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Department of Environmental and Evolutionary Biology, Palaeobotanical and palynological

Presented by

Louise Chassouant

Organic residue analysis in archaeological amphorae

Publicly defended on December 10th, 2021, with the jury composed by:

Pr. Maria Perla Colombini, Full Professor, University of Pisa	**Reviewer**
Pr. Santiago Riera Mora, Full Professor, University of Barcelona	**Reviewer**
Dr Fabienne Olmer, Researcher, Aix-Marseille University	**Examiner**
Dr Nick Schiavon, Assistant Professor, University of Evora	**Examiner**
Dr Carole Mathe, Associate Professor, Avignon University	**Thesis supervisor**
Pr. Cathy Vieillescazes, Emeritus Professor, Avignon University	**Thesis co-supervisor**
Pr. Donatella Magri, Full Professor, Sapienza University	**Thesis co-supervisor**

Résumé

Les travaux présentés dans cette thèse se sont concentrés sur l'analyse de récipients archéologiques. A travers la recherche de marqueurs moléculaires, identifiés par chromatographie gazeuse couplée à un spectromètre de masse et l'observation de restes archéobotaniques, cette étude vise à identifier le contenu originel des récipients étudiés. L'analyse des résidus organiques, aussi bien contenu dans le tesson céramique que dans la couche imperméabilisante à l'intérieur de l'amphore, offre une première lecture de la fonctionnalité de l'objet et de sa contenance. Une importance toute particulière est accordée à l'identification botanique et les techniques de formulation utilisées pour produire une matrice imperméabilisante qui était apposée à l'intérieur de l'amphore. Le volet paléobotanique, principalement axé sur la recherche de pollen, apporte un angle d'analyse nouveau en se concentrant d'une part sur la caractérisation des espèces fossiles environnementales et/ou économiques, et d'autre part sur l'origine botanique des pollens identifiés.

Outre l'optimisation des protocoles existants en matière d'extraction de molécules considérées comme biomarqueurs, cette étude met en avant les bénéfices d'une approche archéométrique multi-analytique à travers l'analyse de différents artéfacts archéologiques provenant de périodes et de contextes hétéroclites. En se concentrant sur l'époque romaine, cette thèse s'attarde sur l'analyse d'amphores à vin et/ou huile provenant des fouilles de l'épave du Planier 3 (France) et de l'ancien ancrage de San Felice Circeo (Italie) avant d'étendre la méthodologie et les acquis sur un vase « verseur » de typologie singulière datant de l'Age du Bronze (Cisjordanie).

Mots clés : Analyse des résidus organiques, palynologie, archéobotanique, protocoles d'extraction, chromatographie gazeuse, spectrométrie de masse, vin, huile, biomarqueur, acide tartrique

Abstract

The work presented in this thesis focused on the analysis of archaeological vessels. Through the search for molecular markers, identified by Gas Chromatography – Mass Spectrometry and the observation of archaeobotanical remains, this study aims to identify the original content of the studied vessels. The analysis of organic residues, both contained in the ceramic sherd and in the waterproofing layer inside the amphora, offers a first reading of the functionality of the object and its content. Particular importance is given to the botanical identification and formulation techniques used to produce a waterproofing matrix that was affixed to the inside of the amphora. The paleobotanical investigation that mainly focused on the search for pollen, brings a new angle of analysis by concentrating on the one hand on the characterization of environmental and/or economic fossil species, and on the other hand on the botanical origin of the identified pollens.

In addition to the optimization of existing protocols for the extraction of molecules considered as biomarkers, this study focuses on the benefits of a multi-analytical archaeometric approach through the analysis of different archaeological artifacts from heterogeneous periods and contexts. Focusing on the Roman period, this thesis focuses on the analysis of wine and/or oil amphorae from the Planier 3 shipwreck (France) and the ancient anchorage of San Felice Circeo (Italy) before extending the methodology and the results to a "pouring" vase of singular typology dating from the Bronze Age (West Bank).

Keywords: Organic residue analysis, palynology, archaeobotany, extraction protocols, Gas Chromatography – Mass Spectrometry, wine, oil, biomarker, tartaric acid

Curriculum Vitae

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Formation

2018 - 2021 **European Joint degree : PhD in Chemistry & PhD in Environmental and Evolutionary Biology**, *Avignon University & Sapienza University*, France & Italy,
· **Research Project : *Organic residue in archaeological amphorae.***
European Joint Doctorate in ARCHMAT (ARchaeological and Cultural Heritage MATerials Science) funded by the European Union's Horizon 2020 under the **Innovative Training Network Marie Skłodowska-Curie grant.** *Supervisors :* Dr. C. Mathe, Pr. C. Vieillescazes & Pr. D. Magri.
Skills : Autonomous project management, scientific writing, literature review, **GC-MS**, **microscopy**

2015 - 2018 **MSc in Molecular and Biological Chemistry**, *EPFL*, Switzerland,
Specialization : Spectroscopy, inorganic and organic chemistry
· **Minor :** Material Sciences
· **Master thesis :** *Determining carboxylic acid concentration in model paints systems.* Understanding of the interactions between linseed oil and zinc oxide related to metal soaps formation (University of Amsterdam - Rijksmuseum). *Supervisors :* Dr. K. Keune & Pr. P. Iedema.
· **Semester Project :** *Characterization of the composition of red footprints from markers.* Study of microtraces of red markers used for street graffiti (EPFL - Criminal Science School UNIL) *Supervisors :* Pr. G. Massonnet & Pr. C. Gervais.
Skills : Autonomous & team work, presentation skills, data analysis, lab skills, **ATR-FT-IR**, **Raman Spectroscopy** and **IR microscopy**

2012 - 2015 **BSc in Chemistry & Chemical Engineering**, *EPFL*, Switzerland.,
3^{rd} year Bachelor International mobility *PUC* Chile

2009 - 2012 **Baccalauréat Scientifique (S) mention Très Bien**, *Lycée Charles Baudelaire*, France,
. Specialization : *Physics & Chemistry*

Experiences

Professional

2015 - 2017 **Teaching assistant**, *Physics ; General and Organic chemistry*, *EPFL*, Switzerland
Skills : Pedagogy, answer queries with precision

Extra-professional

2015 - 2017 **Committee member**, *Festival Balélec & Albaladejo Rugby Club*, Switzerland,
· Signage supervisor for the european largest festival organized by students - Alba Ladies rugby team social manager

2009 - 2016 **Class representatives**,
· 2 years at EPFL · 3 years in high school · 3 years in college
Skills : Responsibility, communication

Publications

· Chassouant, L., Delpino, C., Celant, A. et al., Archaeobotanical and chemical investigations on wine amphorae from San Felice Circeo (Italy) shed light on grape beverages at the Roman time. *PlosOne* PONE-D-21-28574R1 (accepted with minor revision)
· Chassouant, L., Olmer, F., Delpino, C. et al., Protocol Comparison for Organic Residue Analyses from Waterproofing Materials and Shards of Roman Archaeological Amphorae. *Crystals* **2021** 11(11), 1300
· Baij, L., Astefanei, A., Hermans, J. et al., Solvent-mediated extraction of fatty acids in bilayer oil paint models : a comparative analysis of solvent application methods *Heritage Science* **2019** 7 : 31
· Baij, L., Chassouant, L., Hermans, J. Keune, K. and Iedema, P.D., The concentration and origins of carboxylic acid groups in oil paint *RSC Adv.* **2019** 9, 35559-35564

Computer skills

Basic : Mathematica
Advance : LaTeX, Xcalibur, Omnic, CES EduPack, Origin, MS, Inkscape

Languages

French : Mother tongue **English :** Good command **Spanish :** Fluent **Italian :** Intermediate

Personal interest

Social associations, Rugby, Museums
Nature protection, Outdoor sports, Mountain hiking, Climbing

PhD comitee

· **2018 November 28^{th} : 1^{st} year PhD committee, Sapienza University**
Organic residue analysis in archaeological amphorae
· **2019 October 17^{th} : 2^{nd} year PhD committee, Sapienza University**
Organic residue analysis in archaeological amphorae
· **2020 July 3^{rd} : 2^{nd} year PhD committee, Avignon University**
Organic residue analysis in archaeological amphorae

Formation and trainings

· **2018 November $7 - 17^{th}$: Bibliography and scientific diffusion formation**
2 days of formation with an overview of the various possibilities to organize our bibliography and how to diffuse it
· **2019 March $13 - 14^{th}$: Xlstat formation**
2 days of formation on the Xlstat software to understand more on the statistics and its use in archaeological context
· **2019 April $8 - 12^{th}$: Internship at Bibracte (France)**
One week of internship with an archaeologist to investigate amphorae discovered in the archaeological site of Bibracte
· **2019 April $27 - 28^{th}$ & May 2^{nd} : Chromatographic tool formation**
3 days of formation on liquid and gas chromatography with an historical and technical overview and how it can be used in archaeological and cultural heritage context
· **2019 September $2^{nd} - 6^{th}$: Summer School "Diagnosis on Heritage Science : 2. Focus on organic materials in archaeology", Pisa (Italy)**
One week of lectures related to organic analysis in various field of research (archaeological lipids, proteins and DNA, plant macro and micro-remains, wood and waterlogged archaeological wood, dyes, paleopathology). Presentation of several analytical methods (pyrolysis GC-MS, HPLC-MS, radiocarbon dating)
· **2019 Semester III (September – January) : Archaeometry and lab of archaeometry, Sapienza University (Italy)**
One semester of archaeometric lectures with practices on microscope (phytoliths, wood, pollen)
· **2020 January $24 - 30^{th}$: Winter School "Developing entrepreneurial, Project management and Communication transversal skills applied to the Cultural Heritage sector", Evora (Portugal)**
Topics : Managing museum collections ; Project management and international networking on cultural heritage : from LABSTECH to IPERION and E-RIHS and beyond ; Museum and academic research - a fruitful relationship ; Risk prevention for cultural heritage materials and assets ; Digital preservation and dissemination.
Workshops : Science and Communication ; Start-up and enterprise building in cultural heritage
· **2020 May 4^{th} : Inkscape formation**
6h formation on Inkscape software
· **2021 January 19^{th} : Workshop on "Interdisciplinary approaches in scientific research" (online)**
2h workshop from CIVIS agreement between universities (University of Madrid)
· **2021 February 2^{nd} : Workshop on "Social networks, public opinion and science" (online)**
2h workshop from CIVIS agreement between universities (University of Madrid)
· **2021 February $9 - 12^{th}$: Training on "Hints and tips for publishing in ecology, conservation, and environmental journals"**
8h of formation (Sapienza University)
· **2021 February $16 - 19^{th}$: "Grant writing and career perspectives in ecology and conservation science" formation**
8h of formation (Sapienza University)

Formation and trainings (suite)

· **2021 February** 16^{th} **: Workshop on "Transferability and impact of research" (online)**
2h workshop from CIVIS agreement between universities (University of Madrid)
· **2021 March** $9-10^{th}$ **&** $16-17^{th}$ **: Training "Employabilité des doctorants"**
4 days of workshop focusing on Professional project and self-knowledge ; Professional pitch ; CV & Cover letter ; Group recruitment interview ; Individual recruitment interviews ; Salary negotiation ; Professional network development, Social networks
· **2021 March** 23^{rd} **: Workshop on "Publication scientifique"**
1 day of formation on the scientific writing for publishing
· **2021 October** $14-15^{th}$ **: STW1 workshop "Nanotechnology in Archaeological and Cultural Heritage Material Science" (online)**
Workshop on the new methods and materials for the conservation of Cultural Heritage : from renaissance frescoes to modern and art and nanotechnologies applied to the Stone conservation"
· **2021 October** $27-28^{th}$ **: STW4 workshop "Biodegradation and Biotechnology in Cultural Heritage Material Science"**
Practical sessions at the UEVORA Biochemistry labs
· **2021 November** 22^{nd} **: FI MATCH formation**
3h of formation regarding the employability of PhD doctors in industry
Skills developed on how to understand and answer an application form
· **2021 December** $13-15^{th}$ **: STW3 3D technologies Workshop**
3 days of formation on 3D technologies, from definitions and basics to practical lectures on the Archeovision website

Communication

· **2019 May** $7-10^{th}$ **: Technart 2019 (Belgium)**
Organic residues in roman amphorae : a new perspective on ancient trade routes
Chassouant, L., Vieillescazes, C., Mathe C.
· **2021 July** 21^{st} **: Science and Sensitivity 2021 (online)**
Innovating in archaeometry : Combination of organic residue analysis and archaeobotany
Chassouant, L., Vieillescazes, C., Magri, D., Mathe C.

Seminars

· **2019 June** 13^{th} **: Doctoriales 2019 (France)**
Organic residue in roman amphorae
Chassouant, L.
· **2019 July** 1^{st} **: Journée Des Doctorants JDD (France)**
Organic residue in roman amphorae
Chassouant, L.

Dissemination

· **2018 October** 5^{th} **: Science for kids (France)**
Standing and presenting to kids from 6 to 14 years the IMBE research unit on different topics : pigment detection, vegetal model printing
· **2019 February** $27-28^{th}$ **&** **March** $1^{st}-2^{nd}$ **: Research Unit IMBE presentation (France)**
Supporting the IMBE unit and the IRPNC team presentations on the topics of research
· **2021 December** 13^{th} **: Declic 2021 (online)**
Dissemination on research activities and PhD focus to high school students

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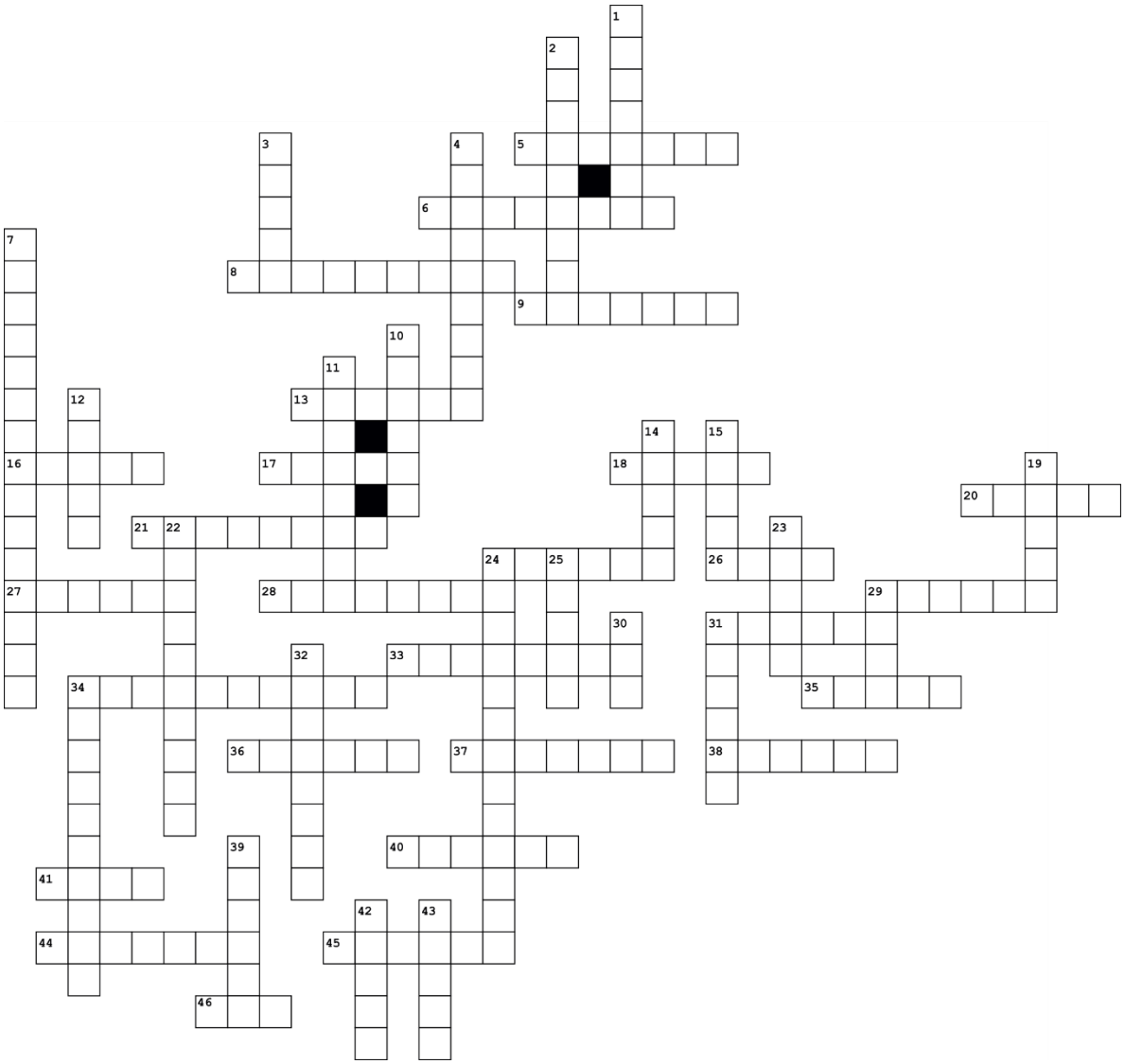
Selon une statistique établie par moi-même, 98% des lecteurs de thèse s'arrêtent à la partie remerciements. Alors pour m'assurer qu'ils passent du bon temps, et parce que les gens cités ci-après connaissent très probablement mon amour pour les blagues et les devinettes, j'ai décidé d'écrire mes remerciements de thèse sous forme ludique. Les personnes sont ainsi décrites dans les pages suivantes.



45 Un immense merci à cette personne qui m'a aiguillée, dirigée, soutenue (et supportée) pendant ces 3 années. Tu as été d'une aide incommensurable, autant quand je passais la porte de ton bureau avec un immense sourire parce que j'avais (enfin) trouvé de l'acide tartrique dans un échantillon que quand je la passais les larmes aux yeux parce que les évènements de la vie font que ! Tu as été un support incroyable et j'ai beaucoup appris à tes côtés. Un grand merci (à ma directrice) pour ton temps, tes conseils et surtout, ton écoute, ta lucidité et ta gentilles dans les moments où ça n'allait pas au mieux.

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28 Un grand merci à notre gestionnaire comptable qui, au-delà de sa grande gentillesse et son sourire agréable, a toujours été d'une aide incroyable quand j'ai eu un souci.

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14 Ahhhhhh ! Je pourrais écrire ça que tu te reconnaitrais de toute façon. Avec une moyenne de deux appels de plusieurs heures par semaine, c'est ma compagnie d'abonnement téléphonique qui doit être contente ! Tu es un petit soleil dans la vie ! Et t'entendre glousser, c'est comme entendre la sonnerie de récréation quand on était à l'école : signe que les choses deviennent intéressantes et qu'on va rigoler ! Cette thèse n'aurait pas été la même sans t'avoir eu aussi proche et aussi loin ! On se revoit vite pour manger pleins de trucs épicés et chopper la turista après que tu sois allée marcher dans le désert !

20 Une autre âme-sœur. Bon je sais, on ne peut en avoir qu'une seule, mais en fait, vous êtes deux ! Merci pour les rires, les sorties, les rires, les activités, les rires, les festivals, les pauses thés, les rires, le sport le matin, les mois de colocation qu'on pourrait dire concubinage presque. Merci pour les plans galères, les tentes sans arceaux, les plans absurdes, les plans pas réfléchis, les concerts les veilles de grands jours. Toujours dans les bons coups =) C'était ouf de faire ma thèse avec toi à côté ! Et dire qu'au début, je m'étais dit que tu avais l'air d'être une fille drôle !

41 Tu venida a Avignon ha sido un regalo increíble. ¡ Gracias por todas las risas, por las experiencias divertidas contigo ! Tus aventuras y tu sonrisa alegraron alcunos meses de la tesis. Awuevo chiquilla !

