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A novel analytical technique for the characterization of ancient wine biomarkers in archaeological pottery

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Article

Keywords: ancient wine biomarkers, mass spectrometry, pyranoanthocyanins, archaeological residue analysis

Posted Date: August 25th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-10541346/v1

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Additional Declarations: No competing interests reported.

Abstract

The identification of wine residues on archaeological materials presents significant analytical challenges due to limited residue, chemical degradation, and contamination during sample collection. Furthermore, conventional biomarkers such as tartaric and syringic acids are ubiquitous in nature and therefore have limited specificity. This study introduces a novel liquid chromatography tandem mass spectrometry (LC–MS/MS) methodological framework optimized for the detection of anthocyanin-derived pyranoanthocyanins, namely vitisin A and vitisin B, as specific molecular indicators of grape wine fermentation. Authentic standards were synthesized from malvidin-3-*O*-glucoside (Mv3G; oenin) and yeast-derived metabolites, purified by preparative high-pressure liquid chromatography (HPLC), and characterized through tandem mass spectrometry. Validation with modern red and white wines confirmed the exclusive presence of vitisins in fermented red wines and demonstrated high recovery rates from spiked ceramic matrices. Analysis of ancient archaeological ceramic samples revealed traces of vitisin A in Roman Imperial Period amphorae from Conimbriga, Portugal, and Sassanian-Early Islamic Period Torpedo Jars from Fulayj, Oman. These findings provide a method for the specific identification of fermentation-derived pigments in archaeological materials. The assay distinguishes between red and white wine residues, and the results can unambiguously confirm the presence of grape fermentation residues.

1. Introduction

The identification of residues of fermented beverages on or in archaeological materials provides a pathway to understanding early agricultural, technological, and social systems. Among such beverages, wine holds a privileged position in the archaeological record due to its association with ritual practices, exchange networks, and the construction of identity in early societies. Yet, the unambiguous identification of ancient wine residues remains a significant analytical challenge. Organic residues preserved on pottery walls or absorbed into the ceramic matrix undergo chemical degradation over time, a process exacerbated by postdepositional alterations, sample age, and storage conditions. In addition, they are often present in trace amounts, making their reliable detection analytically challenging. Moreover, often used biomarkers, most notably tartaric and syringic acids, are present only in trace amounts and lack the chemical specificity required to distinguish grape wine residues from other fermented or fruit-based products. We aim to overcome these limitations by enabling the detection of more specific molecular markers with sensitivity and confidence.

Early analytical approaches to ancient wine included diffuse reflectance Fourier transform infrared spectroscopy (DRIFTS/FT-IR) analysis to identify fermented residues on vessels from the ancient Near East (c. 3500 BCE). The identification of tartaric acid and calcium tartrate within Late Chalcolithic jars provided the earliest molecular evidence of wine production [1]. Later studies on pottery from Neolithic and Chalcolithic contexts in the Near East introduced chromatographic separation with UV-vis adsorption detection in addition to FT-IR [2]. This study demonstrated that tartaric acid, often associated with *Pistacia* resin, can serve as a marker for grape-based fermentation. Subsequent analyses of vessels

from the highlands of the Near East and southeastern Europe confirmed similar organic acid profiles, including evidence from northern Greece showing the earliest, direct prehistoric European wine residues [3–5]. A combination of the above analytical methods was applied in a study on the origin of viniculture in France [6]. These findings established tartaric and syringic acid as foundational biomarkers in archaeological wine research, extending the geographic and chronological understanding of early viniculture.

Previous research from our group established that malvidin, the red anthocyanin molecule detected as syringic acid following controlled alkaline treatment, serves as a robust biomarker for red wine preserved in archaeological ceramics. Using a workflow combining solid-phase extraction (SPE), combined liquid chromatography/tandem mass spectrometry (LC–MS/MS) and targeted multiple-reaction-monitoring (MRM), we demonstrated that malvidin-derived syringic acid can be selectively released, purified, and confidently identified at trace levels. This approach successfully recovered wine-related anthocyanin signatures from Late Chalcolithic (c. 4000 BCE) ceramic residues from Armenia, providing direct chemical evidence for the production and storage of red wine in early highland contexts [3]. This finding was corroborated by the presence of grape rachis in associated archaeological contexts. Biochemical studies have demonstrated that syringic acid is the dominant and predictable degradation product of malvidin under both biological and diagenetic conditions, indicating that its presence reflects anthocyanin breakdown rather than molecular preservation [7, 8].

Subsequent experimental and review studies, however, indicated that these compounds lack sufficient biomarker specificity. Tartaric acid occurs widely in non-grape taxa and is highly mobile in burial environments, while syringic acid is a terminal degradation product of multiple polyphenolic precursors and lignin-derived materials. As a result, their detection alone cannot reliably discriminate grape wine from other fruit products, resins, or environmental inputs [8, 9].

Other DNA and biochemical analyses of ancient residues confirmed that *Saccharomyces cerevisiae*, the yeast responsible for modern winemaking, was already used in vinification more than five millennia ago [10]. This yeast naturally colonizes grape skins and produces pyruvate and acetaldehyde during fermentation [11], two intermediates central to the formation of stable red-wine pigments. These results linked early fermentation directly to the molecular transformations responsible for wine color and chemical preservation. Nevertheless, later evaluations have emphasized that ancient DNA identification of yeasts is also highly susceptible to contamination and differential preservation, underscoring the need for chemically diagnostic fermentation markers [9, 11].

By the early 2000s, advances in chromatographic and spectrometric analysis allowed more detailed identification of preserved wine molecules in archaeological samples. Studies of amphora coatings showed that polyphenols and organic acids diffuse through pitch linings, confirming that diagnostic molecules can migrate through protective coatings and still be recovered [12, 13]. Multidisciplinary analyses integrating combined gas chromatography/mass spectrometry (GC–MS) and palynology detected tartaric, malic, syringic, and pyruvic acids in Roman ceramic wares, but also underscored the

risk of over-interpretation when single compounds are taken as definitive evidence [14]. Recent developments have focused on miniaturized and environmentally sustainable analytical designs. High-sensitivity LC–MS/MS protocols that combine ultrasound extraction with dispersive liquid–liquid micro-extraction now enable reliable quantification of tartaric, malic, and syringic acids in archaeological ceramics at trace levels [15]. While these techniques improve reproducibility and reduce sample consumption, the targeted acids remain too general to confirm true grape fermentation. Distinguishing the molecular signature of fermentation from fruit-derived organic acids has become the central analytical challenge. These organic acids, particularly tartaric and syringic acids, are abundant in multiple fruits and plants, and their high solubility and mobility make them prone to leaching and/or contamination.

Evaluations of methods currently used in the field have concluded that no single biomarker, chemical, or genetic marker provides unequivocal evidence of ancient wine production [9]. These reviews highlighted inconsistent extraction protocols, variable detection limits among laboratories, and the absence of fermentation-specific molecular targets. Even DNA-based identifications of yeast activity remain uncertain due to degradation and contamination. This situation has redefined research priorities to identify chemically specific, fermentation-derived markers, supported by reproducible and quantitative workflows. Comparable multidisciplinary analyses of intact Roman amphorae, combining chemical residue analysis, palynology, and isotopic measurements, have independently confirmed the presence of fermented grape beverages and regional provenance, reinforcing the interpretive value of integrated biomolecular and archaeobotanical approaches [16]. However, the samples often lack a secure archaeological context, limiting the approach to residue analysis of absorbed organic compounds inside the ceramic matrix.

One promising alternative lies in the chemistry of wine pigments. The color and stability of red wine depend on anthocyanins, flavylium-based polyphenols in grape skins, involved in reactions during fermentation to form more complex molecules, including pyranoanthocyanins. Chromatographic studies established reproducible methods for extracting and quantifying anthocyanins from wines [17]. Other biochemical research has shown that under neutral pH and in the presence of microbial activity, these compounds degrade into simpler phenolic acids, which accounts for their poor preservation [7]. Under acidic, anaerobic, and ethanolic conditions, however, they can transform into more stable derivatives. Research into the chemical pathways of these pigments revealed that yeast-mediated reactions involving malvidin-3-*O*-glucoside with pyruvic acid and acetaldehyde generate vitisin A and vitisin B[1], respectively [20–22]. Further studies identified additional polymeric pigments such as portisins, formed during extended oxidative aging, and demonstrated that fermentation conditions and yeast strains strongly influence pigment formation and final wine color [23–26].

