

4.3. Extracting Capacities Comparison on Archaeological Coatings

The protocol comparison applied on coatings encompassed: (i) an alkaline fusion with KOH extraction, (ii) a DCM:MeOH organic extraction, (iii) its coupling with BF_3-catalyzed MW-butylation applied on the dried remaining powder after the organic extraction (2LE-MW) and (iv) the same BF_3-catalyzed MW-butylation but directly applied on the coating matter (R-2LE-MW).

The radar plot (Figure 2) overviews the extractive molecular capacities of the different protocols for each of the eight coatings. Samples Nos. 1014 and 749 have already been discussed through the analyses of their associated shards. Coatings and shards analyses provided identical insights on the nature of the coating and the content even though poor, insignificant molecular differences regarding DHA and DHAM derivatives were observed (Tables 2 and 3).

Table 3. Molecules identified in pitch coatings. Presence (+) and absence (-) of molecular markers under alkaline fusion (KOH ext.), organic extraction with DCM:MeOH, butylation applied on the remaining fraction (2LE-MW) or applied directly on the pitch (R-2LE-MW). The number of '+' refers to the number of molecules present. ac.: acid; OH-DHA: hydroxy-DHA (i.e., 3-hydroxy-DHA; 7-hydroxy-DHA and 15-hydroxy-DHA); Oxo-DHA: 7-oxo-DHA; DiOH-DHA: 7,15-dihydroxy-DHA; Oxo-OH-DHA: 7-oxo-15-hydroxy-DHA. DHAM and oxidized derivatives (OH-DHAM, Oxo-DHAM, DiOH-DHAM and Oxo-OH-DHAM) refer to the same skeleton with methyl ester derivatives instead of the acid function.

Amphora	Protocol	Maleic ac.	Succinic ac.	Pyruvic ac.	Fumaric ac.	Malic ac.	Tartaric ac.	Syringic ac.	Retene	DHA	OH-DHA	Oxo-DHA	DiOH-DHA	Oxo-OH-DHA	DHAM	OH-DHAM	Oxo-DHAM	DiOH-DHAM	Oxo-OH-DHAM
1014	KOH ext	-	+	-	-	-	-	+	+	+	+++	+	+	+	+	+++	-	+	+
	DCM:MeOH	-	+	-	+	-	-	+	+	+	++	-	+	-	+	+++	+	+	+
	2LE-MW	-	-	-	-	-	-	+											
	R-2LE-MW	-	+	+	-	+	+	+											
749	KOH ext	-	+	-	+	+	-	+	+	+	+++	+	+	+	+	++	-	+	+
	DCM:MeOH	-	+	-	+	+	-	+	+	+	++	-	-	-	+	+++	+	-	+
	2LE-MW	-	-	+	-	+	+	-											
	R-2LE-MW	-	+	+	-	+	+	+											
6570a	KOH ext	-	+	-	+	+	-	+	+	+	+++	+	+	+	+	++	-	+	+
	DCM:MeOH	-	-	-	-	-	-	-	+	-	-	-	+	-	+	+	+	+	+
	2LE-MW	-	-	-	-	+	+	-											
	R-2LE-MW	-	-	+	-	+	+	+											
SFC1	KOH ext	-	+	-	+	+	+	-	+	+	+++	+	+	-	+	+	+	+	-
	DCM:MeOH	-	+	-	-	-	-	-	+	+	-	-	-	-	+	+++	+	-	-
	2LE-MW	-	-	+	-	+	+	+											
	R-2LE-MW	-	+	+	-	+	+	+											
SFC2	KOH ext	-	+	-	-	-	-	-	+	+	+	-	-	-	+	-	+	-	-
	DCM:MeOH	-	+	-	-	-	-	-	+	+	-	-	-	-	+	++	+	-	-
	2LE-MW	-	-	-	-	+	+	-											
	R-2LE-MW	-	-	+	-	+	+	-											
SFC3	KOH ext	-	+	-	+	+	+	+	+	+	+++	+	+	-	+	+++	+	+	-
	DCM:MeOH	-	+	-	+	-	-	-	+	+	-	-	-	-	+	+++	+	-	+
	2LE-MW	-	-	+	-	-	+	+											
	R-2LE-MW	-	-	+	-	+	+	+											
SFC4	KOH ext	-	+	-	-	-	-	-	+	+	+	-	-	-	+	++	+	+	+
	DCM:MeOH	-	-	-	-	-	-	-	+	+	-	-	-	-	+	++	+	-	-
	2LE-MW	-	-	-	-	+	+	-											
	R-2LE-MW	-	-	+	-	+	+	-											
SFC5	KOH ext	-	+	-	+	-	-	-	+	+	+++	+	+	+	+	+++	+	+	+
	DCM:MeOH	-	+	-	-	-	-	-	+	+	-	-	-	-	+	++	+	-	+
	2LE-MW	-	-	+	-	-	+	-											
	R-2LE-MW	-	+	+	-	+	+	-											