21 Un grande ringrazimiento al mio amico del microscopio, che ha sempre risposto quando le dicevo « *****, ti posso fare una domanda". E a volte "domandina" quando sentivo che era davvero un sacco di volte che ti fermavi, ti alzavi e che venivi da me a dire "una quercha". Mi ricordo quando sono arrivata nella tua officia, e stavi ascoltando il Vecchio Testamento, con la lista dei "figli di"! Che scherzo! Grazie per avermi insegnato il romano cosi bene, per avermi portata a mangiare la pizza, per avermi fatto complimenti per il mio italiano. Grazie per essere stato un'ancora di salvezza quando ero stufa del microscopio e di questa tesi. Grazie per avermi fatto ridere semplicemente essendo te !

7 Ah là aussi je ne saurais pas trop par où commencer pour remercier cette fameuse bande de joyeux lurons que vous êtes ! Malgré la distance qui limite un peu nos aventures, je suis super contente et je vous remercie mille fois de votre support ! Des rires, des sorties et des WE où on n'a pas inventé des vaccins contre grands choses ! Un grand merci à toi petite blondinette pour ton écoute bienveillante et ta manière vraiment intéressante de voir les choses ! Merci à toi petit Ju-jitsuka pour tes blagues qui me font souvent bien rire et les belles discussions ! Merci à toi petite dame qui va au cabinet tous les jours, pour ton soutien, ton sourire et tes petites blagues quand on les attend le moins. Merci à toi Mr Docteur, je te suis entièrement reconnaissante pour m'avoir permis de relativiser sur la rédaction. Je te dois une fière chandelle parce que je me suis vraiment inspirée de toi pour gérer mon stress et passer du bon temps dans ma rédaction, malgré les efforts. Tu m'as pas mal inspiré sur ce coup-là. Merci à toi petit matheux qui envoie les messages les plus saugrenus de l'histoire des messages, ça me fait toujours bien sourire. C'est toujours un plaisir de discuter et échanger avec toi. Et enfin, merci à toi futur Docteur pour les bons moments passés ensemble ces derniers temps, entre Avignon et la Sicile ! J'aurais parié que tu soutiendrais avant moi, et ça m'avait bien fait stresser de savoir que je serais la suivante après Tom ! Mais j'ai déjà hâte de venir à Londres pour fêter ça !

11 Je ne pouvais pas ne pas te citer dans les remerciements, et tu méritais bien une ligne à ton nom ! Merci pour tous ces moments passés ensemble, que ce soit des WE de nature, de culture et de fêtes. Merci pour les rires, les sorties, les bons repas, les discussions, les débats, les sourires, les petites attentions qui font que le quotidien dans cette thèse a été vraiment chouette ! Merci de m'avoir initiée à la grimpe, de m'avoir écoutée te raconter en long en large et en travers mes podcasts, mes grandes

interrogations, et même mes petites. C'est un peu grâce à toi que je suis venue à Avignon, et ça a vraiment été une belle aventure !

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19 Thank you for your support, your jokes and your smile! It has really great to have you in my back during this thesis, to hear your weird music to concentrate and for the chit-chat in the lab! I still remember the first time we met in the campus, looking at each-other like "Are you the other PhD?". It was really nice to have you in the office to talk, to claim, to cry (sometimes). Thank you for your amazing support.

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10 Thank you for your jokes, your smile, your humor and the really tasty food you offered me to try so many times. Although you did not convince me about Portugal, you tried very hard at least! I hope to see you in India next time!

31 (horizontal) Partie – Revenue - Partie – Puis revenue! C'est mieux que les Feux de l'Amour cette histoire ! J'ai commencé cette thèse avec toi, et tu m'as appris tellement de choses ! Je suis tellement contente de la finir avec toi ! Merci pour les grandes conversations, les pauses-thé, les rires dans le bureau, et les pauses clopes sans clope ! Merci aussi pour les merveilleux conseils qui m'ont évité bien des soucis ! Et enfin, merci pour avoir toujours été une oreille (très) attentive ! Ton rire a vraiment égayé le bureau et tes « Non mais Alice », signe annonciateur qu'on va rire, je les entends encore !

33 Un petit soleil ! Merci pour ta spontanéité, tes histoires drôles et ton écoute avisée de mes aventures, toujours avec un œil critique bien entendu ! Quand je t'ai vu la première fois, j'ai su qu'on allait bien s'entendre les deux ! Je suis juste déçue qu'on n'ait pas commencé cette thèse en même temps, on aurait eu 1 an de plus pour profiter !

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36 Une magnifique rencontre ! J'ai beaucoup appris dans nos conversations où je ne vois pas trop le temps passer, posés sur le canapé en train de manger. C'est un plaisir de te raconter mes petites aventures parce que je sais qu'on va pouvoir en rire !

12 La dernière de notre petit groupe de fille du bureau B201 ! Mon amie des Picons ! Toujours là pour discuter ou aller boire un coup ! Merci 1000 fois pour ces pauses-thé, qui se sont transformées en sorties plus nocturnes avec le temps, mais l'intensité des discussions et le plaisir qui vont avec sont bien restés le même ! Et merci pour cette idée fameuse de mise en page des remerciements !

43 Et merci à toi Jeune zythologue. Allez, c'est bien parce que ça va être publié que je ne fais pas la blague ! Merci pour ta connaissance accrue des boisons maltées, qui m'a régalée pendant plusieurs années ! Sans toi, on n'aurait probablement pas découvert le BD et sans vous (avec 25) et on n'aurait surement pas autant ri ! Mille mercis pour les bonnes blagues 'Bro', les fléchettes, et surtout … Les Blindtests !

31 (vertical) Alors, lui, euh, c'est un ami euh, enfin bon tu vois quoi, qui euh alors, sublime n'importe quel, euh moment, tu vois, et surtout si, en fait, il y a, euh, de la nourriture, enfin, aux alentours ! Alors, merci pour euh, avoir partagé mon stress pendant le premier stage de parapente ! Et alors merci pour avoir été là quand on s'est fait phrasé sans comprendre un mot ! Daje, che ti potevo anche ringraziare in italiano ! Grazie per tutte le volte che sei venuto a prepare il cibo ! Riccordo che teni ancora un bel pezzo di parmeggaino per fare la pasta con le patate, che ci cendra buona sta volta.

16 Rencontre récente sur Avignon : un match parfait ! Merci pour ta bonne humeur, pour toujours noter le détail drôle chez Killian qui va nous fait rire ! Et merci pour ses 10kg de pain ahah !

1 Merci pour m'avoir emmenée grimper à un moment où j'en avais beaucoup besoin je crois. C'est toujours chouette de discuter avec vous (avec 16), et ça a vraiment été d'un soutien incroyable quand ça n'allait pas trop même si je ne le montrais peut-être pas trop…

15 Un petit rayon de soleil dans une tornade matérielle et logistique et un presque-stress de rédaction, voilà ce que tu es ! Sans toi, c'est clair que la rédaction n'aurait pas été aussi amusante et je te suis reconnaissante de m'avoir offert des sorties, des pauses, des petites ellipses temporaires alors que dehors, c'était le feu et la fatigue !

9 My mentor in science, maybe I shall call you this way! Thank you for teaching me all these things in research, for trusting me and letting me improve! You opened me the doors of the labs of the Rijksmuseum, and the conservation workshops, I will always have an incredible memory of it, even if it was 4 years ago! Without you, I probably wouldn't have started a PhD.

13 Un grand merci pour le soutien technique! Un petit couteau-suisse qui a toujours une solution et en plus, avec le sourire ! Tu manques au Campus Agroparc !

26 Un grand merci parce que tu m'as techniquement sauvé la thèse ! Oui oui ! Sans toi, j'aurais pleuré pendant tout l'été la perte de cet ordinateur ! Merci pour toutes ces matinées et toutes ces soirées, où te voir à l'entrée et à la sortie du bâtiment, avec ton sourire, donne le sourire !

24 (vertical) Un ancien membre de l'IRPNC qui joue au va-et-vient lui aussi ! Merci pour m'avoir enseigné pleins de choses pendant les quelques temps où on a pu être au labo ensemble ! Toujours le mot pour rire ce rugbyman !

34 (vertical) Merci à tous les étudiants des labos du deuxième étage, pour les conversations et les petites pauses et les gâteaux ! Entre autres Emilie, Boutheina, Sarah, Léna, Léo, Hugo, Ombeline, Emmanuelle.

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44 Membre actif des voisinades, merci à toi aussi pour ton esprit vif, ta connaissance culturelle et littéraire, et tes bonnes blagues d'italien qui me font rire !

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25 Merci pour m'avoir laissé ta chambre à Lausanne et avoir gentiment choppé le Covid pile quand j'étais venue te voir ! Quel BG celui-là !

29 (vertical) Merci ****-Chou pour être aussi … Chou ! Aussi partant pour tout et aussi désespéré par tout ! Même si je continue à penser que glousser un peu plus te ferait le plus grand bien, c'est quand même bien agréable de discuter avec toi, et d'échanger sur nos podcasts.

23 Merci pour m'avoir accueilli dans ton chez-toi Lausannois et ne pas avoir eu le Covid quand je suis venue te voir ahah ! Toi t'es un vrai BG !

4 et **3** Un grand merci pour votre soutien à distance, ces audios qui nous maintiennent en contant rapproché, les balades sous la pluie et les JO devant la télé avec un thé ! J'ai encore en tête cette fameuse soirée au restaurant cet été, où on a vraiment beaucoup ri des cafetières à presse et des puzzles !

34 (horizontal) Au top cette fine équipe qui est là depuis le lycée ! Merci pour les balades et les looooongues discussions. Et comme on ne se voit pas super souvent, vous m'avez permis de bien visualiser l'évolution de cette thèse et comment c'était l'année précédente !

8 Merci merci merci pour la relecture ! Avec des parties plus drôles que d'autres à corriger j'imagine ! Tu as été top !!!

24 (horizontal) Merci pour m'avoir sauvé tant de fois de ces galères technologiques ! Le vol d'ordi, le disque dur cassé, le dock, Bitlocker… il y en aura eu des péripéties ! Merci pour avoir répondu présent même pendant tes vacances pour calmer mon stress naissant de perte d'ordinateur !

42 Sacrée rencontre dans les airs ! Merci de m'avoir fait voler pour la première, et j'espère, pas la dernière fois !!! Je n'attends qu'une chose : revenir te raconter des petites blagues par talkie-walkie, moi dans les airs, et toi sur la piste d'atterrissage !

29 (horizontal) Parce que je suis sûre que tu jettes un œil de temps en temps, d'où que tu sois ! C'est peut-être toi qui m'as le plus aidée dans cette thèse, malgré toi, en me montrant qu'il y a des choses plus graves qui existent.

37 Et le meilleur pour la fin. Le Larousse vous définit comme l'«Ensemble des personnes unies par un lien de parenté ou d'alliance », mais c'est bien plus que ça. Si je devais écrire moi-même une définition, je dirais plutôt que c'est un ensemble de personnes unies qui ont toujours été là pour moi, que ce soit par des attentions, des conversations ou des moments partagés. Qu'aurait-été cette thèse si je ne vous avais pas eu pour faire passer ma colère, ma tristesse ou ma peine ? Vous avez été un super incontestable et je vous en remercierai à jamais. Merci pour m'avoir écouté au téléphone quand j'avais juste envie de pleurer, pour m'avoir gâté de bons légumes, de délicieuses conserves et de fromage pour que mon cerveau soit bien nourri, merci pour les WE nature, les activités culinaires, les jeux de cartes, les rires qui ne partent de pas grands choses, les histoires drôles autour du thé et des tartes faites par la Reine des tartes. Merci pour votre soutien, impossible à définir avec des mots !

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45 Carole

17 Cathy

2 Donatella

22 Alessandra

27 Céline

32 Federico

38 Chiara

39 Teresa

6 Fabienne

28 Virginie

5 Mariama

14 Fanny

20 Alice

41 Gaby

21 Fabrizio

7 Bretzels & Techno

11 Gauthier

35 Fanny

19 Milan

46 Val

10 Aditya

31 (horizontal) Elodie

33 Amandine

30 Leo

36 Pierre

12 Marie

43 Tophy

31 (vertical) Enrico

16 Sarah

1 Kilian

15 Louise

9 Lambert

13 Karine

26 Saad

24 (vertical) Jean-Baptiste

34 (vertical) Doctorants

18 Manon

44 Etienne

40 Julian

25 Robin

29 (vertical) Jean

23 Maxou

4 et **3** Charlotte et Laura

34 (horizontal) Dream-Team

8 Gabrielle

24 (horizontal) Jérémy

42 David

29 (horizontal) Julien

37 Famille

List of Abbreviations

AcOEt : Ethyl acetate

aDNA : ancient DNA

BSTFA/TMCS : N,O-Bis(triméthylsilyl)trifluoroacétamide / triméthylchlorosilane

$CHCl_3$: Chloroform

DAG: Diacylglyceride

DCM : Dichloromethane

DEE : Diethyl ether

DHA : Dehydroabietic acid

DHAM : Dehydroabietic acid methyl ester

DNA : Deoxyribonucleic acid

DTMS : Direct Temperature-resolved coupled to Mass Spectrometry

EtOH : Ethanol

FA : Fatty acid

FFA : Free fatty acid

GC-MS : Gas Chromatography coupled to Mass Spectrometry

HPLC : High Performance Liquid Chromatography

HPLC-ESI-QToF : HPLC coupled with Electrospray Ionization - Quadrupole Time-of-Flight Mass Spectrometry

KBr : Potassium bromide

KOH : Potassium hydroxyde

MAG : Monoacylglyceride

MeOH : Methanol

MS : Mass Spectrometry

MW : Microwave

NMR : Nuclear Magnetic Resonance

NPP : Non-Pollen Palynomorph

ORA : Organic residue analysis

PTFE : Polytetrafluoroethylene

Py-GC-MS : Pyrolysis Gas Chromatography coupled to Mass Spectrometry

TAG : Triacylglyceride

THF : Tetrahydrofuran

TIC : Total Ion Chromatogram

TMS : Trimethylsilylation

US : Ultrasound

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General introduction

Archaeological problematic

This PhD thesis falls within the promotion of interdisciplinary in the understanding of archaeological and cultural heritages, with the specific aim of developing multi-analytical skills to fairly diagnose ancient materials. The work presented results from the fruitful collaboration between Carole Mathe de Souza, Cathy Vieillescazes and Donatella Magri. With the aim of developing intersectoral knowledge and competences, the research was conjointly carried out at Avignon University and Sapienza University, which possess complementary expertise fields. The organic and analytical parts were developed within the IRPNC (Ingénierie de la Restauration des Patrimoines Naturel et Culturel) lab from the IMBE department (Mediterranean Institute of marine and terrestrial Biodiversity and Ecology) in Avignon. The organic research axis was supervised by my director Carole Mathe de Souza and co-director Cathy Vieillescazes. The lab is specialized in molecular archaeometry, with a particular interest in natural substances present in artistic and archaeological heritage. Through the analysis of color dyes and pigments, easel paintings, mummies balms and ethnic objects, the lab has gained a notable expertise in organic residues, with relevant competences in spectroscopy and chromatography. Natural plants analyses have greatly contributed to the understanding of thermal and photochemical degradation of organic molecular markers and biomarkers from resins, pigments and dyes. Then, archaeobotanical analyses, specifically including palynology, were followed by my co-director Donatella Magri, and admirably supported by Alessandra Celant, both from the Dipartimento di Biologia Ambientale. The long expertise in paleobotany and palynology is included in environmental biology through the analysis from the micro to macroscale. The research is conducted on botanical proxies such as pollen, spores, non-pollen palynomorphs, diatoms, wood fragments, leaves and seeds.

My thesis work focused on the analyses of organic and archaeobotanical residues in archaeological amphorae. The molecular characterization was carried in continuation of Hitomi Fujii's PhD thesis that dealt with the deciphering of amphorae content and coatings, handled at IRPNC and defended in 2018. In collaboration with the archaeologists Fabienne Olmer (France) and Chiara Delpino (Italy), we had the chance to develop our analytical lens on Roman materials from marine context. The samples having spent more than 2000 years under the Mediterranean Sea, the recovery and the analyses of organic residues was all the more challenging. In this regard, we used scientific lens to provide answers to the interrogations raised by the archaeologists. Triggered by the dearth of collaboration between anthropological and scientific disciplines themselves, the problematic was elaborated to overcome the analytical limitations inherent to the objects through a multi-diagnostic study. The aim of this thesis encompassed the development of innovative analytical tools to characterize the content of archaeological amphorae, whether they exhibit visible traces of organic waterproofing. By studying the organic residues of amphorae, we decided to place the archaeological object at the core of the problematic. This choice was taken to integrate them into a broader historical, anthropological and ethno-geographical context. Analyzing micro and macro remains allowed us to better understand the goods exchanged in the past, their origins and their manufacturing processes. In this way, we aimed at unravelling trade routes developed in ancient times and the geographical boundaries of this trade while interpreting the technical prowess and the *savoir*-faire of past civilizations. Understanding the object in its entirety would finally ensures an effective valorization of the object's history and durable conservation. Since objects are singular, it is important to treat them as witnesses of the past, eager to reveal their secrets to future generations.

In this regard, two different approaches are developed hereafter. The chemical analyses of organic residues were carried out at Avignon University. The residues correspond to the microscopic traces and macroscopic remains, preserved in the waterproofing layer of the amphorae or in the archaeological shards themselves. The molecules, extracted from their original matrix by different analytical protocols are then characterized by chromatographic tools. Through comparison with contemporary reference materials, they shed light on the archaeological content, while giving clues on the nature and formulation of the waterproofing coating coming from natural resinous materials, as well as its state of degradation. Palynological investigation was developed and performed at Sapienza University. The pollen contained in the pitch have allowed to support, increment, and even define new horizons in the archaeological interpretation of the studied vases. In the light of the fresh collaboration between these two labs of independent expertise domains, the project aims at developing multi-analytical tools in vessel and ceramic analysis.

ED-ARCHMAT

The research work takes place in a generalized program aiming at developing scientific lens to understand, diagnosis and prevent from degradation archaeological and cultural heritages. The project is supported by the Doctoral School of ARCHMAT (Archaeological and Cultural Heritage in MATerials Science). It accounts for one of the thirteen doctorates funded by European Union's Horizon 2020 research and innovation program (H2020-MSCA- ITN-2017- EJD): Marie Skłodowska-Curie Innovative Training Networks (European Joint Doctorate) – Grant agreement nº 766311 (ESR9).

The research program provided us an astonishing support in terms of cultural and educative opportunities of learning and networking, with a specific focus on trainings and dissemination. With the purpose of bridging gaps in cultural heritage management by providing scientific support, the methodology we are hereby developing focuses on the fundamental shift from a unilateral or multidisciplinary understanding of the archaeological materials to an inter and transdisciplinary approach. Associating independent fields of research together promotes the accumulation of combinative evidence to better address the initial archaeological interrogations. The great collaboration all-over the Mediterranean basin, with university laboratories, archaeologists, researchers, museums and business institutions helped in building, structuring and orienting the research problematic. In order to access a deep knowledge of the studied material, innovative tools that promote cutting edge science through scientific and technological advances were developed. By encouraging a common language between cultural heritage protagonists and scientific, we aim at accessing high technical levels of diagnostic capacities. Aside from the valorization of such a fruitful program, the final axe of development focuses on the dissemination of the out coming discoveries. The transfer of knowledge through entrepreneurial initiatives is encouraged. The dissemination of technical advances together with the promotion of the acquired knowledge need to be spread over to enhance communication between the diverse cultural heritage communities. This way, the conservation of the cultural heritage can be ensured for the future generation.