All these studies established vitisins as specific indicators of red-wine fermentation. Unlike tartaric or syringic acids, pyranoanthocyanins are formed exclusively through yeast-mediated fermentation reactions, and do not occur naturally in unfermented fruits or environmental matrices [21, 22, 25, 27]. Comparative analyses of modern wines revealed structural and concentration variability among cultivars

and fermentation conditions [24]. Meanwhile, kinetic and stability experiments confirmed that pH and temperature significantly influence pigment persistence [28]. Investigations of malvidin-3-*O*-glucoside equilibria across typical wine pH values (3.0–3.6) confirmed that co-pigmentation and pyranoanthocyanin formation dominate near pH 3.3 [27]. Metabolomic analyses of aged wines have shown that condensed pigments and related nitrogenous compounds exhibit long-term molecular stability [8]. Together, these results indicate that pyranoanthocyanins, particularly vitisin A and vitisin B, are among the most chemically stable and fermentation-specific biomarkers of red wine currently known (Fig. 1).

The present study introduces a novel methodological framework, an LC–MS/MS protocol optimized for detecting pyranoanthocyanins vitisin A and vitisin B in both modern and archaeological matrices. By synthesizing authentic standards, refining chromatographic and mass spectrometric parameters, and validating recovery from ceramic extracts, this method provides the first reproducible assay for identifying fermentation-specific red-wine pigments in archaeological contexts. Beyond improving analytical precision, our approach addresses long-standing challenges of specificity, reproducibility, and diagenetic stability, advancing biomolecular archaeology toward more definitive identification of ancient wine residues and more nuanced reconstructions of early vinicultural practices.

2. Results

2.1. Synthesis and characterization of pyranoanthocyanins

Authentic vitisin A and vitisin B were synthesized from malvidin-3-*O*-glucoside and isolated for use as reference compounds. The two products displayed markedly different behavior during semi-preparative HPLC purification. Vitisin A eluted as a sharp, well-defined peak (Fig. 2) whereas vitisin B produced a broad, poorly resolved signal near the end of the gradient (Fig. 3). The overlapping UV–visible absorbance profiles of vitisin B and residual oenin complicated their differentiation by DAD, and the selective collection of the vitisin B fraction. The presence of vitisins in the selected fractions was confirmed by full-scan mass spectrometry. The spectra showed signals at *m/z* 561 for vitisin A and *m/z* 517 for vitisin B, consistent with previously reported values [23, 29]. Vitisin A exhibited a clear baseline separation, indicating high chromatographic resolution and purity. In contrast, vitisin B showed lower conversion efficiency and partial spectral overlap with unreacted malvidin-3-*O*-glucoside. The synthesized compounds were used to build compound-specific MS/MS libraries for subsequent analyses.

2.2. LC–MS/MS method optimization

The analytical method presented here was developed for the qualitative identification of fermentation-derived pyranoanthocyanins in archaeological materials rather than their absolute quantification. This reflects both the difficulty of obtaining analytically pure synthesized vitisin standards suitable for rigorous quantitative calibration and the inherent complexity of archaeological residues, where variable

preservation, extraction efficiency, matrix effects, and diagenetic alteration limit reliable quantification. Accordingly, method development focused on maximizing the specificity and confidence of compound identification through chromatographic separation and tandem mass spectrometric confirmation.

The optimized LC–MS/MS workflow achieved chromatographic resolution and stable ionization of both vitisin A and vitisin B (Fig. 4). Both compounds produced fragmentation patterns consistent with those previously reported for pyranoanthocyanins. Diagnostic multiple-reaction-monitoring (MRM) transitions of *m/z* 561 → 399 for vitisin A and *m/z* 517 → 355 for vitisin B were monitored using a triple-quadrupole mass spectrometer (Supplementary Methods S2). These fragmentation data were subsequently used to develop a targeted MS/MS method on the LTQ-XL linear ion-trap mass spectrometer and served as reference criteria for identifying vitisin A and vitisin B in the archaeological samples [25, 28] (Fig. 5). The resulting mass transitions were reproducible across replicates and matched the synthetic reference spectra. The optimized parameters (Supplementary Table S1) were subsequently applied to the analysis of modern and archaeological samples.

2.3. Validation with modern wines and recovery from ceramic matrix

The characteristic precursor-to-product ion (MRM) transitions of the synthesized standards were established and confirmed using a triple quadrupole mass spectrometer. These transitions were subsequently used to guide targeted LC–MS/MS analysis of archaeological and other samples performed on an LTQ XL linear ion trap. The LC–MS/MS method detected vitisin A and B in all recent and aged red wine samples, but not in white wines, confirming their fermentation-specific formation. To evaluate extraction efficiency from ceramic matrices, powdered modern and archaeological ceramics were spiked with red wine and extracted using the same protocol applied to archaeological samples. Recovery experiments demonstrated efficient extraction of vitisin A, vitisin B, and malvidin-3-*O*-glucoside from the ceramic matrix, with mean recoveries exceeding 80% for all target pyranoanthocyanins (Supplementary Table S4). In addition, both pigments were detected in a 15-year-old additive-free red wine, confirming their long-term stability (Fig. 6).

2.4. Analyses of archaeological samples

Among the 70 powdered archaeological ceramic samples analyzed, only a small number of acidified methanolic extracts exhibited LC–MS/MS peaks attributable to the target compounds. Representative chromatograms from positive archaeological samples, including first- and third-century AD Roman amphorae from Portugal [30] and mid-3rd to late 7th centuries AD ceramic containers from Fulayj, Oman [31], are shown in Figs. 7 and 8. Vitisin A was detected in positive samples from both archaeological assemblages, while Sample No. 3464 from Fulayj additionally showed signals corresponding to malvidin-3-*O*-glucoside and vitisin B (Fig. 8). In each case, the peak co-eluted with the authentic vitisin A standard analyzed within the same analytical sequence. Comparable chromatographic profiles were observed in a few additional samples from both archaeological assemblages. Detailed archaeological

descriptions and contextual information for the Portuguese and Omani materials are provided in the cited studies. The chromatograms are presented here to demonstrate the application of the validated LC–MS/MS method. These results align with previous multidisciplinary studies of Roman amphorae that combined organic residue analysis with archaeobotanical evidence to support the identification of fermented grape beverages [14, 16].

2.5. Differentiation of wine types

Vitisins A and B were detected only in the red-wine controls and archaeological samples attributed to red wine. By contrast, no signals corresponding to vitisin A, vitisin B, and malvidin-3-*O*-glucoside were detected in the aged white-wine control samples (Fig. 9). The combined detection of pyranoanthocyanins and wine-related organic acids therefore supports the differentiation of red- and white-wine residues while providing evidence of fermentation-derived chemical signatures.

3. Discussion

3.1. Analytical performance and reliability

Our LC–MS/MS method enabled the selective and reliable detection of vitisin A and vitisin B, pyranoanthocyanins not targeted in previous analytical approaches [1, 9, 10, 14]. A compound was considered detected when a clearly resolved chromatographic peak with a signal-to-noise ratio greater than 3 co-eluted with the corresponding analytical standard and produced the expected MS/MS fragmentation pattern. Analytical standards were analyzed within each sequence to verify retention-time agreement. The combined use of chromatographic matching and characteristic fragmentation pattern increased confidence in compound identification and reduced the likelihood of false-positive assignments arising from co-eluting matrix components.

3.2. Advantages over conventional biomarkers

Conventional biomarkers such as tartaric and syringic acids have limited diagnostic specificity because they occur in sources other than grapes, and may be affected by degradation, solubility, migration, and postdepositional leaching [1, 10, 14]. In contrast, vitisins A and B are fermentation-derived pyranoanthocyanins formed through reactions of grape anthocyanins with pyruvic acid and acetaldehyde, respectively [5, 9]. Their detection therefore provides more specific molecular evidence of a fermented anthocyanin-containing beverage and, when interpreted together with grape-derived organic acids and archaeological context, strengthens the identification of red-wine residues over unfermented grape juice.

3.3. Addressing methodological pitfalls

The present approach responds to persistent concerns regarding biomarker specificity and evidentiary reliability in ancient wine-residue analysis [9, 32]. By combining fermentation-derived pyranoanthocyanins with grape-associated organic acids and compound-specific LC–MS/MS

identification, the method improves diagnostic specificity and reduces reliance on any single non-specific biomarker. Confidence in compound identification is further strengthened through the analysis of authentic standards, spiked controls, negative controls, and procedural blanks, together with retention-time and MS/MS-fragmentation matching. These measures reduce the risks of contamination, matrix interference, and false-positive assignments while improving analytical reproducibility. The resulting chemical evidence can then be interpreted alongside archaeological and archaeobotanical findings to support a more robust identification of ancient wine residues.