Tartaric acid was successfully identified in all of the eight coatings when the butylation was directly applied on the pitch (R-2LE-MW) (Figure 2). In comparison, when it was applied after organic extraction (2LE-MW), the esterification allowed DBT to be identified from only seven pitch samples. The amphora No. 1014 (Dressel 1) did not exhibit DBT in the butylated fraction, which can be related to the insufficient remaining sample quantity. Indeed, the remaining powders were usually less than 1 mg, a major part of the initial mass being dissolved during DCM:MeOH extraction. Moreover, DBT was identified as traces for all the samples in 2LE-MW, which clearly argued for the limited quantity effect. The protocol was therefore hardly suitable for content identification. Except for the amphorae Nos. SFC1 and SFC3, the alkaline fusion did not allow tartaric acid to be extracted (Figure 2), which mirrors the unsuitability already reported for shards.

Considering fermented markers, pyruvic acid was only identified with butylation, as dibutylcetal (m/z 61, 117, 173) [44]. Maleic acid was never characterized. Succinic and fumaric acids were respectively rarely and never identified with butylation, although they were successfully extracted with alkaline fusion or organic solvent extraction (Table 3). Moreover, KOH fusion allowed eight additional grape-acids (i.e., succinic, fumaric and malic acids) to be extracted from five amphorae (Nos. 6570a, SFC1, SFC3, SFC4 and SFC5) that traditional solvents did not provide.

Syringic acid was not detected in any of the amphorae SFC2, SFC4 and SFC5 (Table 3), hence suggesting a white winemaking process. On the contrary, red beverages were conjectured for all the other samples (Nos. 1014, 749, 6570a, SFC1 and SFC3). Although a two-step protocol with successive alkaline fusion and butylation would favor the identification of free syringic acid, it is technically hardly feasible because the alkaline fusion left an aqueous matrix difficult to dry completely. Remaining water strongly reacts with BF_3, thus inhibiting butylation (data not shown).

Interestingly, diterpenic markers could not be addressed after butylation. Although diterpenic acids should have been recovered as butylated-derivatives, pimarane and abietane seemed to have undergone transformation. Applied on a standard of pimaric acid (Figure 3C), butylation gave rise to a wide distribution of unidentified diterpenic compounds, of which patterns belong to pimarane (m/z 241; 359). The butylation of standard colophony similarly produced unidentified compounds of abietane skeletons (m/z 239; 372) together with the same unidentified molecules already observed with pimaric acid butylation (Figure 3B,C). Induced by the harsh Lewis-acid conditions, diterpenic skeletons were reported to undergo isomerization, skeletal transposition, isomerization, rearrangement and proton migration [45–47]. Moreover, the butylation of standard colophony selectively esterified the diterpenoids present in the resin (Figure 3A,B). Only butyl dehydroabietate and pimarate could be identified (m/z 239; 356 and m/z 241; 343, respectively). The remaining presence of DHA in the butylated fraction outlined the incomplete esterification of diterpenoids.

Aside from showing unequivocal extractive capacities to target tartaric acid when directly applied on the pitch, the butylation is consequently not self-sufficient to describe the resinous material. The protocol must be coupled to broaden the scope of characterization and alkaline fusion coupled to direct-pitch butylation managed to give rise to more quantitative extractions (Figure 2). Emphasizing on coating markers, the numerical difference reverted to the extractive capacities of alkaline fusion versus traditional solvents and homogeneously concerned DHA derivatives (hydroxy-DHA for all the coatings, oxo-DHA for six of them, dihydroxy-DHA for four of them and hydroxy-oxo-DHA for three pitch over eight). Again, the important presence of oxidized Pinaceae diterpenoids highlighted the significant ageing of *Pinus* pitch. Retene and DHAM markers, characterized in all the coatings independently from the protocol employed (Figure 2), attested of Pinaceae wood tar produced under high temperature pyrolytic treatment.