Manuscript construction

The manuscript is structured on the basis of a comprehensive and exhaustive state of the art focused on the three fundamentals of this thesis: the archaeological amphorae, the molecular markers and the pollen markers. The investigated materials are presented in the first chapter through a general overview of the primordial role containers played in the development of trading under the Roman Empire. Archaeological amphorae we worked on are described in their historical contexts from the sunken ship of Planier 3 (France) and the ancient anchorage of San Felice Circeo (Italy).

Molecular and analytical tools that help in interpreting residues contained in the amphorae are detailed in the second chapter. A complete picture of the different types of molecules recoverable in the chromatographic analyses is presented in order to introduce the concept of biomarker. The chapter focuses on the analytical advances through a chronological and historical description of the tools developed, highlighting their advantages and limitations.

Finishing with the introductive chapters, a comprehensive summary of the use of pollen in archaeology is lastly developed. After giving an extensive definition of pollen grains dispersion and preservation over time, methodological approaches are also discussed in the third chapter, from the sample collection to the grain's identification.

The following parts present the outcomes of the thesis. The artefacts investigated are thereupon reported through different thematic, organized in independent chapters, spanning from the presumed archaeological content or historical contexts to the methodological approach developed. Results and interpretations are discussed in the form of scientific articles. The extraction of wine markers from archaeological resinous materials and shards is primarily developed in the fourth chapter, accepted in the special issue of the journal *Crystals (*DOI 10.3390/cryst11111300). It deals with the analysis of wine containers from Planier 3 and from the anchorage of San Felice Circeo. The fifth chapter was accepted with minor revision in the *PlosOne* journal. It details the archaeological value of an interdisciplinary study combining chromatographic and archaeobotanical tools applied on amphorae thought to have contained wine. Although the investigated amphorae belong to the already studied artefacts from San Felice Circeo, the focus was settled on the archaeological and anthropological considerations based on the discovery of *Vitis* pollen grains exhibiting a singular morphology. The sixth chapter deals with the application of the transdisciplinary methodology we initially developed on a very different archaeological context. Leaving the Roman period for the Bronze Age, the analysis of a spouted vase recovered from Jericho (Southern Levant) were interpreted in the light of archaeological medicinal concoctions. The article might be submitted in international journals of archaeometry. Finally, the seventh chapter that encompasses chromatographic analyses of presumed oil amphorae recovered in Planier 3 interrogates on the cultural use of oil at the Roman period. Following this, a general discussion is developed to support the importance of a transdisciplinary methodological approaches to better understand the history of the object highlighting the perspectives of such a fruitful research project.

Chapter 1

Amphorae, fabrication and uses

1.1. Once upon a time: the amphorae

Even if the earliest evidence of jar is dated back to the Neolithic period, the use of amphorae has been developed by the Canaanites around the 15th century BC for ship trading. Due to several logistical advantages in terms of solidity, transport and packing facilities, the Egyptians started to produce their own amphorae a century later. The Phoenician civilization popularized the amphora-shape concept around the Mediterranean Sea. From then on, following Greek and Roman populations developed different amphora typologies, characteristic of the content but also of the manufacture place, the period and/or the civilization (Grace, 1949; Garcia Vargas, 1996; Cipriano and Carre, 1989). Amphorae design thereby evolved to rounder egg-shape bodies in order to optimize sailing goods quantities. For instance, Whitbread reported that Greek ships could contained around 3000 amphorae of 29 liters each in the average (Whitbread, 1995).

The expansion of the Roman Empire corresponded to an intensification of commercial activities throughout the Mediterranean and therefore coincided with the expansion of amphorae manufacture. In order to supply oil, wine and other consumable demands, containers were massively produced to the extent that the Western regions of the Empire were ruling over the production during the late Roman period (Formenti et al., 1978; Manacorda, 1989; Palazzo, 1989). Made of abundant and disposable local clay, they widely contributed to coast local economies. Since the full container was quite heavy, amphorae may have been mass produced nearby and empty transported to harbors where they were filled before shipping. Not only the exported goods but also amphorae themselves were part of the commercial activities (Lyding Will, 1992).

Their inorganic building material, which prevent from breakage, accounts for its slowness to degrade. Hence, amphorae are commonly found in archaeological sites dated from 1500 BC to 500 AC.

1.2. Amphorae typology

Amphorology corresponds to the investigation field dedicated to amphorae morphology studies. Heinrich Dressel first implemented this discipline in 1899 by relating their shape difference and manufacture places. He built up a tailored classification to provide spatial and temporal information on Roman trades. Enhanced by the discovery of many underwater amphorae, the classing discipline however needed to wait for several years to get a real impact. This way, some archaeologists such as Lamboglia or Beltran greatly contributed to the garnishment of the already existing classification (Twede, 2002). One pillar of the identification is the shape identification as shown in Fig. 1.

“Amphora” comes from Ancient Greek: “*amphi*” and “*phoreus*” respectively meaning “both sides” and “bearer”. As presented in Fig. 1, amphorae were built up to stand on a central heavy base, usually called “foot” from which was extended the “body” (or “belly”). The “shoulder” corresponds to the upper part of the body, which slightly bent to connect the neck. A thicker rim circled the top of the neck to control the poured content flow. The ingenuity lies in the presence of two hand holders to link the shoulder to the neck, which widely facilitate the handling. Grabbed by these holders, amphorae were maneuvered by rotational movements to ease the transportation, the pointed base being used as a pivot. They were

sealed with cork stopper or ceramic mortar made of volcanic sand (*pozzolana*) and lime (Lyding Will, 1977).

Amphorae shape is a great symbol of packaging technology inherited from ancient civilizations. As described by Parker and Katszev, the shape may have been adjusted to optimize amphorae stacking and minimize empty spaces in the cargo, starting from the hull to the central part of the hold. Foot bases, necks and bodies were indeed nested with each other in such a packed way that amphorae could not move while sailing (Katzev, 1970; A. J. Parker, 1992).

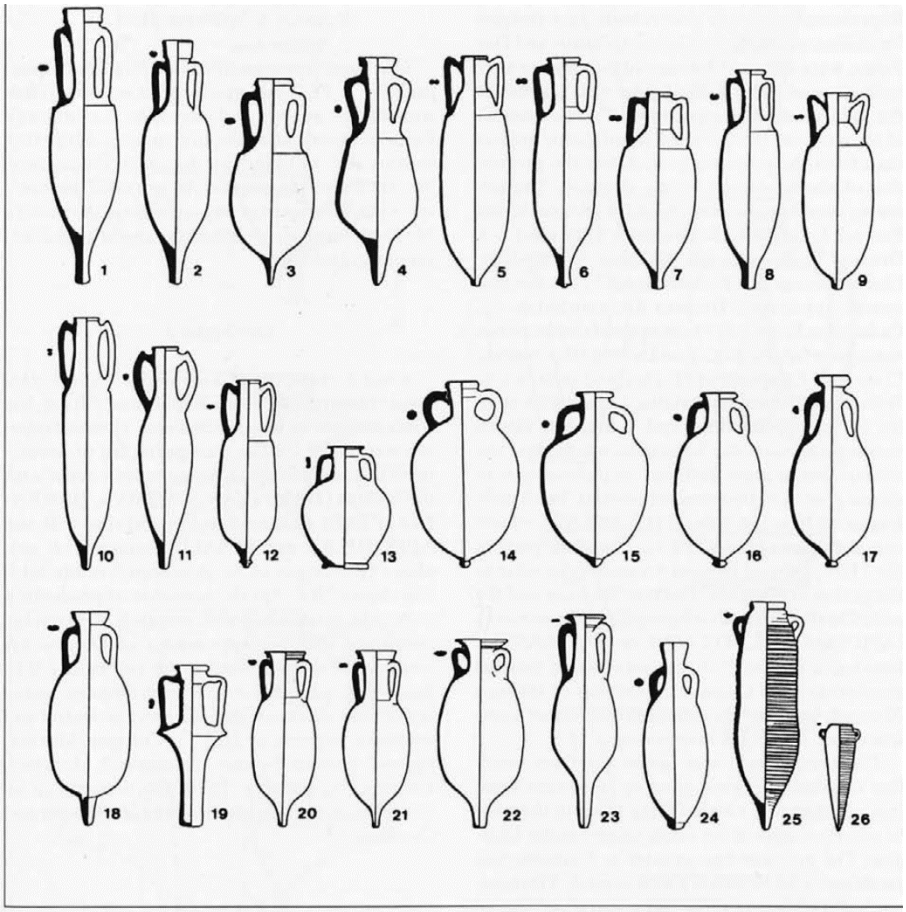

Fig. / Sl. 2: Types of amphora mentioned in the text. / Tipi amfor omenjeni v besedilu. 1 Dressel 1B; 2 Dressel 1 - Pascual 1 3 Lamboglia 2; 4 Dressel 6A; 5 Dressel 2-4 (Koan); 6 Dressel 2-4 (Falernian); 7 Dressel 2-4 (Pompeii); 8 Dressel 2-4 (Tarraconensis) 9 Dressel 2-4 (Gaulish); 10 Dressel 5; 11 Rhodian; 12 Knidian; 13 Gauloise 7; 14 Dressel 25; 15 Brindisi Type; 16 ante 6B 17 Dressel 6B; 18 Haltern 70; 19 Schörgendorfer 558; 20 Dressel 10; 21 Dressel 7; 22 Dressel 9; 23 Dressel 8; 24 Portorecanati 25 Richborough 527; 26 Camulodunum 189.

Figure 1. Most common amphorae shape differences (Bezeczky, 1998)

Commercial activities and amphorae production became so substantial that amphora morphology gave rise to a standardized measuring scale in the Roman Empire. For example, the Greco-Italic style showed up to overcome the need of a common shape between Greco-Roman sailing trades. Every shape evolution resulted from the improvement of the design capacity of the mass ratio. This way, the shape was optimized to allow the container to transport the maximum beverage quantity at once. Usual dimensions

ranged from 45 cm to 1 m. They could weight up to 40 kg and carry until 80 liters (Peacock & Williams, 1986).

1.3. Inscriptions: a precious help to trace back amphorae

Diverse indications from the amphorae can help characterizing jars. Indirect hints such as the shape or the coarse clay composition can shed light on the origin, the site of production as well as the possible content. For instance, wine amphorae were longer and thinner compared to olive jars that had rounder bodies. Elizabeth Lyding Will reports that Roman typologies dated from 30 BC to 400 AC highlighted more the nature of the content rather than the manufacturing region (Lyding Will, 2000).

While ancient jars were only identified thanks to their emblematic shapes, the use of content labels directly stamped or painted on the clay became systematic after 400 BC. Tweede classified them in two categories: the explicit annotations, which clearly provided administrative indications (identity of the manufacture site owner, eventual export duties, volumetric capacities, *etc*.) and the non-explicit ones made of symbols and letters detailing the content (Twede, 2002). This way, stamps and *tituli picti* must be distinguished (Fig. 2 and Fig. 3). While non-explicit indications can be directly stamped on the neck, the holders or even the shoulder with a ceramic mold marker, explicit *tituli picti* were hand painted. Unfortunately, it is not always possible to identify such indications on the archaeological amphorae, especially when the jars are not in optimum conservation states.

Figure 2. Stamp on an amphora found in Planier 3 shipwreck (DRASSM, credit photo: C. Joliot, 2018)

Figure 3. Titulus pictus written on an Ostia type African amphora. "VIR" refers to the red wine content (ui(num) r(ubrum)) and "MPS" may correspond to the wine merchant) (Bonifay et al., 2015)

1.4. Amphora fabrication process

1.4.1. From the coarse clay to amphora

Amphorae are the product of complex shaping, drying and heating processes. The non-respect of the manufacture conditions usually leads to physical weakness and the object may break.

From the fabrication point of view, after the choice and preparation of the raw coarse clay, the material is shaped, the surface may be greased, and the object is dried to prevent from structural damages related to residual water fast evaporation within the matrix during the following firing step. Firing temperature must be high enough to promote evaporation of adsorbed and inter-planar water as well as structural hydroxyl groups (Pollard & Heron, 2008). Roughly estimated between 500°C and 800°C, the temperature strongly depends on various factors such as the raw material, the crystallinity degree and the firing conditions (temperature profile, combustible nature, aerobic conditions) (A. M. Hunt, 2017). Some organic post-firing treatments can finally be applied directly on the hot surfaces or more rarely, after cooling. Several practices have been reported in past ethnographic investigations of internal jar or cooking pot surfaces. Vegetal or animal evidence respectively made of tree sap or fats, milk or even blood have been pointed out (Harry et al., 2009; Shepard, 1956; Skibo, 2013). Diverse coating techniques such as the dipping, the smoking and the spraying or even the rubbing of natural components have also been detailed depending on the liquid or solid state of the treatment (Lea Drieu, 2017; Rice, 1987; Skibo, 2013).

1.4.2. The resin: a natural resource to waterproof the amphorae

In the specific case of amphorae, post-firing coatings were principally developed to overcome the ceramic permeability towards liquid content. Since the porosity was a real issue for sailing activities, waterproofing coatings became necessary. Conventional techniques borrowed from material sciences adequately detailed the extent of the porosity (Allegretta et al., 2017; Harry & Johnson, 2004). In 2000, Stern established the concentration gradients of the fatty acids adsorbed from the inner to the outer part of the Canaanite sherds (Ben Stern et al., 2000). Waterproofing treatments mainly corresponded to the spreading of organic vegetal exudates from resinous trees onto the hot ceramic jar (Charters et al., 1995; Diallo et al., 1995; R P Evershed et al., 1991; Rice, 1987; Ben Stern et al., 2008). Even if organic coatings aimed at waterproofing the surface, some research groups however evidenced its permeation towards the initial content. This way, Romanus *et al.* published on fatty acids and wine markers molecules diffusion through the resinous coating in archaeological amphorae and concluded that wine enhanced lipid permeation (Kerlijne Romanus et al., 2009) and Drieu *et al.* linked the porosity degree to lipids preservation in the surface of potteries (Léa. Drieu et al., 2019).

Furthermore, aromatic plants and seasonings were remarkably frequent to formulate beverages. The earliest biomolecular evidence of plant aromatization belongs to the early Neolithic period in the Middle East and in China and may correspond to their respective plants domestication times (P. E. McGovern et al., 2004; P. E. McGovern & Michel, 1996). Later on, the Egyptians followed by Greek and Roman civilizations developed herbs maceration and spices aromatization for diverse reasons (P. E. McGovern et al., 2009). Pliny the Elder documented the natural substances efficiency to overcome the dreaded “wine disease” that turned wine into vinegar in *Historia Naturalis*. The point was if the resin allowed the tree to be protected from microorganisms’ attacks, similar effects should be observed with beverages. This way, Hippocrates formulated the *vinum absinthianum* by supplying regular wine beverages with medicinal plants to provide it better digestive properties (Caballero et al., 2003). Aside from its bactericidal features greatly detailed by Columella who qualified them as “*mendicamentum*”, another tremendous benefit may arise from flavor enhancement. Natural substances were indeed supposed to cover up both the smell and wine taste. Indeed, terpenic resins acted as curing agent to prevent from moisture and aroma alteration

during sailing periods. The addition anti-bacterial resin hereby tackled the oxidation process responsible for wine color and taste alteration, which considerably extended the suitable wine lifetime. Hence, Zlateva and Rangelov estimated that after a year, the oxygen in the amphora would have oxidized and altered too much the perfume to appreciate the beverage (Zlateva & Rangelov, 2015).

In this field, the terebinth tree mastic has greatly contributed to the reputation of Chios wines. Amphorae coming from the Aegean Sea were usually waterproofed with this particular *Pistacia lentiscus* viscous resin (P. E. McGovern et al., 2003). Some other three *Pistacia* species such as *P. atlantica, P. khinjuk* and *P.* terebinthus also greatly sustained waterproofing treatments, commonly known as Cyprus basalm, Chios turpentine or terebinth (Mills & White, 1989). Broadly speaking, less exquisite terpenoid exudates became regularly used. As for amphorae, the pitch was also locally produced. Hence, depending on the prevalent botanical species, characteristic wood resin pitched the amphorae. Within the Roman Empire, diterpenic resins of *Pinaceae* trees were dominant (Fujii et al., 2019, 2021). In Crete, triterpenic birch bark species were used. Other tree sap like frankincense, fir or myrrh were rife (Izzo et al., 2013; Machenaud, 2017; Mitkidou et al., 2008; Urem-Kotsou et al., 2002).

Besides the resin, other kinds of additives from the natural environment (such as fruits, flowers, roots, leaves and herbs) were macerated, infused or steeped in the beverage to deliver their anesthetic, aphrodisiac or psychotropic characters beyond their aromatic properties (P. E. McGovern et al., 2009; Tonutti & Liddle, 2010).

1.5. Cases of study

The research topic was investigated under two different cases of study, both encompassing Roman amphorae from marine context. We greatly collaborated with Nathalie Huet, research engineer in charge of preventive conservation and furniture management at the DRASSM, who offered us to sample amphorae from the shipwreck “Planier 3”, previously selected by Fabienne Olmer, and with the Soprintendenza Archeologia Belle Arti e Paesaggio per le province di Frosinone e Latina (the local Office of the Italian Ministry of Culture) that provided the access of the amphorae from San Felice Circeo, under the supervision of the archaeologist Chiara Delpino.

After the multidisciplinary methodology was developed on waterproofing coatings, the approach was then extended to inorganic shards. Since strict criteria are necessary to avoid pollen contamination, the transdisciplinary approach required a very well-defined archaeological context vessel. The 'teapot' vase from the Bronze Age at Jericho, excavated in 2018 from the archaeological site of Tell es-Sultan (Jericho West Bank), provided the valuable material to open the methodology to an inorganic matrix.

1.5.1. Shipwreck “Planier 3”

1.5.1.1. Archaeological context

Sank nearby the coast of Marseille (France), the wreck owes its name to the islet of Planier, to the south-west of which the ship was wrecked. Located at the foot of a drop-off on the Pierre-de-la-Bague reef (Fig.

4, adapted from (Branger, 2012)), it is the third one to have been identified in this area of strong maritime current. Discovered in 1961 by Mr Gelindo, it has been added to the long list of wrecks lying in the Mediterranean (Liou & Pomey, 1985). The site constitutes an important element for the knowledge of the maritime trade and the productions of the Late Roman republic. Four underwater expeditions under the direction of the *Département des Recherches Archéologiques Sous-Marines* (DRASSM) took place from 1968 to 1975. The wreck exhibited two parts: the upper one represented a 'bed' of amphorae lying on the sand, and the lower part with pieces of wood that originally formed the hull of the ship (Tchernia, 1968). With the help of the *Archaeonaute,* the DRASSM research vessel, excavations began in June 1968 under the direction of André Tchernia and ended in 1974 with Patrice Pomey. According to the dendrochronological study of the spruce of the boat's pre-cavity, Marina Branger established the construction of the ship between 180 and 60 BC (Branger, 2012; Pomey & Guibal, 1995).