3.4. Preservation and diagenetic implications

The detection of vitisins in archaeological ceramic samples indicates that these pigments, despite their polarity, persist within ceramic matrices over extended time periods. Their retention may be promoted by interactions with mineral surfaces, entrapment within ceramic pores, or transformation into more stable pigment forms, although these mechanisms require experimental confirmation. The detection of vitisins in a 15-year-old wine sample further demonstrates their persistence during prolonged wine aging but does not, by itself, establish their stability under burial conditions. Metabolomic analyses of century-old wines have shown the preferential long-term survival of condensed pigments and nitrogen-containing compounds, providing additional support for the potential persistence of pyranoanthocyanins [33]. Nonetheless, their long-term diagenetic pathways remain to be experimentally characterized, particularly regarding degradation products and potential interconversion with other polyphenols. In addition to vitisins, other stable anthocyanin-derived pigments such as oxovitisins and portisins have been identified in aged wines and may represent additional targets for archaeological detection due to their exceptional chemical stability [34, 35]. Experimental studies demonstrate that oxovitisin-type pigments exhibit superior resistance to pH variation, thermal degradation, and sulfur dioxide bleaching, suggesting strong archaeological preservation potential [36].

3.5. Archaeological significance

The identification of vitisin A in Roman- and Sassanian-period ceramic samples provides compound-specific evidence consistent with the former presence of fermented red-grape products. When considered alongside grape-associated organic acids and the archaeological context, this fermentation-derived marker strengthens the interpretation of the detected residues as red wine. The distinct chemical profiles observed for red- and white-wine reference samples further demonstrate the potential of this approach to support differentiation between wine types in archaeological materials. Such distinctions may contribute to more detailed investigations of ancient wine production, consumption, storage, and exchange. By integrating compound-specific LC–MS/MS identification with contextual archaeological evidence, this study provides a reproducible framework for improving the interpretation of ancient wine residues.

4. Materials and Methods

4.1. Reagents and standards

Analytical grade methanol, acetonitrile, formic acid, pyruvic acid, acetaldehyde, and hydrochloric acid (37%, ~ 12 M) were obtained from Thermo Scientific (Waltham, MA, USA). Malvidin-3-*O*-glucoside (oenin) was purchased from PhytoLab (Vestenbergsgreuth, Germany). Daidzin was purchased from Tokyo Chemical Industry (TCI, Tokyo, Japan). Deionized water (18.2 MΩ·cm) was obtained from a Milli-Q purification system (Millipore). Authentic vitisin A and vitisin B reference standards were synthesized in-house from malvidin-3-*O*-glucoside as described below.

4.2. Synthesis of pyranoanthocyanins

Vitisin A and vitisin B reference compounds were synthesized under controlled laboratory conditions following modified oenological reaction pathways described previously [23, 29]. For vitisin A, malvidin-3-*O*-glucoside (567.5 µg, 1.15 µmol) was dissolved in deionized water adjusted to pH 2.6 with hydrochloric acid, giving a final reaction volume of 2.0 mL. Neat pyruvic acid (4.00 µL, 57.5 µmol), corresponding to a 1:50 molar ratio relative to Mv3G, was added. The reaction mixture was incubated in the dark at 37°C for 5 days to promote condensation between the flavylium nucleus of the anthocyanin and pyruvic acid [23].

For vitisin B, malvidin-3-*O*-glucoside (483 µg, 0.98 µmol) was dissolved under identical conditions. Neat acetaldehyde (2.75 µL, 49.0 µmol), corresponding to a 1:50 molar ratio relative to oenin, was added, and the reaction mixture was incubated in the dark at 37°C for 10 days to promote formation of vitisin B. Under these acidic hydroethanolic conditions (pH approximately 2.6–3.2), acetaldehyde reacts at the C-4 position of the anthocyanin, followed by cyclization to form the corresponding pyranoanthocyanin derivative, vitisin B [29].

Reaction progress was monitored by semi-preparative HPLC-DAD, and fractions were collected at 1-min intervals for subsequent off-line LC–MS and MS/MS characterization. Complete chromatographic conditions are provided in Supplementary Methods S1. Product formation was assessed semiquantitatively by comparing the extracted-ion chromatographic peak area of each vitisin with that of residual oenin. Because compound-specific MS response factors were not determined, the resulting peak-area ratios were interpreted as relative MS responses rather than measures of molar conversion or chemical purity. Under the synthesis conditions used, vitisin B exhibited a lower relative MS response than vitisin A.

The fractions showing the greatest apparent enrichment of the target compounds were retained as reference materials. The compounds produced diagnostic transitions at *m/z* 561 → 399 for vitisin A and *m/z* 517 → 355 for vitisin B, consistent with the loss of a hexose moiety (Supplementary Table S1). The synthesized reference compounds were subsequently used to establish compound-specific MS/MS reference spectra for method development and analysis of the archaeological samples.

4.3. Modern wine and ceramic reference materials

Reference wines were analyzed to validate biomarker specificity and assess the detection of wine-aging markers in modern samples. These included a commercially bottled red wine (Shawn Red Blend; Trader Joe's, USA) and two approximately 15-year-old traditionally produced homemade wines: a red wine made from the indigenous Albanian grape variety *Shesh i Zi*, and a white wine made from Riesling grapes. Both homemade wines were produced without added sulfites or preservatives and served as naturally aged reference materials.

For extraction recovery testing, 2 g of pre-cleaned powdered ceramic from both commercially available unglazed terracotta and archaeological ceramic sherds from the Late Neolithic site of Kallamas, Albania, were each spiked with 1 mL of the commercial red wine, dried overnight at room temperature, and subsequently extracted using the same protocol described for the archaeological samples. Archaeological blanks consisted of uncoated ceramic fragments from Kallamas that were confirmed to be free of visible residues prior to analysis.

4.4. Archaeological samples

A total of 70 archaeological ceramic samples were analyzed, including Roman amphora fragments from Portugal and southern France, Sassanian-period ceramics from Fulayj, Oman, and Neolithic ceramics from Albania. The Albanian samples were included as comparative archaeological materials to evaluate the method and replicate previous residue analyses. Before sampling, the sherds were brushed to remove loose surface material, sonicated in ultrapure water, and air-dried in a laminar-flow hood. The dried ceramics were powdered using an agate mortar and pestle, and approximately 2 g of powder from each sample was used for extraction.

4.5. Extraction protocol

Approximately 2 g of ceramic powder from each sample was transferred to a glass tube. Daidzin (100 pmol in 10 µL of ethanol) was added as an internal control before extraction. The samples were treated with 5 mL of methanol/water/hydrochloric acid (50:50:0.1, v/v/v), thoroughly mixed, and centrifuged at 2,000 × *g* for 15 min. The supernatants were transferred to clean tubes, and the remaining ceramic material was extracted a second time with 3 mL of the same solvent mixture. Following centrifugation under the same conditions, the supernatants from both extractions were pooled. The pooled extracts were dried in a vacuum concentrator for 4 h and reconstituted in 100 µL of methanol/water/formic acid (95:5:0.1, v/v/v). After thorough mixing, the samples were centrifuged at 16,000 × *g* for 5 min. The resulting supernatants were transferred to polypropylene autosampler vials, and 25 µL was injected for LC–MS/MS analysis.

Extraction performance was evaluated using powdered pottery samples spiked with synthesized reference compounds of malvidin-3-*O*-glucoside, vitisin A, and vitisin B before extraction. Spiked samples were processed using the complete extraction protocol described above and analyzed by LC–MS/MS under identical instrumental conditions. Recoveries were calculated by comparing the peak areas obtained from the extracted spiked samples with those obtained from reference solutions

prepared directly in solvent. Mean recoveries and coefficients of variation from three independent replicate extractions are reported in Supplementary Table S4.

4.6. LC–MS/MS analysis

Chromatographic separation was achieved using a Dionex UltiMate 3000 UHPLC system equipped with a GL Sciences InertSustain Phenyl analytical column (2.1 × 100 mm, 2 µm). Water and acetonitrile, each containing 0.1% formic acid, were used as mobile phases A and B, respectively. The complete chromatographic conditions are provided in Supplementary Methods S3. Targeted MS/MS analysis was performed using a Thermo Scientific LTQ XL linear ion-trap mass spectrometer operated in positive ESI mode. Product-ion spectra were acquired for vitisin A, vitisin B, malvidin-3-*O*-glucoside, and daidzin. Diagnostic product ions at *m/z* 399.17, 355.08, 331.10, and 255.0, respectively, were used during data analysis. Detailed source settings, CID conditions, collision energies, and scan ranges are provided in Supplementary Methods S4 and Supplementary Table S2. Additional MS^3 experiments were performed for malvidin-3-*O*-glucoside, vitisin A, and vitisin B; the corresponding acquisition conditions and observed product ions are reported in Supplementary Table S3.