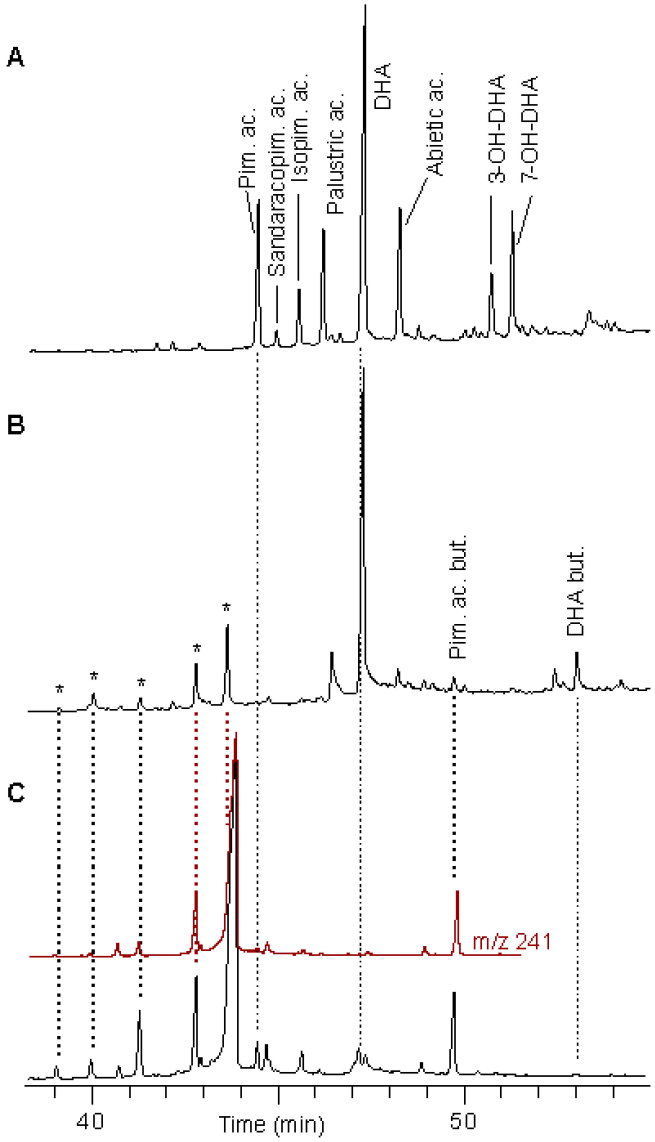

Figure 3. TIC Chromatograms. (**A**) Standard colophony extracted with DCM:MeOH; (**B**) standard colophony extracted with MW-assisted butylation and (**C**) standard pimaric acid extracted with MW-assisted butylation with m/z 241 chromatogram. ac.: acid; but.: butylated; *: unidentified compound.

5. Conclusions

Beyond the successful MW parameters set to esterify molecules, the analyses show different trends. On the one hand, the butylation of standard acids familiar of wine composition is successfully achieved. Grape-acids were recovered as butylated products using MW. Indeed, the energy conduction of MW-assisted reactions being different from reflux [38], it is essential to verify the molecular integrity. On the other hand, even though butylation undoubtedly promoted tartrate extraction from ceramic and coating materials, the protocol, when solely applied, did not provide a representative picture of archaeological artefacts, specifically for coating material characterization. Although MW are widely encouraged to reduce reaction times and costs, butylation has to be strengthen up using another extractive protocol. For the analyses of shards, organic extraction with traditional solvents followed by esterification were remarkably complementary, offering more reliability for the identification of grape-derivatives in terms of number of extracted molecules and retene extraction.

In parallel, the butylation protocol, initially developed for inorganic potsherds, was successfully adapted to organic coatings, hence ensuring a reliable tracing of archaeological

grape-beverage directly from the coating. Alkaline fusion allowed diterpenic markers to be extracted, concluding on the nature; the formulation and the ageing of the coating matter, as well as it suggested the presence of fermentation markers. Although a two-step protocol would prevent from free-acid misinterpretation, further investigation is needed to overcome BF_3 reaction with water.

Except for the amphora from Chios coated with pine pitch, all the other artefacts were waterproofed with *Pinus* wood tar. All the samples validate the presence of archaeological grape contents, which could have been wine, vinegar or any other fermented derivatives [3]. Even though Mañà C2 (No. SFC3) are mostly considered for oil or fish preserves, the identification of wine markers may be interpreted in the light of a reutilization, that needs to be further investigated. Among the frequent typologies mentioned for wine trading, the presence of red beverage was confirmed in Dressel 5 (No. 6545), hitherto barely studied.

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Abbreviations

DBT	Dibutyl tartrate
DCM	Dichloromethane
DHA	Dehydroabietic acid
DHAM	Dehydroabietic methyl ester
DiOH-DHA	7,15-dihydroxy-dehydroabietic acid
DiOH-DHAM	7,15-dihydroxy-dehydroabietic methyl ester
KOH	Potassium hydroxide
OH-DHA	Hydroxy-dehydroabietic acid (3-hydroxy-dehydroabietic acid; 7- hydroxy-dehydroabietic acid; 15-hydroxy-dehydroabietic acid)
OH-DHAM	Hydroxy-dehydroabietic methyl ester (3-hydroxy-dehydroabietic methyl ester; 7-hydroxy-dehydroabietic methyl ester; 15-hydroxy-dehydroabietic methyl ester)
Oxo-DHA	7-oxo-dehydroabietic acid
Oxo-DHAM	7-oxo-dehydroabietic methyl ester
Oxo-OH-DHA	7-oxo-15-hydroxy-dehydroabietic acid
Oxo-OH-DHAM	7-oxo-15-hydroxy-dehydroabietic methyl ester

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