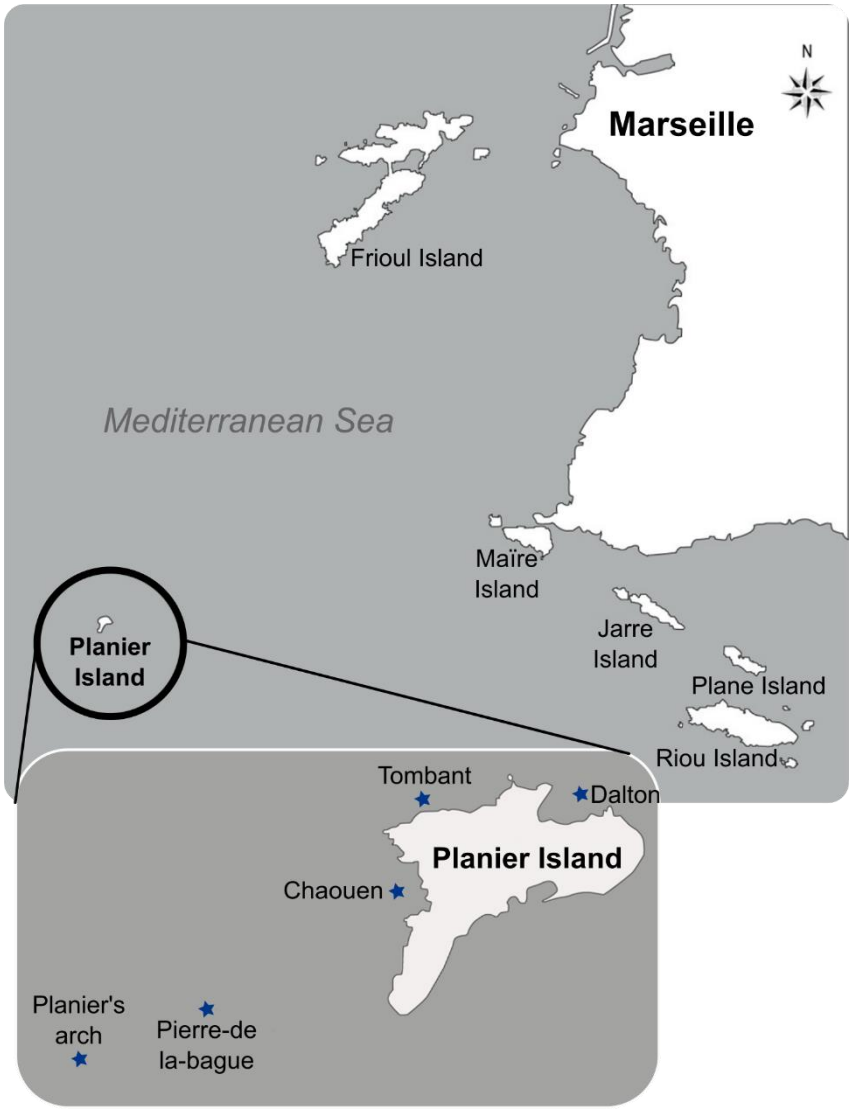

Figure 4. Location of the Planier 3 shipwreck in the Mediterranean Sea

The description of the Planier's cargo is withdrawn from Branger's report (Branger, 2012). It contained a majority of Dressel 1 (169 artefacts including necks, handles, feet and bodies, which may correspond to 42 amphorae) and Lamboglia 2 (68 artefacts including rims, necks and handles which may correspond to 23 amphorae). A large quantity of pozzolan stoppers were recorded, making the Planier 3 cargo the largest discovered to date. Other wrecks such as the Madrague de Giens (Hesnard & Gianfrotta, 1989) or the Dramont A (Benoit, 1962) wreck had initially reported the presence of these stoppers. Made from cork, previously inserted into the neck of the amphora and covered with lime produced from pozzolan, they were used to seal the Dressel 1. It is not uncommon to observe stamps on their external mineral surface. 66 objects have been referenced, hence highlighting the underestimation of the Dressel 1 quantity established by Branger. 6 Brindisi amphorae, each stamped, were recorded alongside ovoid amphorae. Under the ovoid name are grouped morphologically looking-like amphorae. Their identification and provenance remained however uncertain. The amphorae exhibited a large body, beaded edges and round section handles. 39 artefacts (corresponding to 17 amphorae) have been identified as ovoid, among which 'collared' and 'rimless' amphorae. Several groups of stamps are then observed, including '*M Tuccius Galeo*', 'AEC' ligatured and 'CESTI'. A significant number of Greek amphorae were noted, including Cos amphorae (17 reconstructed handles indicating a minimum of 9 amphorae), an amphora from Chios and a Rhodian amphora. 5 Iberian amphorae were referenced, including 2 Oberaden 74, 2 Dressel 7-11 and 1 Lomba do Canho 67 (LC 67). Finally, there is a long list of fragments excavated but not attributed to a given typology. Indeed, even if the analysis of ceramic pates allowed considerable progress in the definition of typologies, its limitation however lies in the absence of a universal photographic catalogue. Defining the manufacture paste cannot always allow the attribution to a well-described typology. Thus, no less than 54 fragments could not be attributed, including 4 amphorae supposedly of small size, 3 amphorae with rounded edges, fragments of unidentified edges, handles with round, oval, ribbed or undefined sections, sometimes with lower or upper attachments, and amphora feet, mostly with flat bottoms.

Furthermore, mineral pigments from Pozzuoli were recovered from the excavations (Tchernia, 1968). Indeed, Tchernia's excavation reports mentioned realgar, a red pigment extracted from arsenic sulphide mineral, also reported as "*sandaraca*" by Pliny the Elder[1], that might come from the Solfatares volcano, located close to the Campanian city (Branger, 2012). Then, very dense cylinders identified as "litharge" were recovered. Interestingly, Dioscorides[2] reported the presence in Pozzuoli of ancient productive hearths. Finally, blue frit balls identified as Egyptian blue were recuperated in significant quantities. The colored pigment certainly came from the city of Pozzuoli, where the production is located. Indeed, *Vitruvius*[3] reported in *De architectura*, the installation in the Campanian region of *Vestorius*, then considered to be the sole producer of Egyptian blue in the Roman (Cavassa, 2018).

Another sign of important economic influence is related to the numerous stamps observed from the cargo. Stamps of Brindisium, or from the slaves of Mallaolus as well as from the illustrious *M. Tuccius Galeo* clearly indicated Italian origins (Tchernia, 1968). At the departure of Brindisium with the shipment of *M Tuccius Galeo* stamped amphorae, the ship would have made a stopover in the Sinus Tarentinus to

[1] Pliny the Elder, *Naturalis Historia*, XXXV, 39
[2] Discorides, *De Materia Medica*, V, 87
[3] Vitruvius, *De architectura*, VII, 11

load Lamboglia 2. Assumption of a second stopover in Pozzuoli seems appropriate regarding the typical dyes aforementioned, that are produced in this region. Finally, the ship had set course for the Narbonnaise before the wrecking. The Planier 3 shipwreck is even more interesting since it has benefited from a precise dating, based on factual events, amphorae typologies and precisely referenced stamps. The presence of Campanian B ceramics of Morel 71 and Lamboglia 5-7 had opened the way to a first dating in the 1st century BC, before being deeply detailed through the observations of C. Goudineau. Indeed, by attributing the ceramic material to "pre-arretine" productions, he tightened the period to 50-45 BC (Goudineau, 1980; Goudineau et al., 1968). Another striking fact resulted from the *M. Tuccius Galeo* stamp exhibited in several amphorae. *Tuccius* was a close friend of Cicero, as attested by a 47 BC letter in which he reported his decision of accepting *Galeo* inheritance[4], who passed away in 47 BC. Even if no facts are established, the coincidences are disturbing and led to believe that *Tuccius* and *Galeo* were the same person (Tchernia, 1968). Consequently, Tchernia assumed the sinking earlier to *Galeo*'s death, putting then an end to the marketing of the eponymous amphorae (Desy, 1987; Tchernia, 1968).

The interesting heterogeneity of Planier 3's cargo highlights the great wealth that the ship must have represented for its time. Indeed, the presence of different typologies, among which the production of Dressel 1 and Lamboglia 2 are largely geographically referenced during the Roman period, gave insight on the ship course (Hesnard et al., 1989; Tchernia & Olmer, 2004). The numerous stamps fit perfectly into the study and understanding of maritime flows and Mediterranean trade (Branger, 2012). The amphorae from Cos, loaded onto the ship in Italy, attested commercial links between the Adriatic ports and the eastern Mediterranean. Chios, Rhodian and Hispanic amphorae, found in lesser extent, could have had a utilitarian function on the ship. Indeed, only 7 amphorae of these types have been attested. This low representation could be explained by its use to feed the crew with *garum*, fish sauces and oil, during the journey. However, despite the great wealth and the important economic representation that the cargo ship may suggest, the publications referring to it are, for the best of our knowledge, limited to the sole excavation report of Tchernia and by the Branger's master in 2012.

1.5.1.2. Archaeological amphorae

Although the great diversity in terms of amphorae has been developed earlier, this section will only focus on the typology that have been selected for the analytical corpus. Investigated artefacts are presented in Table 1. Amphorae and stamps drawings, withdrawn from the Branger's thesis (Branger, 2012) are presented in the appendix 1.

[4] Cicéron, *Att*icus, XI,12, 4

Ref.	Picture	Typology	Sample information	Black coating	Pozzolona stopper	Shard	Hypothesized content
6545		Dressel 5	Kos amphora, Greece			X	Wine
6828a		Lamboglia 2	Adriatic coast, Italy			X	Wine
6828b		Lamboglia 2	Adriatic coast, Italy			X	Wine
6828c		Lamboglia 2	Adriatic coast, Italy			X	Wine
6565		Lamboglia 2	Adriatic coast, Italy			X	Wine
6566		Lamboglia 2	Adriatic coast, Italy			X	Wine
1014		Dressel 1B	Tyrrhenian coast, Italy Pozzolana stopper	X	X	X	Wine
749		Lamboglia 2	Adriatic coast, Italy	X		X	Wine
6570a		Dressel 1B	Tyrrhenian coast, Italy	X			Wine
6793		Lamboglia 2	Adriatic coast, Italy			X	Wine
6904		Chios amphora				X	Wine

Ref.	Picture	Typology	Sample information	Black coating	Pozzolona stopper	Shard	Hypothesized content
6561		Ovoid	Adriatic coast, Italy CAEC stamp Caduceus symbol			X	Olive oil
747		Ovoid	Adriatic coast, Italy			X	Olive oil
561		Ovoid	Adriatic coast, Italy CAEC stamp Caduceus symbol			X	Olive oil
6849b		Ovoid	Adriatic coast, Italy			X	Olive oil
6781a		Early African	Tunisia			X	Olive oil
6823		Early African	Tunisia			X	Olive oil
6849		Early African	Adriatic coast, Italy			X	Olive oil
579		Brindisium amphora	Adriatic coast, Italy			X	Olive oil
1005		Early African	Tunisia			X	Olive oil
598a		Ovoid	Adriatic coast, Italy CAEC stamp Caduceus symbol			X	Olive oil
728		Ovoid	Adriatic coast, Italy Stamp "APOLO'.CAL" (or GAL)			X	Olive oil

Table 1. Amphorae sampled from the shipwreck Planier 3

1.5.2. Archaeological site of San Felice Circeo

1.5.2.1. Archaeological context

The archaeological site of San Felice Circeo is located in the Latina province (Lazio), 90 km southeast of Rome (Fig. 5, adapted from (Grimaldi & Spinapolice, 2010)). The Roman settlement was built on a rocky plateau, between the plain that connects Circeo to Terracina on the one hand and the foothills of the Circeo promontory on the other. The ancient city extended over a few hundred metres (150x200 m) at an altitude of about 100 m (Fig. 6). The urban architecture developed in the 19th century bears witness to the remains of the Roman era by including the main axes in the current layout, in particular the *decumanus* axis, nowadays represented by the *Corso Vittorio Emanuele*, which belongs to the Roman period (de Rossi, 1973; Ronchi, 2017).

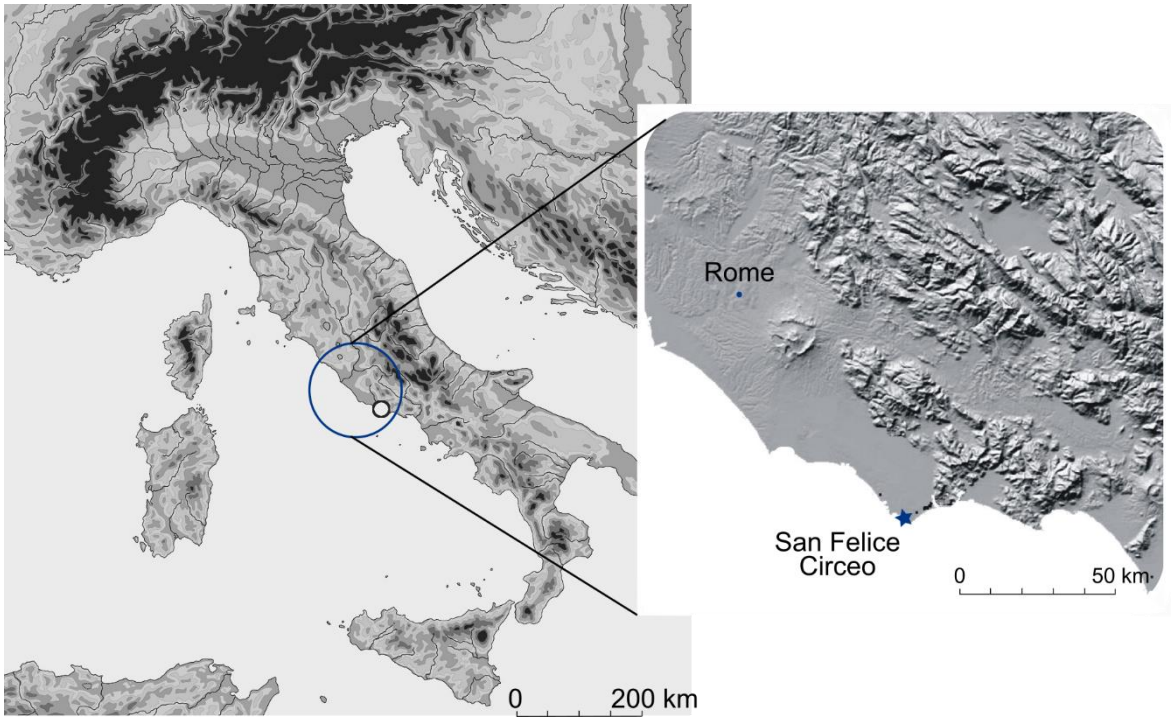

Figure 5. Location of San Felice Circeo

Although the city was occupied by the Volscians before the installation of the Roman colony in 393 BC (as attested by Diodorus Siculus[5]), no visible traces of this occupation remain. Indeed, the great walls and the acropolis at the top of the promontory were erected by the Romans. The settlement of the Circeii, which was disputed for a long time between the Volscians and the Romans, justified the special "Latin-right" the city suffered for years. The first historical writings testify to the sending of colonists to Circeo, led by Tarquinio il Superbo, with the aim of setting up a policy of organization, referred as "praesidia Urbi futura terra marique[6]". It is well established that in 340 BC the city belonged to the Latin League. This

[5] Diodorus Siculus, *Bibliotheca historica*, XIV, 102
[6] Livy, *Ab Urbe Condita Libri*, 56, 3

status implied, among others, a military and pecuniary duty towards the Empire, without enjoying the advantages and privileges of the Roman citizenship. This intermediate position led the habitants of Circeii to revolt against Rome in the first half of the fourth century BC. It is not excluded that this insurrection was fomented by the descendants of the Volscians, partially present in the Circeii and its surroundings. At the international level, the ratification of bilateral agreements between Rome and Carthage in 509 BC ensured the economic development of the city by protecting it from foreign invasions for the next two centuries. Indeed, the two capitals of the Empire agreed on regulating their respective interests by limiting their geographical expansion. This mutual arrangement came to an end in 209 BC, during the Second Punic War, when the Carthaginian army led by Hannibal's brother was sent into Italian territory. According to the historical writings of Livy[7], Circeii was one of the 12 colonies to rebel against the Rome's additional demand for military contributions. The colony was heavily punished, 6 years later, by the injunction to double the contingents of soldiers and provisions requested from the Empire. The lack of historical sources does not allow a precise understanding of Circeo's occupation until the end of the Republic's status. Although Circeii was granted Roman citizenship during the 1st century BC, the colony remains absent from historical literature. This new statutory acquisition marked the beginning of the urban development of the city, with the construction of villas and residential complexes under Roman influence. The city therefore became a place of frequentation of the Roman politicians.

The port area was a major asset that ensured the connection with foreign cargoes. The existence of ancient anchorage is supported by the ancient mouth of the Ufente river, that would have supplied water for the city development (Cancellieri, 1986). The landing of goods was then relegated to support local trading by terrestrial meant or foreigner marketing by sea exportation throughout the Empire.

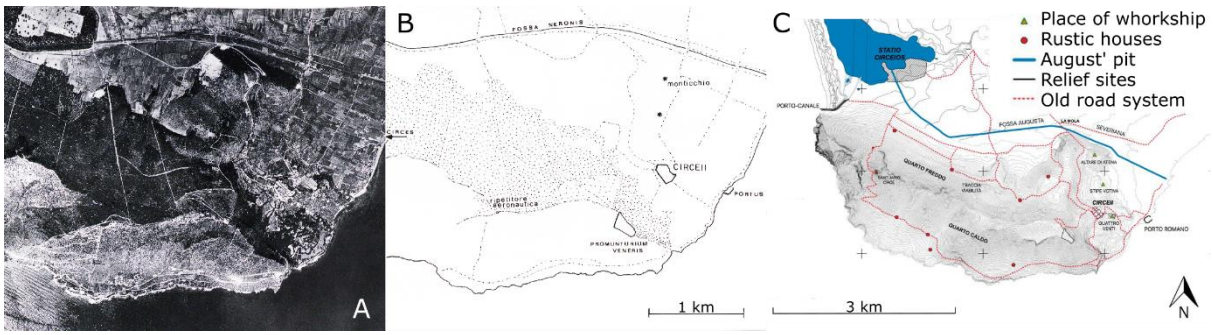

Figure 6. San Felice Circeo typographies from: A; B. The oriental sector of San Felice Circeo with polygonal organisation of the actual city (on the right), the antic anchorage (on the bottom) and the Neronis pit (on the top) (Quilici & Quilici Gigli, 2005)*; C: Circeo promontory (Ceruleo, 2021).*

Thanks to the dominant position of the promontory of Circeo on the Tyrrhenian Sea (about 500 m far from the coastal border), the city enjoyed an important strategic advantage in terms of trade but above all in terms of protection against invasions. Circeo was included among the Roman cities with a strong maritime and commercial influence following the Roman-Carthaginian treaty[8]. Indeed, the promontory protected against north-west and north-east winds and allowed ships to anchor close to the Circeo coasts.

[7] Livy, *Ab Urbe Condita Libri,* 27, 9 and 29, 15

[8] *Polibio, III, 22, 4-13*.

The port area was a major asset that ensured the connection with foreign cargoes. The existence of an ancient anchorage is confirmed by the ancient mouth of the river Ufente (now located in the vicinity of Torre Vittoria), which would have provided water for the development of the city (de Rossi, 1973; Quilici & Quilici Gigli, 2005). The landing of goods was then relegated to supporting local trade by land or foreign trade by sea export throughout the Empire.

1.5.2.2. Archaeological amphorae

A cemetery of archaeological artefacts has been uncovered following the severe winter storm of 2018 that hit San Felice Circeo. The objects were discovered at a short distance from the coast, half metre below the regular level of the sand in a trench covering approximately 100 m².

Among the several objects uncovered were ancient anchors made of stone, wood, lead and iron, cannons, muskets, remains of ancient ships and amphorae. The objects are dated to the 6th century BC, but also to medieval and Roman periods. The 5 amphorae investigated in the thesis were all recovered from the same excavation campaign (Table 2). The amphora no. SFC1 shows a *titulus pictus* labelled "L.M." that could revert to the name of one of the two consuls in office, annually elected (i.e. Lucius Marcius Censorinus in -149; Lucius Mummius Achaicus in -146; Lucius Marcius Philippus in -91 or Lucius Manlius Torquatus in -65).