4.7. Quality control and data analysis

Each analytical sequence included solvent blanks, extraction blanks, and ceramic procedural blanks. Co-elution tests were performed by spiking archaeological extracts with the corresponding reference material at a standard-to-extract ratio of 1:100 (v/v). Co-elution was supported by an increase in the intensity of a single chromatographic peak without peak splitting or the appearance of a shoulder. Compound identification required a clearly resolved peak with a signal-to-noise ratio greater than 3, a retention-time difference within ± 0.05 min of the corresponding reference material, and the presence of the diagnostic product ions established from the reference MS/MS spectra.

4.8. Contamination control

All sample preparation was conducted in a clean laboratory designated for organic-residue analysis. Glassware was solvent-rinsed before use, and the agate mortar and pestle were thoroughly cleaned between samples. Personnel wore nitrile gloves and clean cotton laboratory coats throughout sample preparation. Reference materials and archaeological samples were prepared separately, and procedural blanks were included to monitor contamination introduced during sample handling and extraction. Sample extracts were analyzed in randomized order to reduce the influence of systematic analytical drift.

5. Conclusions

This study developed an LC–MS/MS approach targeting the pyranoanthocyanins vitisin A and vitisin B as fermentation-derived biomarkers in archaeological wine-residue analysis. Synthesis of reference compounds, optimization of chromatographic and mass-spectrometric conditions, and analysis of modern reference materials established compound-specific retention times and MS/MS product-ion profiles. Identification was based on chromatographic agreement with the reference materials,

diagnostic product ions, co-elution tests, and a signal-to-noise ratio greater than 3. By targeting fermentation-derived pyranoanthocyanins alongside grape-associated organic acids, the method provides greater diagnostic specificity than approaches relying solely on nonspecific organic-acid markers.

The detection of vitisin A in Roman- and Sassanian-period ceramic samples demonstrates the potential persistence and analytical recovery of this compound from archaeological matrices. When interpreted together with grape-associated organic acids and archaeological context, its presence provides evidence consistent with fermented red-grape products and strengthens the identification of red-wine residues. The distinct profiles observed in modern red and white wine reference samples further indicate the potential of this approach to support differentiation between wine types in archaeological materials.

Future research should evaluate the preservation and degradation of vitisins under controlled burial conditions and extend the method to other stable anthocyanin-derived pigments, particularly oxovitisins and portisins. The synthesis and characterization of additional reference compounds, together with analysis of broader and independently documented archaeological sample sets, will expand the molecular evidence available for investigating ancient wine production, storage, consumption, and exchange.

Declarations

Competing interests

The authors declare no competing financial or non-financial interests. None of the authors have any commercial affiliations, financial relationships, or personal associations that could be perceived to influence the results or interpretation of this research.

Author Contribution

G.E. conceived and designed the study, developed the LC–MS/MS analytical workflow, synthesized and characterized vitisin A and vitisin B standards, performed data acquisition and interpretation, wrote the main manuscript, and prepared the figures and tables. K.F.F. supervised the analytical chemistry component, advised on tandem mass-spectrometric optimization, contributed to the interpretation of chromatographic and spectrometric data, and assisted in manuscript revision. H.B provided archaeological context, curated sample provenance, and contributed to the interpretation of the archaeological and cultural significance of the findings. J.W. contributed to method validation and instrumental performance evaluation and assisted in manuscript revision. J.C. provided and documented archaeological samples, contributed to contextual interpretation of their provenance and preservation conditions, and advised on the integration of archaeological and chemical data. All authors

discussed the results, contributed to writing and editing, and approved the final version of the manuscript.

Acknowledgement

This research was supported by the Pasarow Mass Spectrometry Laboratory and the Cotsen Institute of Archaeology at the University of California, Los Angeles. The authors thank the directors of the Cotsen Institute, Willeke Wendrich and Jason De Leon, for their invaluable support of the project. We are especially grateful to Hilli Weinberger, Haxhi Elezi, Nexhip Elezi, and Namik Bezati for their contributions.

Data Availability

All LC–MS/MS raw data, calibration files, spectral libraries, peak-integration data, and compound-identification data generated or analyzed during the current study are available from the corresponding author upon reasonable request. Processed chromatograms are included in the main article, and additional methodological and analytical information is provided in the Supplementary Information.

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Footnotes

[1] To avoid nomenclature ambiguity, it should be noted that the trivial names “vitisin A” and “vitisin B” have also been used for structurally unrelated resveratrol oligostilbenes from *Vitis* species [18,19]. In this manuscript, “vitisin A” and “vitisin B” refer exclusively to the pyranoanthocyanin wine pigments derived from anthocyanins during red wine fermentation and aging (Figure 1).

Figures

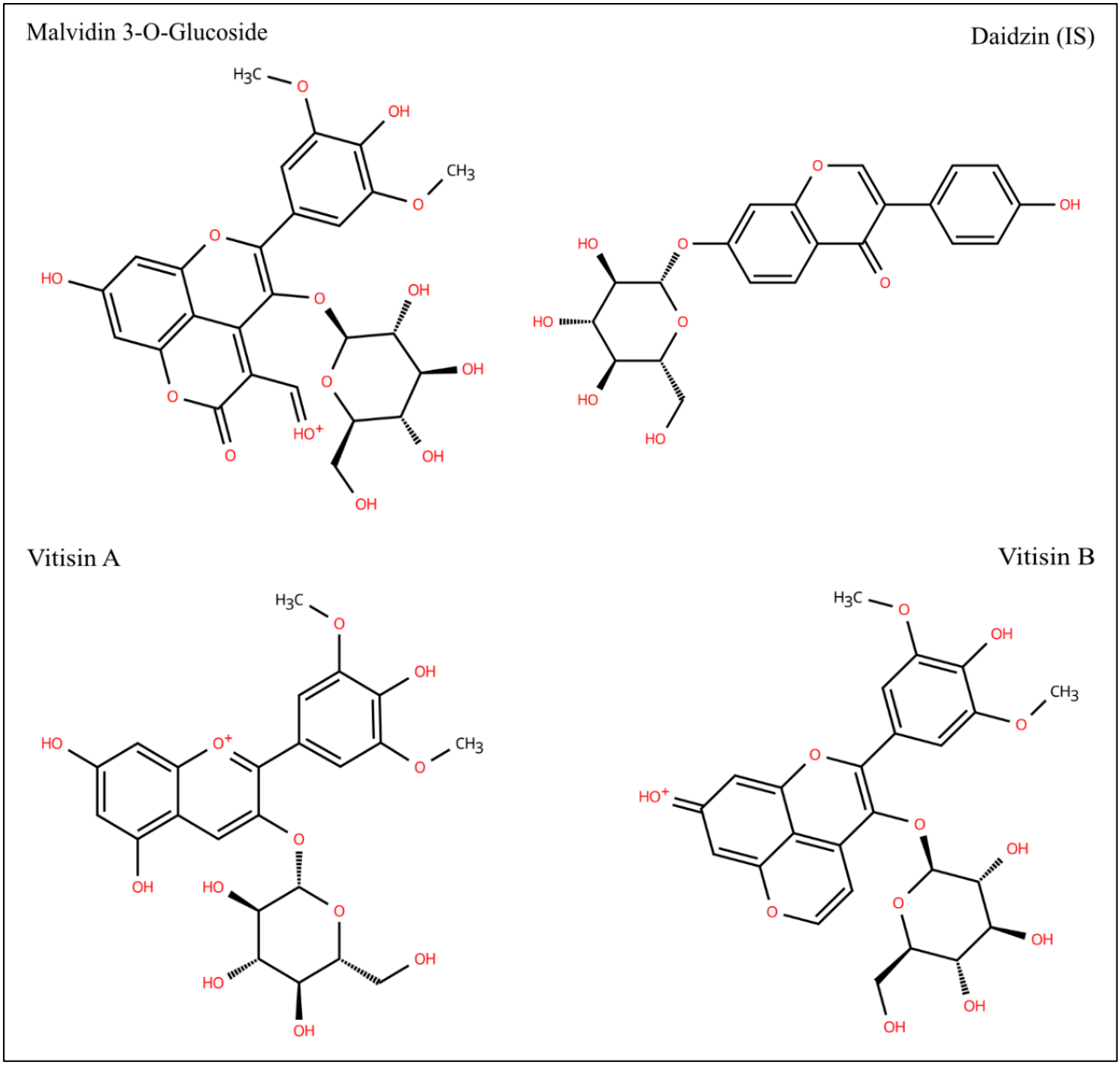


Figure 1

Chemical structures of malvidin-3-*O*-glucoside (Mv3G; oenin), vitisin A, vitisin B, and daidzin (internal control).