1.5.3. Archaeological site of Jericho

1.5.3.1. Archaeological context

Tell es-Sultan is located in the archaeological site of Jericho in Southern Levant (31°52'15.99" N; 35°26'39.32" E). Several excavation campaigns led by European archaeologists were launched from the beginning of the 19th century. They allowed the successive uncovering of important monumental foundation of the rampart and the necropolis of Jericho (Wright, 1957; Garstang, 1934). Using site stratigraphy analysis, the occupation of the site could be dated back from the Early Bronze Age to the Roman period (Kenyon, 1993).

In cooperation with Sapienza University of Rome and the local Palestinian National Authority (Department of Antiquities and Cultural Heritage of the Ministry of Tourism and Antiquities (MOTA_DACH), Italian excavation paved the way for the excavation management since 2017, with a specific regard on fortifications and residential quarters (Nigro et al., 2011). During the 2019 excavation campaign, the vessel pot was uncovered from a collapse layer of the rampart stone in the small village extensions towards the flanks of the tell-es Sultan.

Ref.	Picture	Typology	Sample information	Black coating	Pozzolona stopper	Shard	Hypothesized content
SFC1		Late Greco-Italic / Dressel 1A	Tyrrhenian coast, Italy Cork stopper	X	X		Wine
SFC2		Dressel 1A	Tyrrhenian coast, Italy	X			Wine
SFC3		Mañá C2		X			Wine Garum
SFC4		Greek-Italian		X			Wine
SFC5		Lamboglia 2	Adriatic coast, Italy	X			Wine

Table 2. Amphorae sampled from San Felice Circeo

1.5.3.2. Archaeological amphora

The vase labelled was functionally surnamed “teapot” due to its specific typology with an upper hole-mouth with vertical lug-handle (Table 3). The ceramic clay is reddish-brown, and the vase measured 12 cm height. The vase was dated back to the Early Bronze Age, ca. 2300 BC (Montanari, 2019).

Ref.	Picture	Typology	Sample information	Black coating	Shard	Hypothesized content
TS.19.TrIII. 2000/1		'Teapot'	Tell-es Sultan Jerico, Southern Levant Bronze Age		X	Undefined

Table 3. Amphora sample from Tell-es Sultan

1.6. Summary

Amphorae greatly symbolized the capacity of ancient civilizations to adapt, develop and take advantages of the environmental conditions. Developed to optimize the filled space in cargos, they highly contributed to the expansion of the commerce in the Bronze Age. Commonly found in archaeological excavations, they illustrate a considerable challenge to unravel ancient practices in terms of trade routes and commercial goods. Bearing several characteristic and anthropic information, they still represent a precious and promising source for anthropologic and ethnographic investigations. From the scientific point of view, the resinous materials used to waterproof the inner surfaces greatly highlight these adaptive abilities to overcome physical issues as well.

In this regard, 27 archaeological artefacts were investigated in the thesis. The study includes the analysis of 8 resinous coatings and 22 shards, of which the original content was hypothesized by archaeological consideration of the clay and the shape of the container. 3 objects for which the coating was visible with naked eyes, were sampled together with the associated shards to corroborate the outcomes and strengthen the archaeological interpretations. 16 artefacts were analyzed under the archaeological primary assumption of fermented grapes derivatives, probably wine while 11 shards were analyzed in the light of oil content, again speculated by the archaeologists.

Chapter 2

The organic residues analysis: a state of the art

2.1. Identified substances within the pottery ceramic

Over the last century, the contribution of science and more specifically chemical investigations at the molecular level have greatly influenced the archaeology. Advances in research, both in terms of analytical technique development and characterization capacities have enabled the traceability of many contents. In particular, the identification of fatty acid compounds from plant oils (Maria Perla Colombini, Giachi, et al., 2005; J. Condamin et al., 1976) and animal fats from terrestrial or marine context (Copley et al., 2004; R. P. Evershed et al., 2008; E. Ribechini et al., 2009; K. Romanus et al., 2007; Steele et al., 2010), but also the botanical characterization of resins among natural exudates from all over the world (Mills & White, 1977; Erika Ribechini et al., 2008; Urem-Kotsou et al., 2002) and plants or animal waxes (Charters et al., 1995; Garnier et al., 2002; Martine Regert et al., 2003).

2.1.1. Biomarker concept

In order to assess the nature of the content, Evershed *et al.* popularized what they called the *archaeological biomarker concept* (Richard P Evershed, 1993). Currently used in the geochemistry to characterize bitumen composition, the concept lies on the presence or absence of some molecular markers whose carbon structure or distribution among the molecular content matched with the archaeological compound ones. Rightly named “chemical fingerprints”, those components correspond to organic features whose molecular structures are not highly altered with time. They are usually preserved from microorganisms or bacterial deterioration activities (Philp & Oung, 1988). Surviving over centuries, they can greatly give insight to the initial composition thereby (Maria Perla Colombini, Modugno, et al., 2005; Martine Regert, 2011; Erika Ribechini et al., 2008).

Two identification pathways have been developed (Richard P Evershed, 1993; Carl Heron & Evershed, 1993). While some molecules are highly specific and facilitate direct content hypothesis, some other characterizations rather depend on the molecular distribution. The molecule itself does not directly illustrate the composition, but associated to other compounds, they can nevertheless provide indication. Some patterns hence became relevant to decipher the archaeological composition acting as chemical indirect markers. In the specific case of ceramic reutilization, the widened molecular profile generally complicates the identification. Fortunately, recycling amphorae was unlikely although it occurred frequently for vessel pottery (Pecci et al., 2017). However, it remains primordial to not only focus on the target indication but to insert it in a broader picture to systematically measure the related archaeological, botanical, palaeocological means to prevent overinterpretation.

Chemical marker, described hereafter, are generally classified into biomarkers, markers confirming natural degradation or ageing, markers accrediting anthropic degradation and markers attesting contamination.

2.1.1.1. Biomarkers

The identification step is driven by the presence or absence of biomarkers. At the molecular level, not only the organic skeleton is informative but also its stereochemistry, the presence of unsaturations, their locations and quantities. Any atomic or structural particularities became relevant and might be significant. Heteroatoms and functional groups such as alcohols, ketones, hydrocarbons and fatty acids are of a great support in the identification task. The assignment uncertainty degree decreases with the rareness of the identified targets. For example, di- or triterpenoic acids thoroughly highlight the botanical nature of the resin at the genus level, acting as a chimiotaxonomic guide (Font et al., 2007; Urem-Kotsou et al., 2002; Zareva & Kuleff, 2010).

2.1.1.2. Natural degradation markers, sustainability and preservation

The fundamental benefit of the biomarker concept lies in the ability to structurally target a molecule as an exclusive characteristic toward species or compounds despite the variations it could have undergone. Recognizing altered molecular patterns also means obtaining information not only on the natural origin of the compounds but also on the degradation conditions involved. This way, biomarkers can offer an additional degree of diagnosis.

By introducing the biomarker concept, Evershed *et al.* also recommended the careful interpretation of the markers regarding their natural degradation pathway possibilities. Indeed, it is often possible to observe slightly altered structures rather than the original ones. As described previously, various transformation pathways could occur to modify the molecular pattern of biomarkers, spanning from microbial and enzymatic deterioration to atomic changes through oxidative reactions. Chemical and biochemical transformations could be stimulated by archaeological conditions of storage. For example, light exposure, aerobic conditions, humidity and temperature can strongly affect biomarkers (R P Evershed et al., 1991; Martine Regert, 2011). Based on tailored replica ceramics, Evershed *et al.* studied the oxidative aerial effect on acyl lipids conservation over time and concluded to faster degradation under oxic environments (R. P. Evershed et al., 2008). Archaeological jars recovered from tombs under anaerobic and isothermal conditions usually display good preservation state. Arid climates such as desert sites, where the moisture content is specifically low guarantee molecular preservation preventing from microbial development.

Aside from the chemical nature and physico-chemical properties that largely affected the lifetime of organic residues biomarkers, several factors could account for biomarkers preservation against microbial degradation (Carl Heron & Evershed, 1993). Two environmental aspects need to be distinguished. Firstly, the presence of mineral matrices and ceramic porosity can notably enhance molecular stability over archaeological times. Indeed, organic residues adsorbed in the porous ceramic layer remain therefore less available because harder to reach for microorganisms (Eglinton & Logan, 1991). Additionally, the organic matrices seemed to act as a physical barrier. For instance, resin or bitumen are good example of residues preservation because microorganisms could not attain them and thereby they prevent from organic residues decay (Font et al., 2007; T.F.M. Oudemans & Boon, 1991). In archaeometry, lipids, in the broadest

sense, are the predominant marker family. The long carbon chain is responsible for their hydrophobic properties and low reaction abilities. Secondly, some biomarkers can strongly interact with mineral cations from the ceramic to form salts. This environmental bonding possibility can partially ensure their preservation. This is the case for the tartaric acid, usually preserved as tartrate salts in ceramic shards (Léa Drieu et al., 2020; P. McGovern et al., 2017; P Ribéreau-Gayon et al., 2006).

2.1.1.3. Anthropic degradation markers, an interesting signature of human activities

In the early days of archaeometry, some experimental studies led by Charters, Evershed *et al.* became pillars of the field, notably by establishing the beginnings of scientific input. The main purpose was a deeper understanding of the molecular modifications undergone through heat treatment on the natural organic matter. They performed laboratory experiments on replica ceramic potteries to simulate archaeological vessel uses and extended it to excavated jars (Charters et al., 1995, 1997; R. Evershed et al., 1995; Raven et al., 1997). They referred to these organic altered features as anthropic markers. They notably give insight on human practices and manufacture technologies applied to the matter to change its physical properties (R. P. Evershed et al., 2008; R P Evershed et al., 1991).

Some great examples emanate from harsh heating treatments implemented on resin. High temperature of approximately 300°C represented enough energy to promote molecular markers of the natural exudates to stabilize upon aromatization as well as losing some functional groups. Resulting markers showed a coherent carbon skeleton, slightly but significantly modified from the precursor ones. For example, retene and 28-norolean-17-en-3-one compounds have been identified from respectively diterpenic resin from Coniferous and triterpenic resin from *Pistacia* spp. (Fig. 7) Their occurrences in resin indicate a considerable heating during their preparation (Mills & White, 1977, 1989; B. Stern et al., 2003).

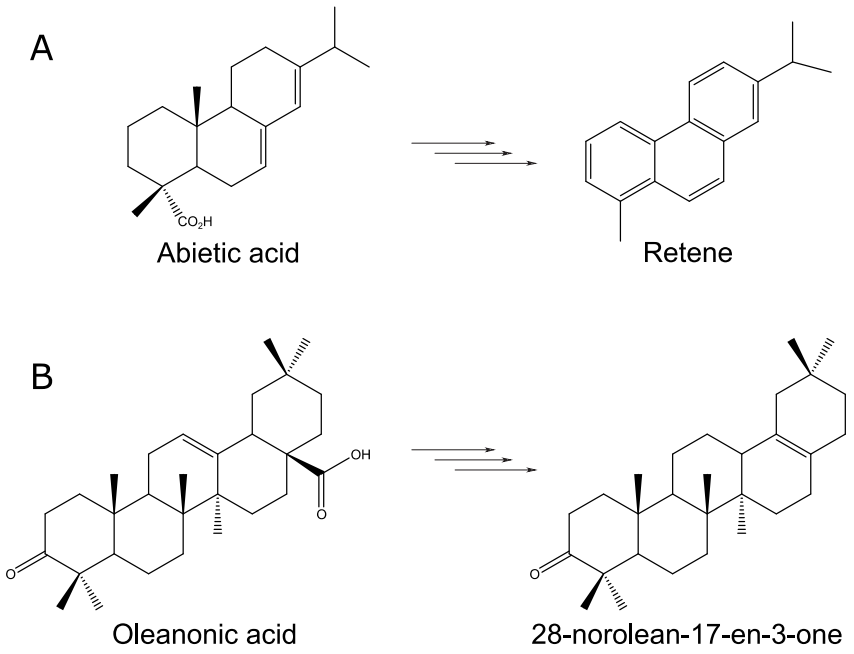

Figure 7. Markers of heating treatment preparation for the diterpenic compound of abietic acid (A) and the triterpenic compound of oleanonic acid (B)

2.1.1.4. Contamination markers

The last marker category represents molecular patterns which presences are not straightforwardly linked to archaeological queries. For example, pollution or migration markers might come from molecular exchanges with the sediments surrounding the object before excavation (Martine Regert, 2011). Indeed, it is becoming more and more common for archaeologists to simultaneously sample soils that are directly in contact with the artefact in order to sustain blank measurements for further analysis (Koh & Betancourt, 2010; P. McGovern et al., 2017). This way, any leaching into the environmental media can be assessed.

Another aspect of contamination arises from bacteria and microorganisms' development in shards. Fatty acids interpretation must be careful regarding the fat sources, since almost every organism daily produces them. Moreover, the hypothesis of post-appearance of bacteria that would have consumed the organic remains contained in the shard cannot be neglected and would lead to false positive and incorrect conclusions (Eerkens, 2007). For example, branched odd carbon chain may correspond to ruminant or non-ruminant animals but can originate from bacterial activities as well (K. Romanus et al., 2007). Small organic acids (e.g., succinic, fumaric acids) can diagnosis prehistoric fermentation as they can originate from the citric acid cycle, a series of metabolic reactions used by aerobic organisms that aims at releasing the energy stored from the degradation of fats, proteins and carbohydrates compounds.

The last source of contamination marker discussed here corresponds to human interfering while dealing with archaeological objects. This way, phthalates are frequently detected in chemical analysis due to the plastic use during the object sampling and storing. The structure being close to tartaric acid, phthalates can act as precursors when using acid conditions for the extractive treatment although Drieu *et al.* (Léa Drieu et al., 2020) verified the acid absence from pure standard of phthalic acid and plastic bags. Some

contaminants can appear during the chemical extraction of the material, from solvents for example, even though analytical grades are preconized, or from filter cartridges made of plastic derivatives. Additionally, the occurrence of cholesterol and other squalene precursors can arise from ungloved handling. The contamination might go through physical contact without good protection. Besides the fact that squalene is a characteristic organic compound produced by the sebaceous gland (Picardo et al., 2009), its important poly-unsaturation feature would indeed not survive over archaeological centuries. Sterol presences must be interpreted with caution, in particular when abundantly detected. Considered as biomarkers of plant oils due to their natural presence therein, they can become contamination markers when present in large extent since their regular distribution in plants is not so important. Additionally, some fungal markers such as ergosterol can help in noticing contamination from soil fungi or yeast (Stephanie N. Dudd et al., 1998) as well as characterizing alcoholic fermentation (Isaksson et al., 2010). In general, lipid compounds such as fatty acids, wax esters, squalene, sterols and its ester derivatives must be considered with prudence since human skins can produce them (Richard P Evershed, 1993).

2.1.2. Terpenes

2.1.2.1. Definition and structure

To protect themselves from external attacks, resinous plants can produce viscous exudates to dress their wounds, singularly characterized by their chemical composition. Resins are better itemized as oleo-gommo-resins. “Oleo” points out their high fragrant features providing antibacterial properties and corresponds to the essential oil fraction made of mono- and sesquiterpenoids, respectively made of 10 and 15 carbon atoms. “Gommo” invokes their sticky aspect related to the presence of many polysaccharides chains and “resin” describes their molecular organic composition represented by di- (C_{20}) or triterpenoids (C_{30}) depending on the nature of the resinous materials (Fig. 7). In archaeological samples, it is unfortunately rare to notice oleo and gommo fractions. The former is composed of volatile organic compounds that disappeared with time. The latter is hydrosoluble. Consequently, the resin fragment, which displays the best molecular stability, remains the more relevant to characterize in order to decipher their botanical and geographical origins as well as the technologies involved to prepare them.

From a chemical point of view, resins exhibit great molecular pluralities, which make their characterization even more complex. Since terpenes are not playing any role in plant cell growth, they are considered secondary metabolites. Terpenes are biosynthesized by plants through the “head to tail” assemblage C_5 isoprene units. Formed chains are then arranged in rings (Fig. 8).

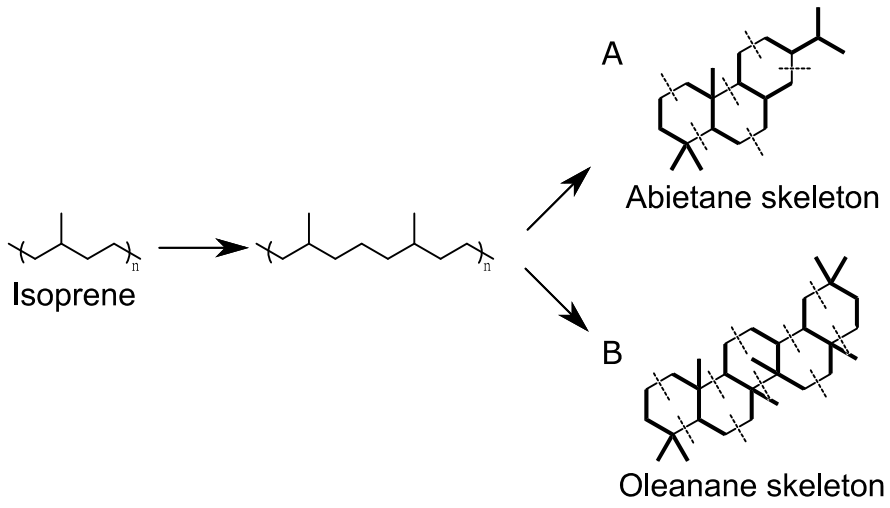

Figure 8. Example of diterpenic (A) and triterpenic (B) skeletons synthesized from isoprene C_5 units (represented by the dashed lines)

In this thesis, we mainly focused on diterpenic molecules since almost all the amphorae studied came from regions where coniferous resins of Pinaceae and Cupressaceae were prevalent. We therefore developed more their relative biomarkers bearing in mind the possible presence of triterpenes.

2.1.2.2. Natural distribution of diterpenic resins

Diterpenic resins mainly originate from the Gymnosperm branch, more specifically produced by conifers although Leguminoseae and Fabaceae families from Angiosperms can also synthesize them. Among the conifers reign, Pinaceae and Araucariaceae are the most frequent families in the world event though Cupressaceae, Taxaceae and Podocarpaceae roughly account for more than 340 registered species (Bailly, 2015; Langenheim, 2003). In Mediterranean regions however, diterpenic resins predominantly correspond to rosin and sandarac, respectively emanating from Pinaceae and Cupressaceae families. Their molecular markers are both composed of bicyclic or tricyclic carbon structures stimulated by carboxylic acids and alcohol functions. Fig. 9 illustrates their three representative patterns: abietane, pimarane and labdane skeletons.

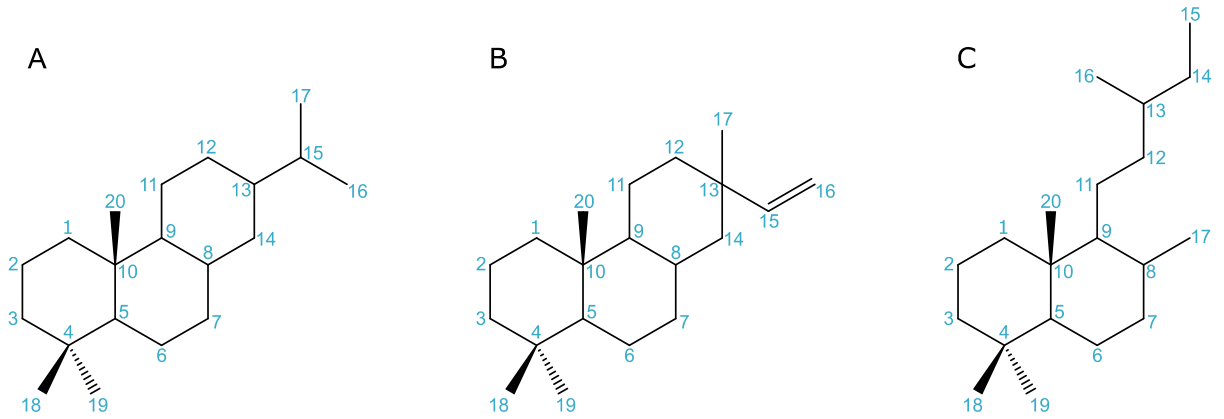

Figure 9. Abietane (A), pimarane (B) and labdane (C) skeletons with numbered carbons

Distinguishing them is possible at the molecular level (Langenheim, 2003; Mills & White, 1994). Despite the ubiquitous nature of these markers, the chemotaxonomic characterization of plant families is diagnosed either by the quantity of markers present or by the absence of certain others. In this way, abietane and pimarane skeletons are considered the main markers of Pinaceae resin (called colophony or rosin). Abietane derivatives are generally present in greater extent compared to pimaranes. Dehydroabietic acid (DHA) and 7-oxo-DHA (Fig. 10) account for the typical markers of the rosin and the oxidation of the resin.

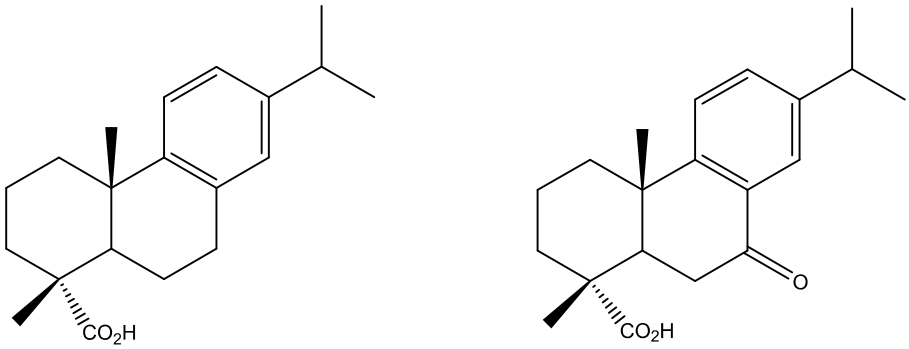

Figure 10. DHA and 7-oxo DHA, respective markers of Pinaceae resin and oxidized Pinaceae resin

For its part, sandarac made of Cupressaceae is essentially characterized by pimaranes and labdane structures, with significant amount of sandaracopimaric acid, agathic acid and *trans* and *cis*-communic acids (Fig. 11) (Azémard et al., 2017). The presence of conjugated diene in the labdane framework induces polymerization via cross-linking, cleavage reactions, isomerization and oxidation. Aged resins of Manila copal and sandarac frequently exhibit low molecular weight polymers (Scalarone et al., 2003). For instance, sandaracopimaric acid originates from 12-acetoxy sandaracopimaric acid.

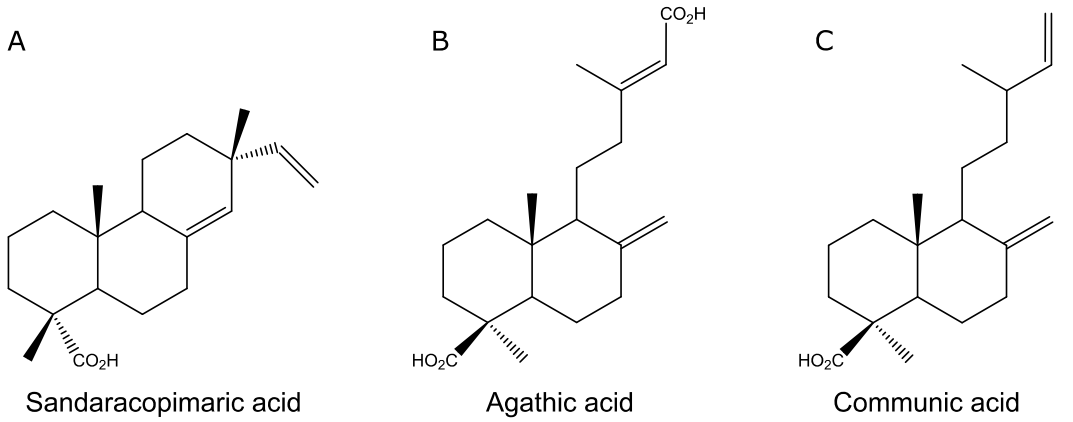

Figure 11. Pimarane (A) and labdane (B-C) skeleton markers of Cupressaceae resin

Likewise, some phenolic compounds derived from oxidation and rearrangement of abietane skeletons such as ferruginol, totarol, sempervirol and sugiol are considered as Cupressaceae biomarkers (Fig. 12) (Steigenberger, 2013).

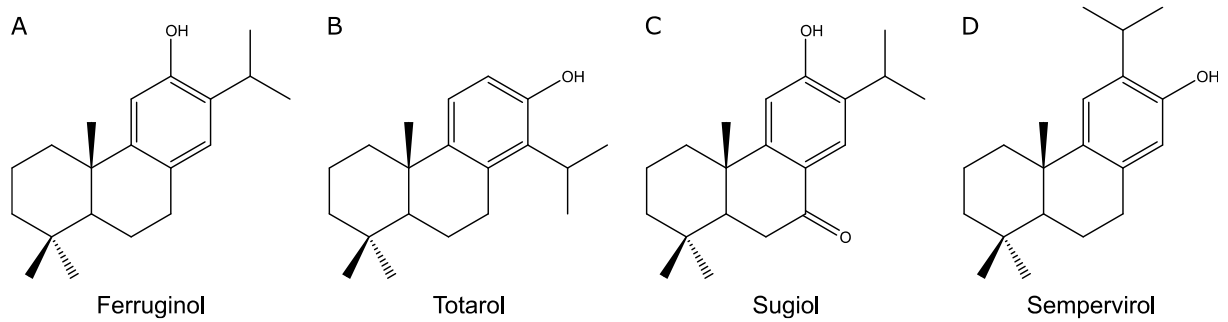

Figure 12. Oxidized phenol markers of Cupressaceae resin

2.1.2.3. Degradation pathways

Besides the botanical indication provided by the presence of characteristic frames, expression of past technologies to acquire the resin can be traduced by natural and anthropic markers related to abietane skeleton transformation under specific conditions. Abietanes represent the main source of archaeological information since the pattern is the most reactive one. Indeed, one structural information is primordial to notice. Abietanes exhibit diene conjugation compared to pimaranes. This conjugation is notably the chemical reason for the great reactivity of abietanes towards alterations or heat treatments. Pimaranes that do not have this conjugation because of a quaternary carbon then present a more restricted set of markers (Scalarone et al., 2003).

Once the resin exuded from the tree, the diterpenic molecular composition starts to change with aerial oxygen. Isomerizations occur to stabilize abietic (**A1**), palustric (**A2**), neoabietic (**A3**) and laevopimaric (**A4**)

acids (Fig. 13, adapted from (Mezzatesta et al., 2021a)). Among them, abietic acid is the most prevalent isomer. The natural degradation may be assimilated to the ageing of the resin, triggered by the oxidation of the conjugated diene of the abietic acid to form DHA (**A5**) (Mills & White, 1994). Several further oxidations occur then to integrate oxygen via hydroxy or ketone groups. The exact mechanism remains obscure, but it shall involve peroxide formation, loss of water and/or even peroxide reduction. Oxygenation takes place at three likely reactive positions (3, 7 and 15), giving rise to 3-hydroxy-DHA (**A8**), 7-hydroxy-DHA (**A6**), 15-hydroxy-DHA (**A9**) and 7-oxo-DHA (**A7**). In turn, molecules can keep going on with oxidation and isomerization to form dihydroxy-DHA and hydroxy-oxo-DHA, respectively named 7,15-dihydroxy-DHA (**A10**) and 15-hydroxy-7-oxo-DHA (**A11**) (Fujii et al., 2021; Serpico & White, 2000; van Den Berg et al., 2000).

Another part of the reaction pathway concerns anthropic heating treatment. Above 300°C, DHA is transformed in norabietatriene and norabietatetraene species (usually 18 or 19-norabietatriene (**A14** and **A15**)) via the removal of acidic function. The resulting compounds are stabilized through partial or complete aromatization, giving first rise to tetrahydroretene (**A18**) and finally retene (**A19**). Simultaneously, DHA dehydrogenation generated dehydrate-DHA formation, which forms tetrahydroretene after aromatization of the intermediate (Serpico & White, 2000). Likewise, dehydroabietane (**A12**) can generate simonellite (**A13**) by aromatization and finally generate tetrahydroretene and retene upon decarboxylation (Bailly, 2015).

At last, when the tar was prepared by pyrolysis of the coniferous tree, DHA is esterified by the methanol present in the wood slowly released by the raise of temperature, producing methyl dehydroabietate (DHAM). It is interesting to notice that these methylated compounds are subject to similar oxidative pathways as DHA (Pollard & Heron, 2008).

As presented above, the wide variety of potential chemical reactions illustrates the ability of abietanes to provide archaeological evidence. First, the botanical source is deducted from the molecular composition and the occurrence of abietadienic, pimaradienic or labdadienic species. Indeed, the presence of oxidized DHA is then indicative of the natural degradation process followed by the resin over time. Stable markers such as 7,15-dihydroxy-DHA and 15-hydroxy-7-oxo-DHA make it possible to describe the ageing of the pitch as well as environmental factors such as oxygen exposure. Additionally, the presence of highly aromatic molecules such as retene clearly points out artificial transformations triggered by human activities, suggesting high temperature heating during preparation. Finally, methyl ester compounds shed light on the pyrolytic manufacture treatment from the resinous softwood.

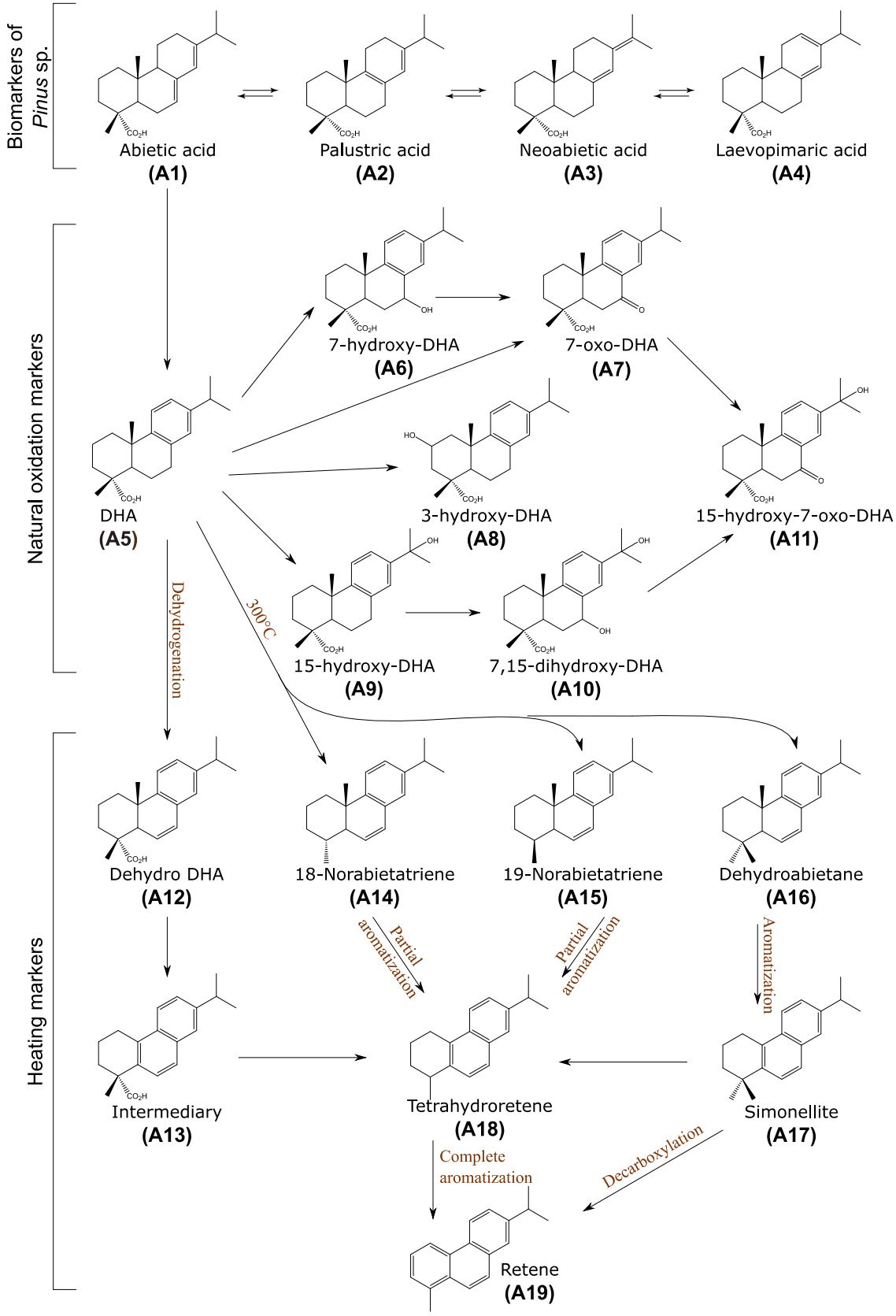

Figure 13. Abietane markers degradation pathway from a Pinaceae resin

2.1.2.4. Distinguishing resin form pitch and tar

The archaeological difference between resin, pitch and tar can be transposed to chemical queries. The main difference lies in their molecular composition. Nevertheless, how do they evolve under formulation and heating practices? Thermally altered features have been mentioned previously to distinguish them. However, caution regarding the interpretation is necessary and DHAM becomes a biomarker of formulation via wood distillation only when hydrocarbon polycyclic derivatives are also characterized. Indeed, even if phenanthrenes, norabietatrienes and simonellite are markers of artificial activities, their production requires high-energy, usually provided by intensive heating under anaerobic conditions (Rageot, 2015).

To describe plant substances from the Pinaceae family, the term *resin* covers the natural exudate of Gymnosperm trees. When the resin has undergone heating treatment, the common appellation becomes *pitch*. Lastly, the *wood tar* corresponds to the pyrolysis of the wood with the resin.

2.1.3. Wine residues

2.1.3.1. A brief introduction to wine

Wine remains are one of the most promising content to study in order to gain knowledge on ancient trade routes and Mediterranean economy (Pecci, Cau Ontiveros, et al., 2013). Besides the great analytical challenge to face, fermented grape characterization might greatly supply practical and philosophical queries spanning from the ancestral methods to prepare wine to the religious, social, political or hedonic aspect it might confer (Maria Rosa Guasch-Jané, Andrés-Lacueva, Jáuregui, et al., 2006). Indeed, wine has been identified in Shedeh jars found in the Tutankhamun's Tomb in Egypt, which greatly highlight its divine bestowed aspect. The earliest method to attest wine presence were going through amphorae typologies. Various morphologies dated from the 5th century BC to the 1st century AC have been mentioned for containing wine. Within the following non-exhaustive list, wine residues have been notably detected from *Corinthian* amphorae coming from Greek islands of Corfu and Mende, Roman *Dressels* and *Late Roman* or *African Keay* amphorae (Foley et al., 2012; Fujii et al., 2019; Pecci et al., 2010, 2017; Martine Regert et al., 2003; Woodworth et al., 2015).

From the historical point of view, wine assumption is also relevant to chronologically address the domestication of grape cultivars. Earliest evidence therefore became more important since they also drew back in time our knowledge about the population abilities to take advantage of their environment. Based on molecular markers and supported by palynological and xylological observations, McGovern *et al.* recently recorded the earliest evidence of wine from an Early Neolithic pottery roughly dated to 6000 - 5800 BC (P. McGovern et al., 2017). Both excavations took place in the northern regions of the Zagros Mountains in Caucasus. From there, they suggested that large-scale winemaking might have been initiated (P. E. McGovern, 2013). Even if no relevant proof of domesticated grape cultivars has been evidenced in these sites, the domestication hypothesis relied on the fact that archaeological artefacts have been found out of the extension area of wild plants. Indeed, wild and cultivated *Vitis* species usually

develop in different environments from each other (Bouby et al., 2013). The cultivated *V. vinifera* is supposed to emanate from its wild *V. vinifera* subsp *silvestris* ancestor (Louis Levadoux, 1956). Despite the contribution of various investigative fields including natural sciences, history and archaeology, the origins of the cultivated vines, as well as the conditions involved in the extension and development of its cultivation in the Mediterranean basin are far from being perfectly understood. However, it remains important to notice that several studies of different cultivated species find their roots in the Caucasus regions, where the observed morphological and genetic diversity among wild species and local cultivars is significantly ample (Coito et al., 2019; Milanesi et al., 2016; This et al., 2006; Zohary, 1995).

Back to the historical context, grape markers have also been detected from Neolithic potteries from the 5th millennium BC in the archaeological site of Dikili Tash in Greece (Garnier & Valamoti, 2016; S. M. Valamoti et al., 2007). Chemical traces of fermented grapes were found in Ancient Egyptian vessels, roughly dated to 3000 BC (Maria Rosa Guasch-Jané et al., 2004). Domesticated cultivars have been identified by carpological analysis of grape pips. Grape cultivars have been attested in the 3rd millennium BC in Minor Asia, Southern Greece (Banilas et al., 2012). The early cultivation in Cyprus corresponds to the same period, when the island was under Phoenician's domination (Grassi et al., 2003). Slowly spreading over, wine production has also been evidenced by the presence of cultivar vines in 2200 BC in Crete to finally expend westwards from Northern Italy to Southern France, Spain and Portugal two centuries later under the influence of the Roman Empire (Arroyo-Garcia et al., 2006; Coito et al., 2019; Hardie, 2000; Hopf, 1991; P. E. McGovern et al., 2004).

The identification of archaeological wine is the result of multidisciplinary studies. Morphological analysis of pips and woods, molecular characterization and finally genetic analysis represent the three pillars of current scientific investigations. In addition to the archaeological contributions abovementioned, the chemical characterization of wine may corroborate with the study of grapevine domestication and cross-conclusions might be therefore drawn. It however remains primordial to consider the grapevine domestication as a complex phenomenon, multi-located and slow processing (Bouby et al., 2013; Soultana Maria Valamoti et al., 2020). Indeed, the emergence of alternative hypothesis regarding the multi origins of vineyard domestication is widely supported by genetic and morphometric evidence. Evolution of DNA possibilities and routine genetic sequencing brought on light the relevant contribution of local wild species in the genome of the modern cultivars. Autochthonous are additionally identified all over the Mediterranean basin (Cargnello et al., 1980; Lombardo et al., 1978; Maletić et al., 2015). Northern Greece, Sardinia but also the Iberian Peninsula have been mentioned to be the epicenters of vineyard domestication along with the Caucasus (Arroyo-Garcia et al., 2006; Bouby et al., 2013; Grassi et al., 2003; Myles et al., 2011; Ucchesu et al., 2015; Soultana Maria Valamoti et al., 2020).

2.1.3.2. Composition

The chemical composition of wine, to say the least, is complex. While the major components remain water and ethanol, hundreds of other compounds are also present. Among them, some key organic acids and polyphenol patterns, more specifically phenolic acids and anthocyanins will be discussed further since they represent the major degradation markers to trace back wine. These biosynthetic components explain the great diversity in terms of aroma, color, and wine taste. Phenolic compounds greatly balance the beverage astringency, bitterness, sourness and account for the body aroma (Jackson, 2008).