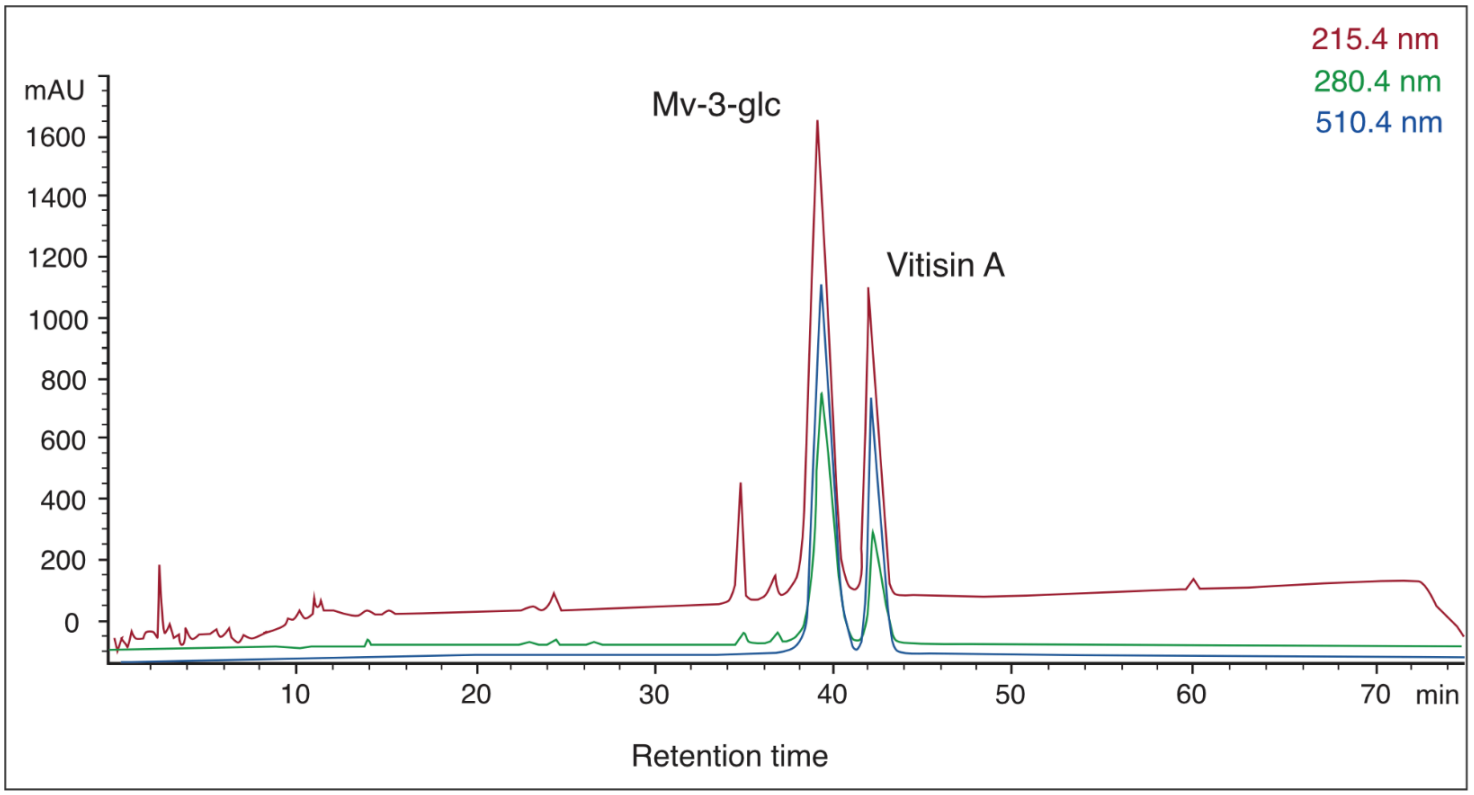


Figure 2

Preparative HPLC-DAD chromatograms of synthesized vitisin A recorded at 215.4 nm (red), 280.4 nm (green), and 510.4 nm (blue). The chromatograms demonstrate baseline chromatographic separation of unreacted malvidin-3-*O*-glucoside from the purified vitisin A product.

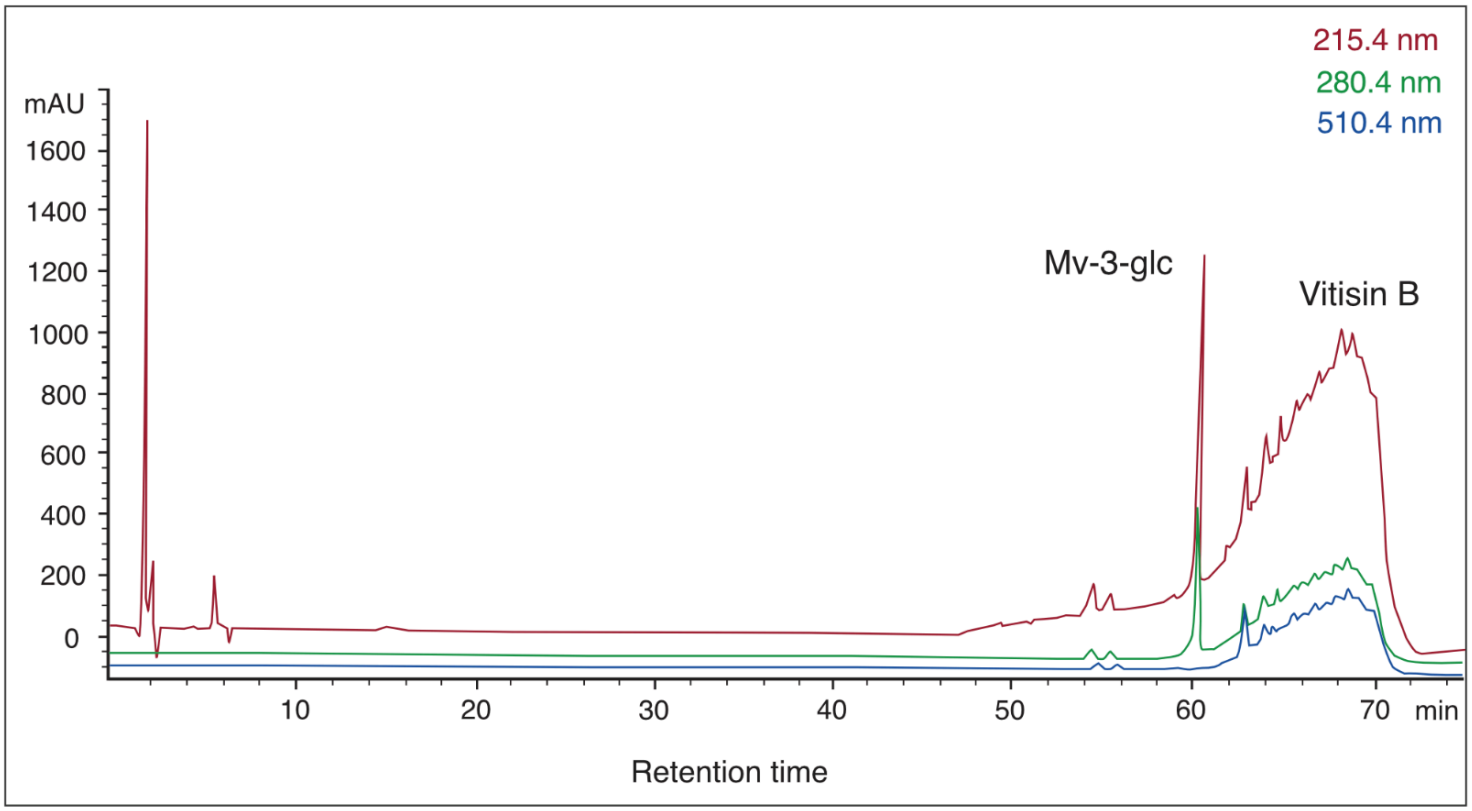


Figure 3

Preparative HPLC-DAD chromatograms of synthesized vitisin B recorded at 215.4 nm (red), 280.4 nm (green), and 510.4 nm (blue). The chromatograms show the near-baseline separation of vitisin B from the precursor malvidin-3-*O*-glucoside.