2.1.3.2.1. Phenolic acids

Chemically speaking, phenolic acids correspond to molecules whose organic structures present an aromatic ring substituted with functional groups such as hydroxyl, ester or methyl ether. Plants are rich of these non-flavonoid acids, conferring those flavors and gustative characters. The astringency is for instance due to the dimerization of specific phenolic acids giving rose to hydrosoluble tannins (Garnier, 2003). While monomeric flavonoids influence wine bitterness, some phenolic acids such as caffeic or *p*-coumaric acting as H^+ donors account for wine sourness (Salameh et al., 2008). Only the case of grape and vine derivatives composition will be developed since this thesis mainly focused on wine characterization.

As illustrated in Fig. 14, polyphenols are classified upon their lateral carbon chain number into benzoic acids (single carbon), phenylacetic acids (two carbons) and cinnamic acids (three carbons with central *trans* unsaturation). Gallic and *p*-coumaric acids are respectively the most abundant benzoic and cinnamic compounds in grape and vine. The latter is usually present through its esterified form, bound to tartaric acid.

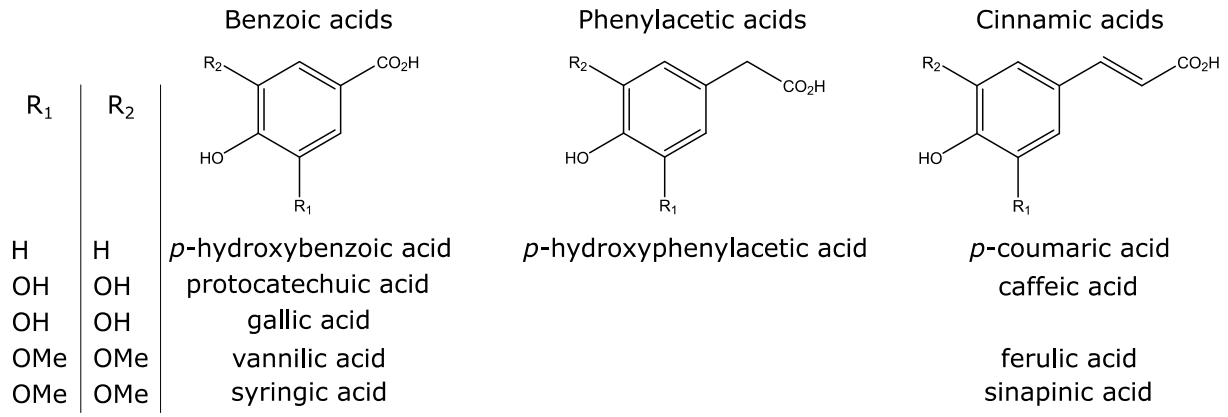

Figure 14. Molecular structure of polyphenols (Garnier, 2003)

Phenolic compounds are mainly constrained in the flesh of the fruits, where they are biosynthesized. Their concentrations increase with time and ripening of the fruit before being consumed as a precursor for the formation of flavonoids, which marks their decline (Belitz et al., 2009). After being grabbed and transformed, polyphenol concentrations keep evolving and can decrease with time upon their antioxidant actions towards lipids auto-oxidation. Easily oxidized, polyphenols can indeed be consumed by capturing peroxy radicals. Alternatively, the concentration can also increase by oxidative flavonoid degradation while wine is ageing or by oxidative enzymatic reaction occurring to adapt and facilitate the alcoholic fermentation (Edwin N. Frankel, 2010; Pascal Ribéreau-Gayon et al., 1998).

Wine ageing coincides with the oxidation of polyphenols and notably unsaturated cinnamic acids triggered by radical reactions. One striking observation arises from the ability of resulting compounds to polymerize with anthocyanins and other flavonoids, giving rise to insoluble high molecular weight polymers (Garnier, 2003).

2.1.3.2.2. Anthocyanins

Anthocyanins are hydrosoluble pigments that belong to the oxidized organic structure of flavonoids. In red wine, they largely influence the mouth-fullness perception of the beverage (Vidal et al., 2004). Composed of 15 carbons arranged all over the tricyclic pattern, the electronic arrangement within the framework of the molecule confers some absorptive properties. The A ring acts as chromophore while the heteroatoms and functional groups substituting the B cycle allow higher delocalization (Fig. 15). The central ring can be easily acylated, which increases the anthocyanins stability through π- π interactions between the aromatic rings henceforth closer to each other. Thereby, it promotes better protection towards oxidation and contributes the color stability (Darias-Martín et al., 2002). This way, anthocyanidins react with glucose units to form anthocyanins referred as 3-glucosides.

R_1	R_2	
OH	H	Cyanidin
OH	OH	Delphinidin
OMe	H	Peonidin
OMe	OH	Petunidin
OMe	OMe	Malvidin

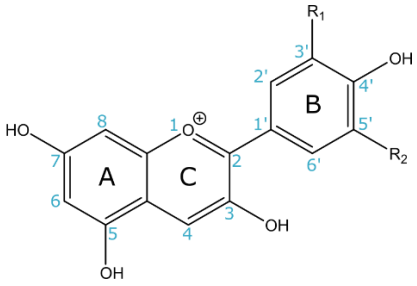

Figure 15. Main anthocyanidins of grapes

Among the several anthocyanidins reported, grapes are composed of cyanidin, delphinidin, petunidin, peonidin and malvidin (Fig. 15). The latter is actually the more abundant, representing up to 90% of the anthocyanidins content in some species (Garnier, 2003).

Anthocyanins are responsible for fruits tints. Colors nicely vary based on the electronic molecular environment from red coloration in acidic conditions to green-yellowish hues in neutral solutions and become colorless when the molecule is completely reduced in alkaline circumstances. Due to their high electronic density, anthocyanins can condensate with electrophilic molecules. First, monomers of condensed tannins can arise from direct reaction with flavanols. Additionally, tannins oligomers also result from indirect condensation via compounds that have been previously oxidized or decarboxylated, such as acetaldehyde, pyruvic acid or even vinylphenol. Naturally present in the wine, they are good candidates for electrophilic addition, raising the opportunity to improve the pyranoanthocyanin stability by the formation of condensed tannins (Fulcrand et al., 1998; Romero & Bakker, 2001). Wine ageing thereby corresponds to molecular changes within the wine composition through condensation of anthocyanins to form less colored but more stable derivatives (Berrueta et al., 2020).

The formation of condensed tannins from anthocyanin structures leads to a decrease in their concentration within the wine, consequently promoting tannins singularities to each wine. In terms of taste, it is important to note that grape variety plays a great deal on the quantity of tannins. Because of

this important capacity to polymerize, it seems unlikely to characterize anthocyanins in archaeological potteries. A first indication of their absence directly comes from the bland color of the wine.

2.1.3.2.3. Organic acids

Organic acids are prevalent in wine and largely account for the appreciated sourness of beverages, generating disagreeable acidity that covers the other wine flavors (Polaskova et al., 2008). Among the recurrent organic acids reported in grapes (Fig. 16), tartaric acid remains the most abundant one from the unripe grapes to the must (Margalit, 2012). Significantly biosynthesized by the *Vitis* vine, it has an essential influence on wine taste and can deteriorate flavors when highly concentrated (Sass-Kiss et al., 2008). It is important to note that malic, citric and tartaric acids account all together for many of the acidic grape components. Unfortunately, malic and citric acids are naturally present in living organisms. Citric acid is highly involved in organism growth since it has a crucial biochemical and metabolic role in the Krebs cycle. The excess of malic acid can induce unpleasant wine acidity that can be tackled by malolactic fermentation, producing lactic acid out of malic acid. Their wide distribution does not make them suitable biomarkers.

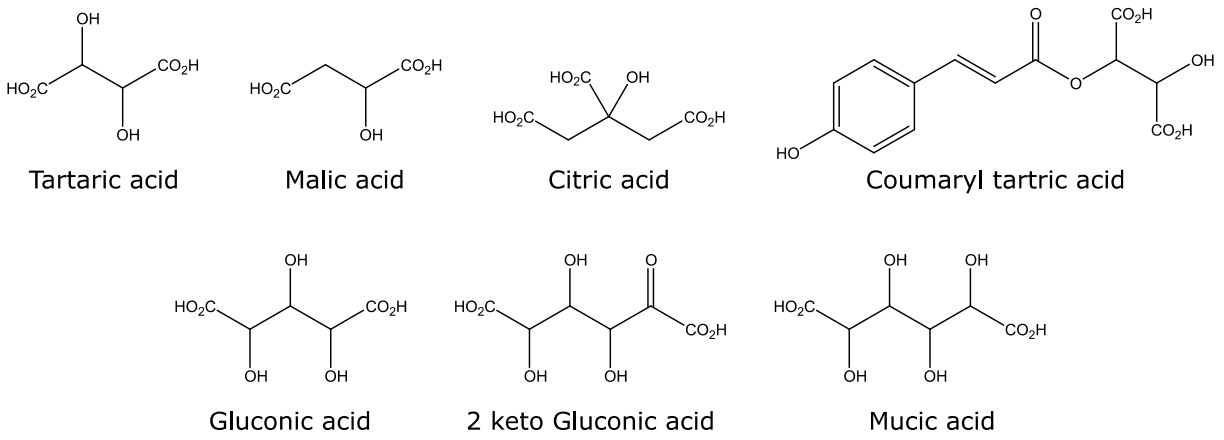

Figure 16. Essential anthocyanidins of grapes

Additionally, the oxidized aldehyde or primary alcohol functions of ketose may indicate that grapes or must were rotting. Resulting gluconic acid and its derivatives are markers of noble or gray rot. Ketoglutaric acid has been mentioned to nicely enhance wine's flavor by bonding with free sulphur compounds and consequently reducing distasteful perfumes (Arias-Gil et al., 2007).

The molecular composition of wine, as well as the composition of grapes, is not stable upon time. Both keep evolving after winemaking. Illustrated in Fig. 17, several acids are directly involved in the fermentation process. Even if pyruvic acid is unlikely to find in wine, its crucial precursor role in cell metabolism needs to be noticed. For example, it gives rise to lactic acid through enzymatic reductions. The resulted stereoisomers can either emanate from bacterial or yeast origins (Swiegers et al., 2005). Likely, oxaloacetic acid comes from carboxylation of pyruvic acid; acetic acid arises from enzymatic

decarboxylation of the same precursor. Succinic and fumaric acids do not originate from pyruvic acid but are rather involved in the Krebs cycle and independently synthesized during lipid metabolism. In wine beverage, succinic acid nicely points up wine's flavors by providing bitter notes arousing salivation (P Ribéreau-Gayon et al., 2006). Most of the acids present in wine or during the winemaking process are highly functionalized through hydroxy and acid functions, justifying their reactivity and highlighting wine flavors modification through ageing. This poly-functionality also explains their polar and hydrosoluble features.

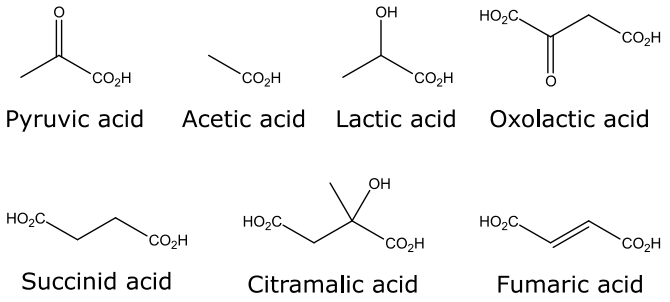

Figure 17. Principle organic acids produced during fermentation

2.1.3.3. From grape to wine

From the chemical point of view, winemaking modifies the chemical composition from grape berries to fermented grape beverages. Compounds such as organic acids, sugars, phenolic and nitrogenous derivatives and minerals are subject to evolve, hence giving wine its color, aroma and its complex taste (Jackson, 2008). Aroma, initially developed in the fruits, are modified with fermentation and keeps evolving with ageing and wine maturation. According to Smith *et al.*, molecular evolution can be classified into 4 groups (Smith et al., 2015). First are the unchanged molecules arising from grapes and present in wine without being modified. They mainly account for grape taste, such as rotundone that gives wine its pepper aroma (C. Wood et al., 2008). Their concentration in aged wine directly arises from berries growing conditions (sun exposure, environmental humidity and temperature, berry maturity before harvesting, *etc*.). The second group concerns grape compounds that undergo chemical modification during the technical winemaking steps (i.e., the grape crushing) without important metabolic alteration. C_6-alcohols (that induce grassy/herbaceous fragrances), benzene and monoterpenes contained in the grape skins, belong to this class (Peng et al., 2013). Responsible for the floral and citrus taste, terpenols are either present as free molecules or bound to sugar units (namely glycosides). Such bonding does not allow the flavor to develop. Bound configuration are considered as aroma precursor since they inhibit olfactive characters until the glycoside bond is hydrolyzed under acid or enzymatic attacks (Mateo & Jiménez, 2000). The third group deals with molecular compounds arising from biological activities, either yeast or bacteria. It mainly regroups FA such as acetic acid, esters and several alcohols. During the winemaking process, malolactic fermentation produces lactic acid through the impulsion of bacteria, and yeast in lesser extent. The decarboxylation of the malic acid, responsible of displeasing strong flavors, is transformed into lactic acid that has a sweeter character (P Ribéreau-Gayon et al., 2006). Moreover, esters that confer wine its fruity fragrances, are not originally present in the fruits and get synthesized during the fermentation when FA react with alcohols. For instance, ethyl esters such as the isoamyl acetate (3-methylbutyl acetate) that is recognized for its banana tasty character comes from the esterification of

acetic acid with alcohol produced during the fermentation. The last category specifically targets compounds responsible for the wine color that are also produced by metabolic activities of yeast or bacteria. It includes thiols, tannins and polysaccharides. Anthocyanins, mentioned above, promote reddish coloration. Although they are not stable over a long time period, they disappear slightly and let the tannins take precedence over the coloration (Šuklje et al., 2016). During the winemaking process, this last category of compounds might arise from enzymatic reactions, oxidation or pre-fermentation of the crushed skins in the must.

Early on fermentation was surely triggered by the addition of fruits into the beverages (Nigro & Rinaldi, 2020). The assumption comes from the recent discovery of *Saccharomyces cerevisiae*, the yeast responsible for fermentation, into the pollinator's guts (Meriggi et al., 2020; Stefanini et al., 2016). Insects could therefore transfer the yeast to the fruits while pollinating, hence giving a valuable way to control the spontaneous fermentation induced by the reaction with the initial sugar contained in the fruit meshes. Alternatively, honey has been highly mentioned to trigger the fermentation process as well, since pollinator insects surely provided the *Saccharomyces cerevisiae* yeast (P. McGovern et al., 2017; P. E. McGovern, 2013). Egyptian used honey to modulate the beverage taste: when added before the fermentation was achieved, it raised the alcohol degree while it rather sweetened wines when added at the end of the fermentation (Rösch, 2005). Besides the taste benefit, honey provided a great deal to control the fermentation process.

2.1.3.4. Tartaric acid: a suitable biomarker?

Chemical characterization of wine in archaeological artefacts is nowadays routinely achieved through the biomarker concept, alike the identification of resin. Nevertheless, one central query remains how viable are the markers for wine traceability.

Among the wide scope of organic molecules present in wine, tartaric acid has been greatly awarded to evidence grape beverages since it is indeed one of the most abundant organic acid naturally present in grapes (Maria Rosa Guasch-Jané et al., 2004; Maria Rosa Guasch-Jané, Andrés-Lacueva, Jáuregui, et al., 2006). It is also interesting to note that grapes ripeness, varieties as well as their origins influence its concentration (Ben Stern et al., 2008).

Tartaric acid potential as biomarker arises from its high concentration in grape towards other plant material. Two cases need to be distinguished. First, the molecule is synthesized with the metabolism of ascorbic acid in many vegetables and fruits. However, and therein lies all the subtlety, it is present in lower extent (roughly 100 mg/L compared to approximatively 5 g/L in grapes) (Singleton, 1996). Other edible products are consequently not supposed to release enough acid to be remarkably extracted during the analysis, especially if they underwent degradation. Contrariwise, producing wine out of grapes does not consume tartaric acid, affording fermented beverages to remain with significant acid concentration (P Ribéreau-Gayon et al., 2006). Secondly, some exotic fruits such as tamarinds, yellow plums or pomegranates display high concentrations of tartaric acid (Barnard et al., 2011; Hasnaoui et al., 2011; Rao & Mathew, 2012). Notwithstanding, it would be very unlikely from an archaeological point of view to find them in Mediterranean amphorae dating from Roman times. Indeed, interpreting analytical results requires caution. Archaeological considerations must be included to not misunderstand the meaning of

the conclusions. In spite of this, it is important to notice that some uncertainties may origin from the fact that neither wild nor modern plants have been quantified (Barnard et al., 2011; Léa Drieu et al., 2020).

Although tartaric acid is not exclusively but mainly produced by *Vitis* species, it displays some drawbacks regarding its physico-chemical properties. Present in acidic form (TH_2) in aqueous solution, the polar molecule is grandly soluble in water which clearly reduces its subsistence over archaeological times. Despite the formation of tartrate (TH^-) or bitartrate (T^{2-}) salts with surrounding alkaline cations that slightly decreases its water solubility, the acid marker can leach out by water ground percolation (Barnard et al., 2011; P Ribéreau-Gayon et al., 2006). Its presence or absence might not be sufficient to draw relevant interpretation. Especially in ground context, surrounding sediments should also be analyzed regarding the hydrosolubility of tartaric acid since water runoff might have dissolved it and promoted its migration (Barnard et al., 2011).

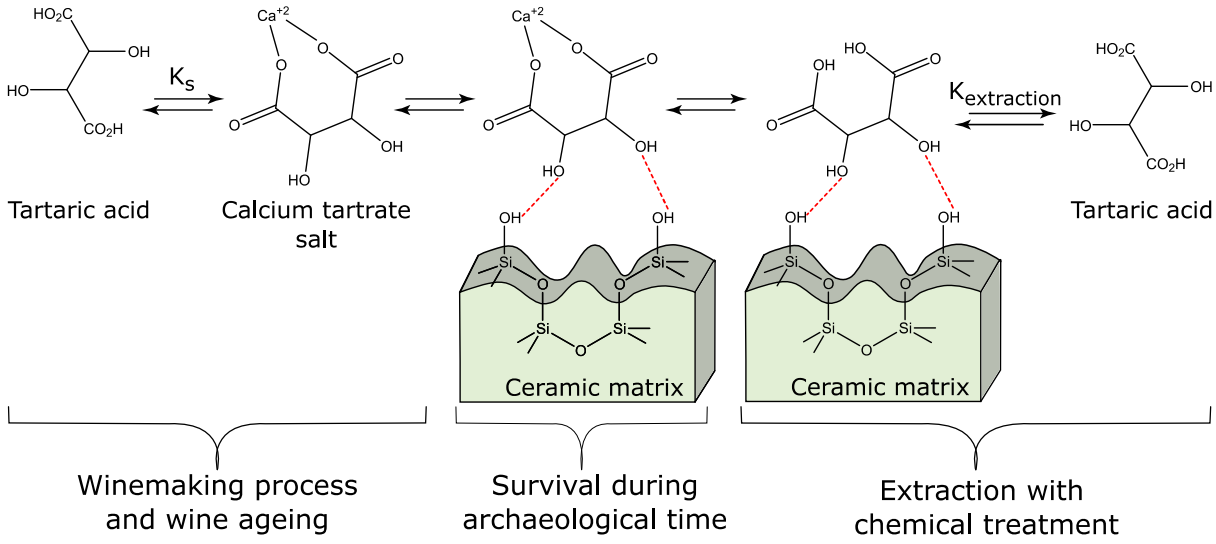

Figure 18. Equilibrium states of precipitation and adsorption of tartrate salts with the ceramic matrix from the winemaking process to the extraction in the lab

In wine artefacts, salts are unlikely to find since crystallized structures could not pass through the organic layer of pitch. However, tartaric acid molecules might have got across due to their small sizes and chemically bound with the ceramic matrix. Indeed, ceramics present the advantage of being silicate-rich favoring intramolecular hydrogen bonding with the acid. Acting as a Lewis acid, the polar character of the dihydroxy dicarboxylic acid is enhanced by a rich electronic density enabling the molecule to strongly interact with electrophile components from the matrix (Garnier & Valamoti, 2016; Michel et al., 1993b). The formation of tartrate salts is thereby the best transformation perspective to allow the marker to persist. The precipitation as salts is triggered by the bonding with alkaline ions or alkaline earth metals. Recurrent species are mono potassium (THK), dipotassium (TK_2), calcic ($TCa\text{-}4H_20$) or even mixed potassium and calcium salts (T_2CaK_2) or calcium tartaromalate salts (Pascal Ribéreau-Gayon et al., 1998). By adsorption on the inorganic matrix, the salts formed are then preserved from leaching, turning tartaric acid into a suitable biomarker (Fig. 18, adapted from (Garnier, 2003)). Several investigations have

highlighted the great ability of tartaric acid to subsist, versus other small organic acids naturally present in wine (Pecci, Cau Ontiveros, et al., 2013; Pecci, Giorgi, et al., 2013).