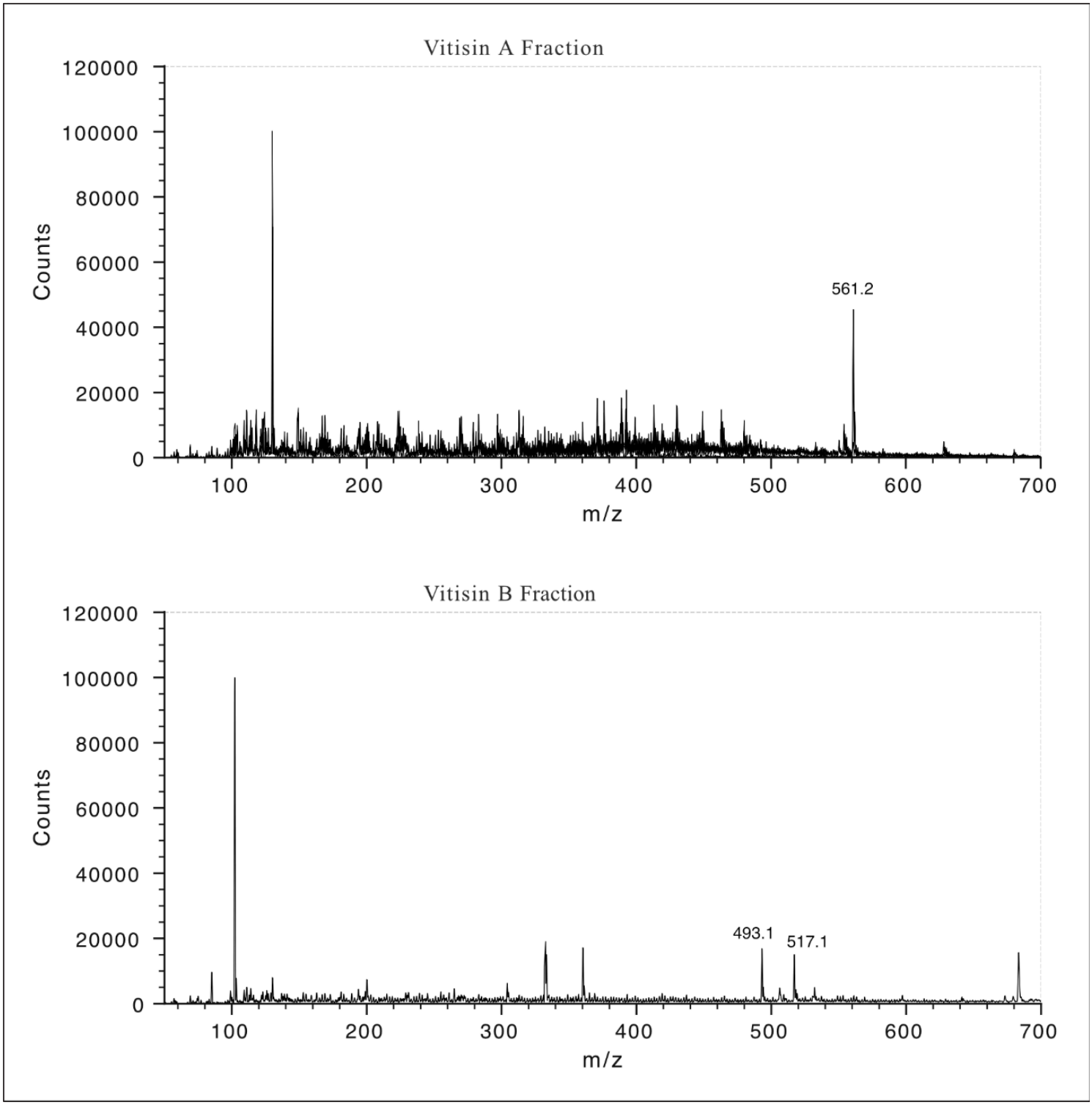


Figure 4

Full-scan positive ion mode LC-MS spectra of the purified fractions containing (A) vitisin A and (B) vitisin B. The protonated molecular ions of vitisin A (*m/z* 561.2) and vitisin B (*m/z* 517.1) are indicated.

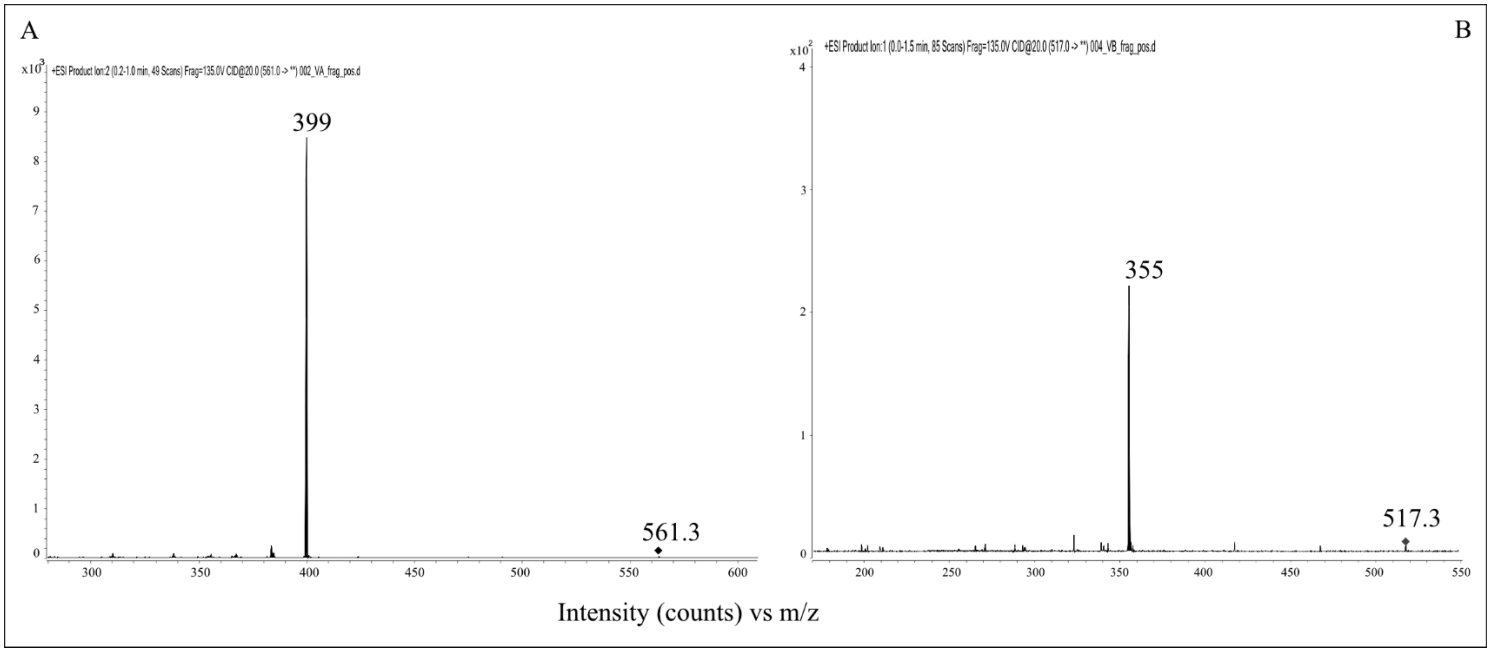


Figure 5

Representative positive-ion LC–MS/MS product (fragment) ion spectra of (A) vitisin A (*m/z* 561.3 → 399.0) and (B) vitisin B (*m/z* 517.3 → 355.0). The dominant product ions used for compound identification and subsequent targeted MRM analyses are indicated.

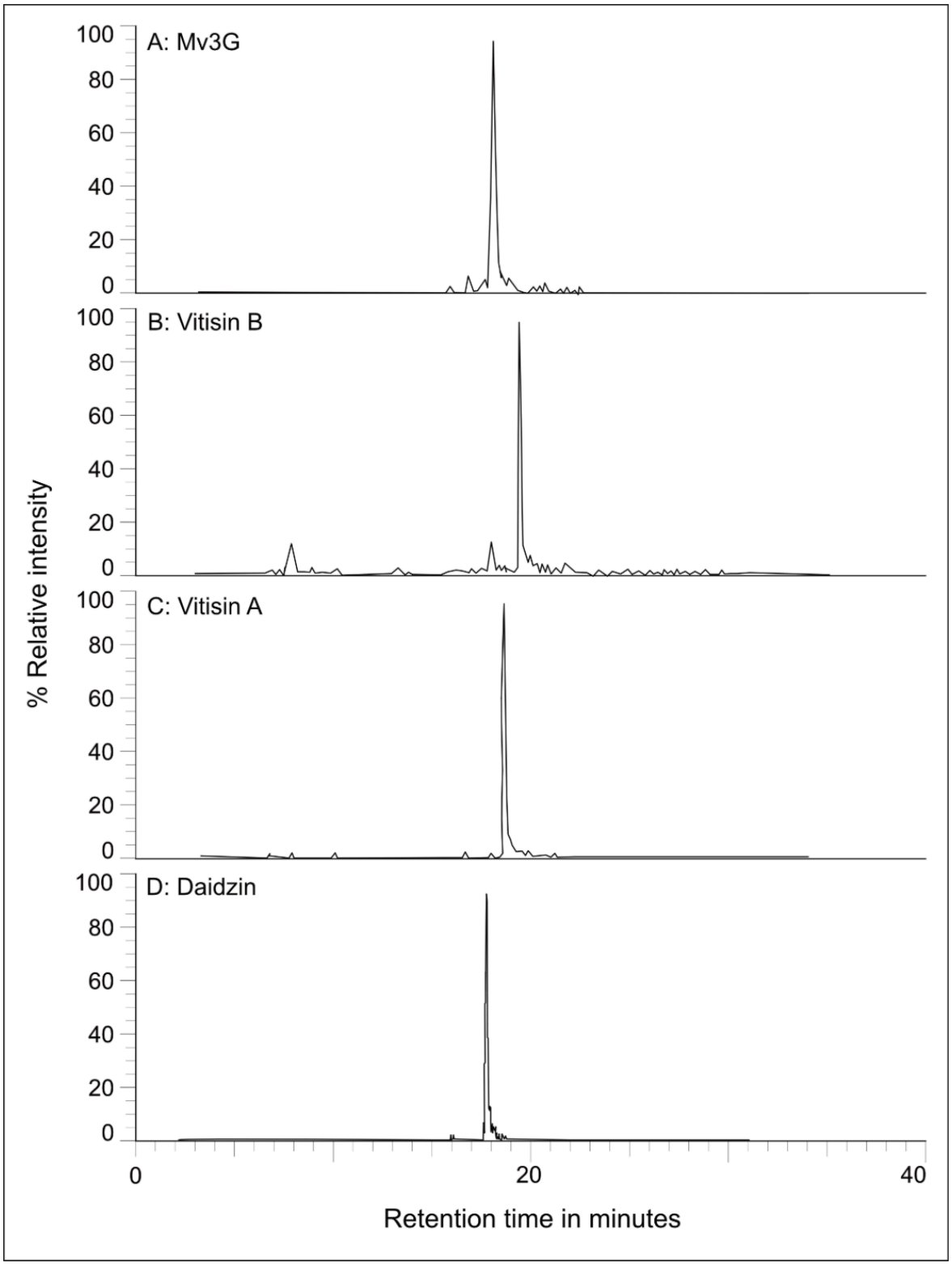


Figure 6

Representative LC–MS/MS chromatograms of a modern red wine extract showing the detection of malvidin-3-*O*-glucoside, vitisin B, vitisin A, and the internal control daidzin under the optimized targeted LC–MS/MS method.

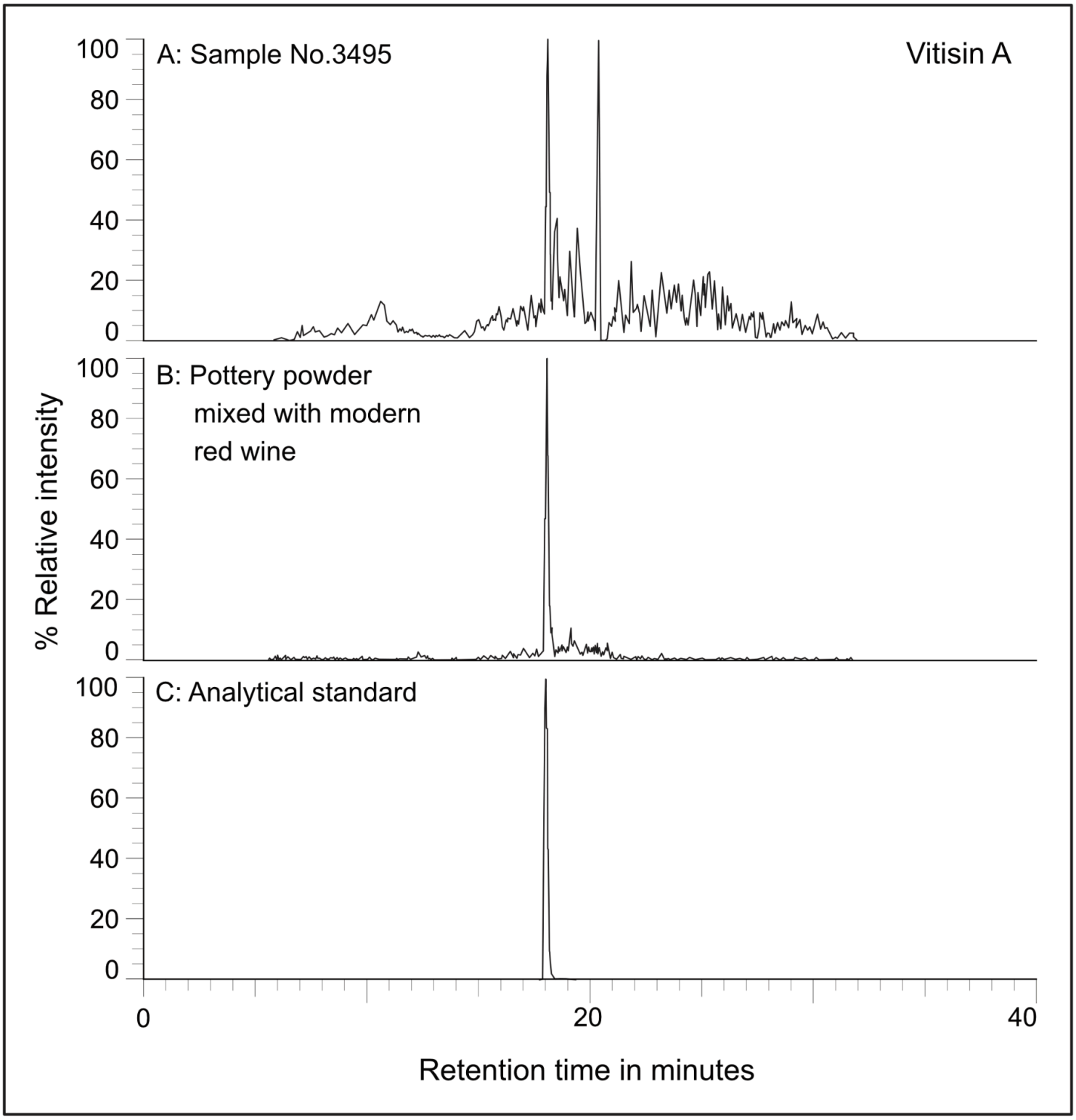


Figure 7

Representative LC–MS/MS chromatograms of vitisin A (*m/z* 561 → 399) from archaeological Sample No. 3495, recovered from a first-century AD Roman amphora at Conímbriga, Portugal. The chromatograms were generated using the targeted LC–MS/MS method described herein and show (A) the archaeological sample, (B) a positive control consisting of pottery powder mixed with modern red wine, and (C) the authentic analytical standard. The vitisin A peak in the archaeological sample co-eluted

with the analytical standard, supporting its assignment. Reproduced from Ref. [30] under the Creative Commons Attribution 4.0 International License.

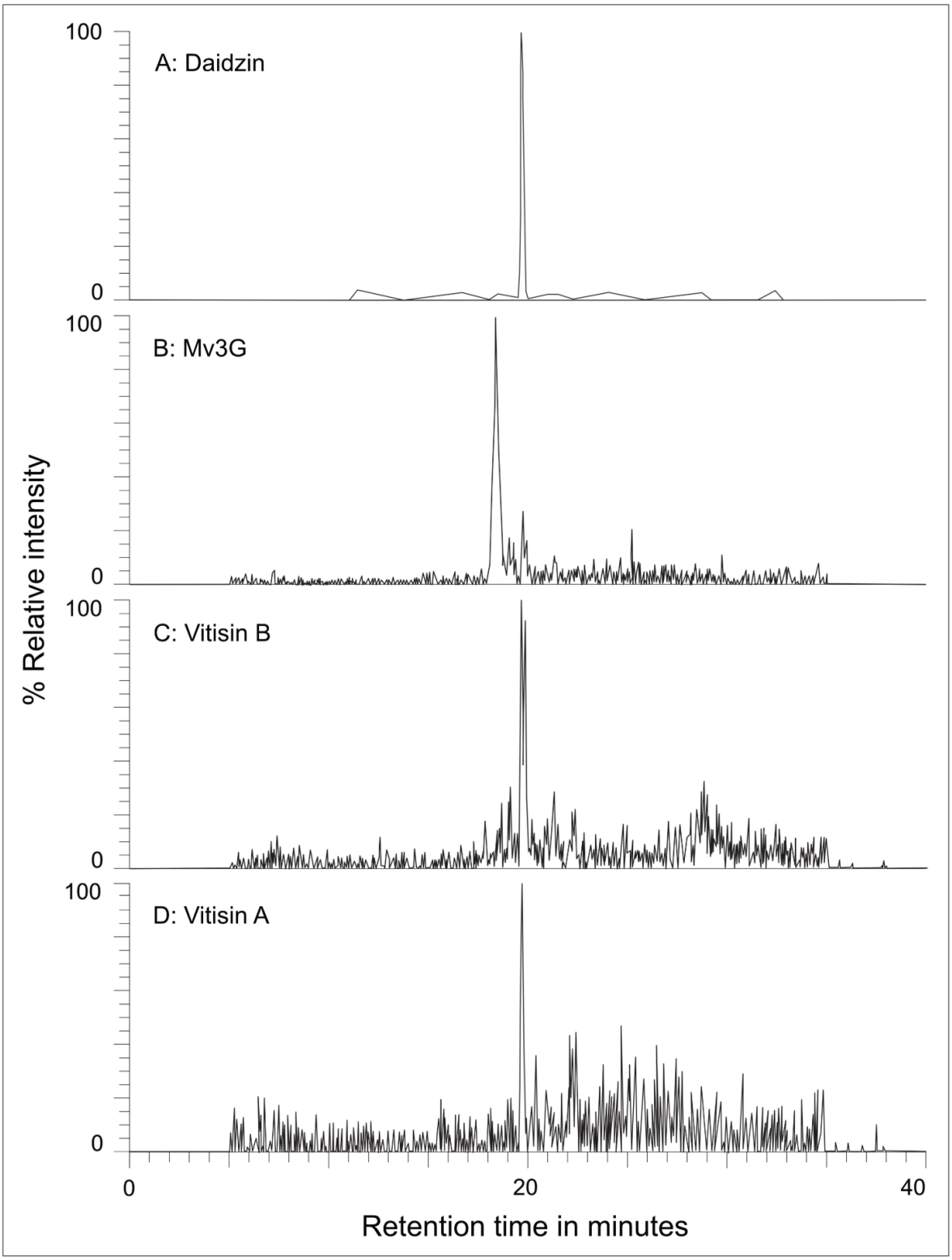


Figure 8

Representative LC–MS/MS chromatograms of archaeological Sample No. 3464 from a Sassanian-period vessel recovered at Fulayj, Oman, showing the monitored precursor-to-product ion transitions for (A) the

internal control Daidzin (*m/z* 417 → 255), (B) malvidin-3-*O*-glucoside (*m/z* 493 → 331), (C) vitisin B (*m/z*517 → 355), and (D) vitisin A (*m/z* 561 → 399). The chromatograms were generated using the targeted LC–MS/MS method described herein. Adapted from Ref. [31] under the Creative Commons Attribution 4.0 International License.

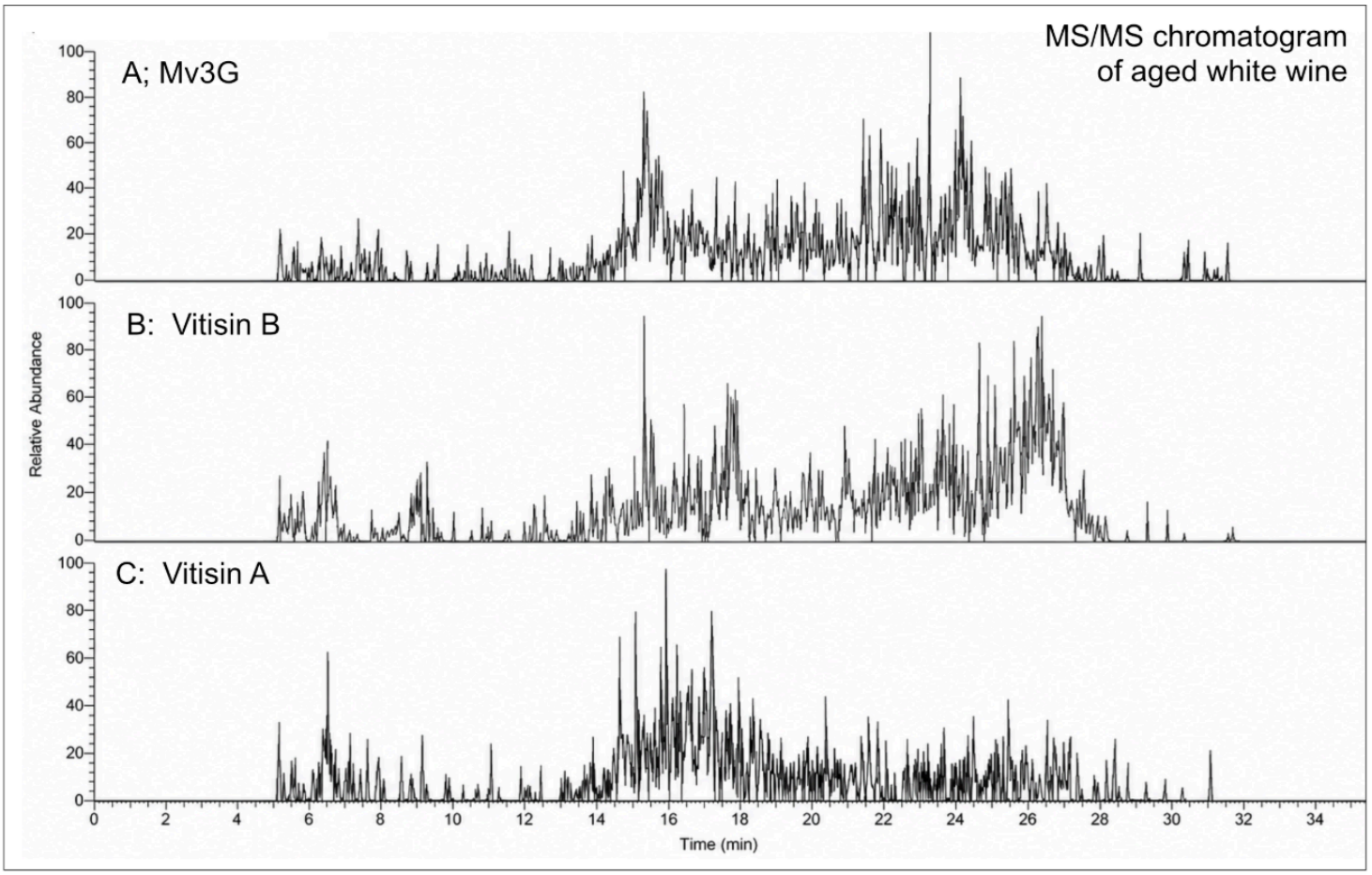


Figure 9

Representative LC–MS/MS chromatograms of an aged white wine control showing the monitored precursor-to-product ion transitions for (A) malvidin-3-*O*-glucoside (*m/z*493 → 331), (B) vitisin B (*m/z* 517 → 355), and (C) vitisin A (*m/z*561 → 399). The chromatograms were generated using the targeted LC–MS/MS method described herein. No detectable signals corresponding to malvidin-3-*O*-glucoside, vitisin A, or vitisin B were observed, supporting the differentiation of white- and red-wine residues.

Supplementary Files

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