Finally, rigorous care regarding sampling and storing protocols must be pointed since tartaric acid can arise from plastic bags phthalates (R. P. Evershed et al., 2008).

Considering what we know, tartaric acid can thus be considered as a reliable marker although its presence does not exclusively prove the former presence of alcoholic beverage. It does instead evidence grape derivatives such as juice, raisin fruits, grape syrup or defrutum (Garnier, 2003; Garnier & Valamoti, 2016). Considering amphorae characterization, the archaeological context is primordial for archaeometric analysis and should never be discarded from analytical conclusions.

Palaeoenvironmental considerations, anthropologic and ethnographic indications are indivisible from the object history. The artefact must be considered as a whole. As a pledge of quality, conclusions must be drawn after interpretation, put in a broader picture and supported by archaeological insights.

2.1.4. Lipids

In the broadest sense of the term, lipids can be defined by their molecular structures made of carbon and hydrogen units, giving them a lipophilic character that increases with the length of the carbon chain and the absence of functional groups. Following the scientific appellation of *lipid*, this thesis will also restrict its use and meaning to that of *fatty acid*.

In the chemical sense of the term, lipid definition helps characterizing the chemical composition of fresh oil, as described below. It essentially corresponds to a mixture of triacylglycerides (TAG). In less extent, some other compounds such as sterols can be present.

Fatty acids are common compounds in living organisms. They have been reported in vegetal oil composition, animal fats but also in the grape composition.

2.1.4.1. Triacylglycerols and fatty acids

2.1.4.1.1. Definition and structure

Structurally speaking, a TAG presents a glycerol skeleton linked to three fatty acid (FA) units with ester bounding instead of hydroxy functions (respectively represented in black and red in Fig. 19). They qualitatively and quantitatively account for the major components of fats and oils of natural origin. In the case of vegetable oils, FA can be saturated or unsaturated. Conversely, fats of animal origin are generally saturated in order to promote better fluidity (Nicholson & Shaw, 2000). Unsaturated fatty acids, mostly have double bonds in *cis* position. Indeed, *trans* position is very little represented, with the exception of certain products such as dairy products or ruminant animal fats (Bastien, 2011). For the following part, only the chemistry of vegetable oils will be detailed since amphorae are more likely to have contained

vegetable fats, contrary to vessel potteries that could contain animal or body fats for cooking (Lea Drieu, 2017; K. Romanus et al., 2007).

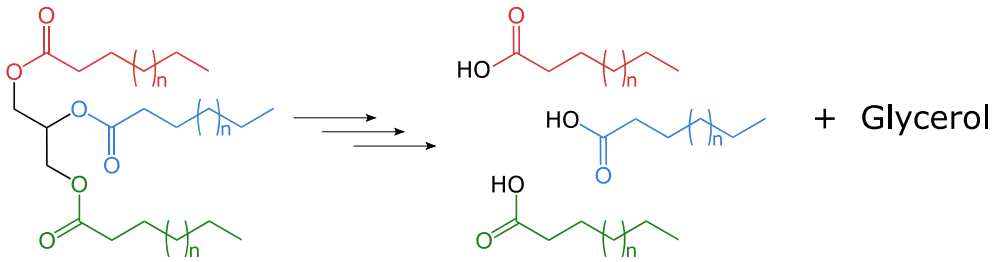

Figure 19. TAG unit degraded into free fatty acids and glycerol

Among the main FA present in oil, the linear structure comprises an even numbered carbon skeleton ranging from 14 to 18 carbon atoms (Table 4). Up to three unsaturations can be present along the FA chain (Fig. 19). FAs with odd-numbered chains of carbon atoms is mainly synthesized by microorganisms, while unsaturated/polyunsaturated FA are rather common in plants and aquatic animals.

Fatty acid	Carbon chain number	Unsaturation number	Abbreviation
Myristic acid	14	0	$C_{14:0}$
Palmitic acid	16	0	$C_{16:0}$
Palmitoleic acid	16	1	$C_{16:1}$
Stearic acid	18	0	$C_{18:0}$
Oleic acid	18	1	$C_{18:1}$
Linoleic acid	18	2	$C_{18:2}$
Linolenic acid	18	3	$C_{18:3}$

Table 4. Structural characterization from the main FA of vegetable oils

Due to the ester instability towards archaeological times, TAGs are subject to degradation via hydrolysis. As described by Dudd et al, the loss of a first fatty acid is quickly followed by the hydrolysis of the others two (Stephanie N. Dudd et al., 1998). The consequence is twofold: the formation of TAG derivatives such as diglycerol (DAG) and monoglycerol (MAG) and the release of the associated fatty acids. More than DAG or MAG, resulting free fatty acids (FFA), considered as natural and anthropic markers of degradation, are more likely to observe in amphorae organic residues. Alternatively, FFA come also under degradation owing to their structure, notably unsaturated FFA. Degradation reactions are detailed below.

2.1.4.1.2. Degradation reactions

A non-exhaustive set of degradation products is illustrated in Fig. 20 (adapted from (Mezzatesta, 2019)). Among the many potential reactions in the reign of polyunsaturated linear molecules, only the more

foreseeable in archaeological context will be detailed: the oxidation through auto-oxidation and β-oxidation and dihydroxylation. Other FA degradation have been mentioned, such as FA reduction triggered by microorganisms which takes place in anaerobic context, the formation of long-chain ketones via dehydration and decarboxylation under heating above 300°C, and the formation of ω-(*o*-alkylphenyl)alkanoic acids from polyunsaturated FA heated to 270°C (Lea Drieu, 2017; R. Evershed et al., 1995; R. P. Evershed et al., 2008; R P Evershed et al., 1991). However, they remain rare within archaeological oil amphorae since they require specific reaction conditions unlikely to have occurred.

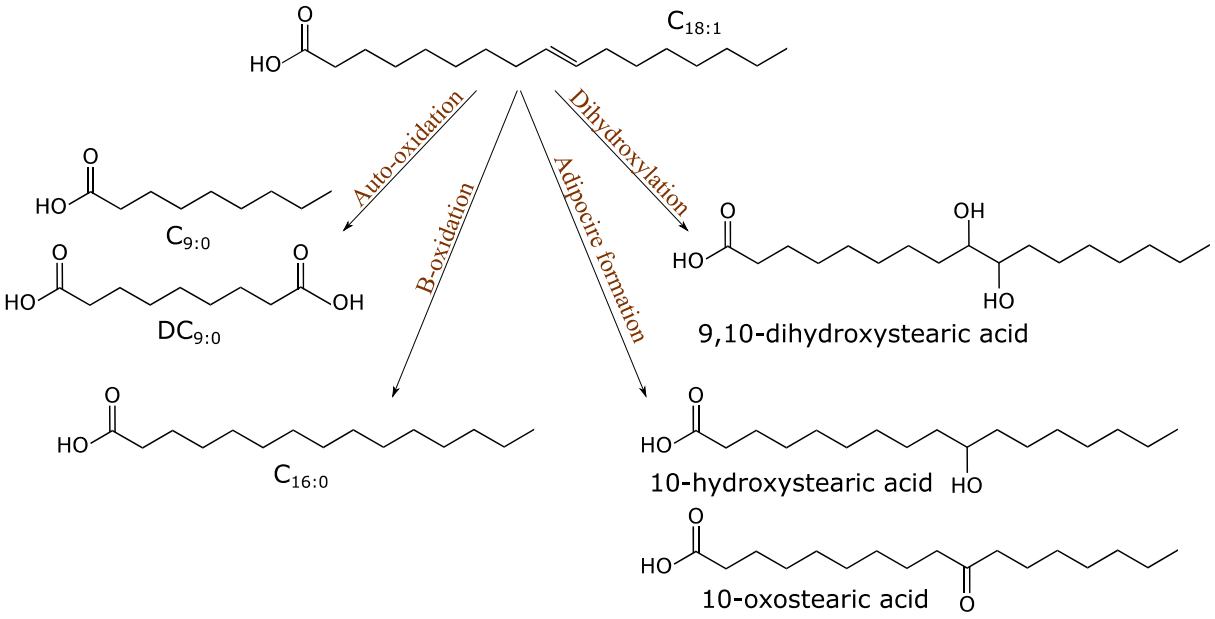

Figure 20. Main degradation pathways of fatty acids in archaeological context

2.1.4.1.2.1. Unsaturated fatty acids auto-oxidation

Auto-oxidation outlines the natural breaking down of the FA chain number through oxygenation of the unsaturated bonds. The reaction involves radical initiation and hydroperoxide formation. Due to its high instability, they finally give rise to splitting into diacids, formation of short-chain mono and diacids, dimerization among other side-products (Copley et al., 2005; Hansel et al., 2011).

Several structural and environmental factors can affect the rate of degradation. Among them, polyunsaturation and longer carbon chain increase the mechanism (Eerkens, 2005). Even though auto-oxidation occurs at room temperature, the process becomes more critical with light exposure and raised temperature. Likewise, some metallic ions can act as catalyzers, which is important to notice since amphorae are built from inorganic matter (Aillaud, 2001; Lea Drieu, 2017).

Novelty in deciphering the initial nature of FA arose from the retro analysis of the carbon position submitted to degradation. This way, the diacid chain length highlights the position where the peroxide reacted and consequently, the initial position of the unsaturation. The carbon chain number therefore gives information on the parent molecular structure. The oleic acid ($C_{18:1}$) degradation into azelaic acid ($C_{9:0}$) is a great example of this retro-analysis and clearly demonstrates the relation among them through

oxidative cleavage of $C_{18:1}$. Additionally, Copley *et al.* evidenced the direct formation of α,ω-diacids from unsaturated FA within archaeological potteries by qualitative correlation of isotopic analysis (Copley et al., 2005).

2.1.4.1.2.2. β-oxidation

β-oxidation features the segmentation of FA carbon chains, inducing a decrease in chain length. Even though the mechanism can affect linear and branched FA, unsaturated linear skeletons are more sensible to β-oxidation, especially for long carbon chains (R P Evershed et al., 1991). The biological mechanism triggered by the acetyl-coenzyme A, is operating in aerobic conditions (Den Dooren De Jong et al., 1961).

2.1.4.1.2.3. Unsaturated fatty acids hydroxylation

Monosaturated FA are also subjects to dihydroxylation. As described earlier for the oxidative cleavage and splitting of the carbon chain, vicinal diols take place on both sides where the unsaturation was initially located (K. Romanus et al., 2007). For instance, Colombini *et al.* brought to light the affiliation of α, ω-dicarboxylic and ω-hydroxycarboxylic species towards the unsaturated of the same chain length by degrading intentionally gondoic ($C_{20:1}$) and erucic acids ($C_{22:1}$). They also confirmed the formation of dihydrxyocarboxylic acids identifying 11,12-dihydroxyeicosanoic acid and 13,14-dihydroxydocosanoic acid from their acid precursors (Maria Perla Colombini, Giachi, et al., 2005).

Although FA are omnipresent in natural edible substances, their poly or monounsaturated structures for some do not ensure their preservation over archaeological periods. This is the reason why organic residue analysis must consider the potential degradation endured in order to better evaluate the originally present products. However, the oxidation of original fatty acids induces a quantitative loss of lipids. In addition, by-products of degradation are also less hydrophobic (in particular by reduction of carbon chains or by addition of functional groups) which makes their presence rarer in non-arid archaeological environments.

2.1.3.3. Sterols

2.1.4.1.3. Definition, structure and natural distribution

In archaeology, sterols refer to triterpenic molecules exhibiting a functionalized sterane backbone (Fig. 21a). They are an integral part of the composition of natural plants or animal substances. One of the common animal sterols is cholesterol (Fig. 21b). Contrarywise to the animal kingdom where sterols have C-27 structures, plants show C-29 patterns phytosterols such as campesterol, stigmasterol and β-sitosterol which are predominant (Fig. 21c, d and e).

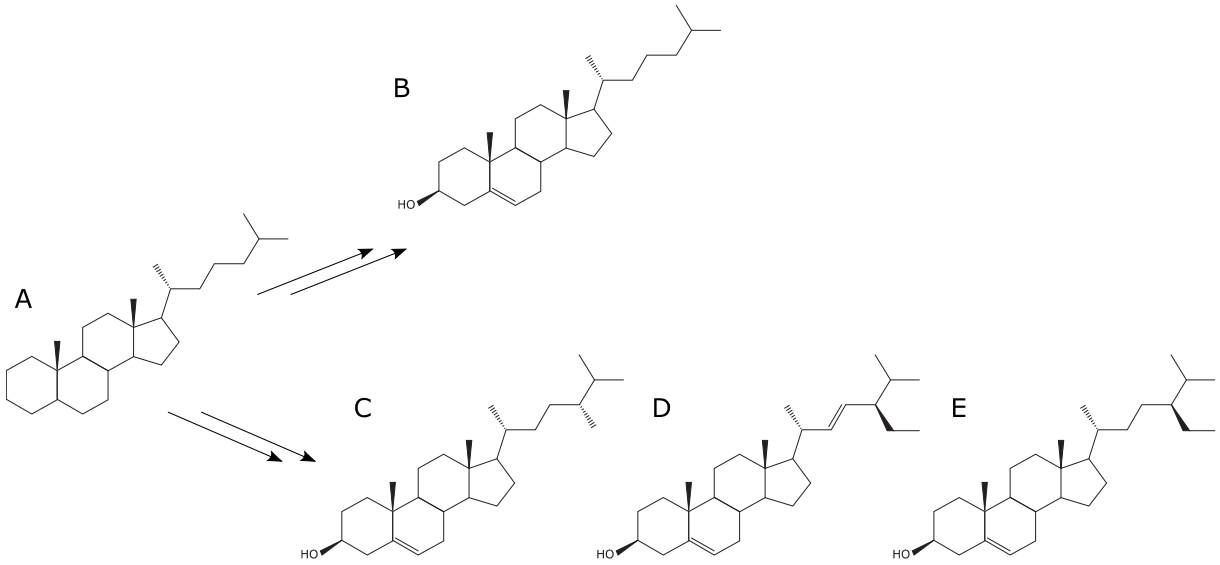

Figure 21. Sterane skeleton (A) into cholesterol (B); campesterol (C); stigmasterol (D) and ß-sitosterol (E)

2.1.4.1.4. Degradation pathways

Constrained by their molecular structure, sterols are relatively stable over time. Indeed, it is not unusual to observe campesterol, stigmasterol or β-sitosterol when analyzing organic residues of pottery that have contained vegetable oils (Erika Ribechini et al., 2008; Spangenberg et al., 2014). However, sterols can be subject to oxidative degradation by ambient atmosphere when natural substances are heated. Even though cholesterol is likely to find in analysis, its interpretation needs to be careful regarding possible human contamination.

2.2. Analytical methods

2.2.1. History: Organic analysis residues introduction

Organic residue analysis (ORA) is strongly related with the biomolecular approach (detailed in 2.1.1. Biomarker concept). It deals with the capability of analytical techniques to extract, at the molecular level, organic residues trapped in their original matrix, that can be organic or inorganic. Such molecules cannot be observed through traditional analytical method since they would not have survived archaeological times without being physically and/or chemically protected (Richard P Evershed, 2008). Following the biomolecular approach, organic residues translate archaeological information regarding the original compounds. Dealing with the laborious task of understanding the ceramic becomes fundamental at the molecular level since residues can be altered by human activities and burial conditions (Bethell et al., 1994; Gülaçar et al., 1989). Estimation up to 80% of the absorbed molecular residues are supposed to survive in the ceramic wall surface (Charters et al., 1993; Ben Stern et al., 2000).

Although the organic matter was early on lusted by archaeologists because sometimes visible to the naked eye, the development of more advanced analytical techniques allowed the matter to reveal its secrets. In the 1900s, the organic surface of ceramics as well as organic fragments recovered from sediments during archaeological excavations were initially characterized by their visual and physico-chemical properties, such as the melting point or viscosity, *etc*. (Lucas, 1914). The use of 'spot tests', such as the Feigl test for tartaric acid (P. E. McGovern, 1997; Michel et al., 1993b), and the characterization by chemical reaction (Cotte, 1917) led to certain advances, nowadays controverted due to their imprecision and low selectivity (Léa Drieu et al., 2020; Ben Stern et al., 2008). Mainly based on the presence/absence of compounds, these techniques ultimately reflected very little of the original material.

Then, the middle of the 20th century has seen the emergence of spectroscopic techniques, notably infrared, Raman and nuclear magnetic resonance approaches. Shedding light on the bulk composition, spectroscopic techniques were on the cutting edge of innovation and turned to be greatly used to assess the fingerprint of the overall material. For instance, calcium tartrate had been identified in Haji Firuz potsherds thanks to the carboxylate infrared bonds, hence highlighting the presence of archaeological grape-derivative content (P. E. McGovern & Michel, 1996; Michel et al., 1993b). Likewise, other attempts have been supported by diffuse-reflectance infrared spectroscopy coupled with liquid chromatography with ultraviolet detection (P. E. McGovern, 1997; P. E. McGovern et al., 1999, 2004). Although strongly questioned today because of the additional nature of the infrared spectroscopy and the complexity of targeting specific bands indicative of specific compounds in molecular mixtures, it was nevertheless perceived as a major advance in the ORA field at this time (Léa Drieu et al., 2020; Garnier & Valamoti, 2016; C Heron et al., 1991; Ben Stern et al., 2008).

Taking precedence over spectroscopic methods, the emergence of chromatographic tools in the 20th century led to great outcomes. Selective and sensitive enough to target organic residues, they started to be routinely used on archaeological artefacts. (J. Condamin et al., 1976; P. E. McGovern, 1997). The analysis of medieval ceramics demonstrated the capacity of the porous material to absorb organic matter on the one hand, and its capacity to conserve it on the other. (J. Condamin et al., 1976; Jeanne Condamin & Formenti, 1978). The presence of organic residues could not be attributed to the environment, given the different molecular distribution and quantities involved (C Heron et al., 1991). From then on, the absorption of various natural substances, such as vegetal and animal fats have been tested simultaneously to molecular characterizations of natural substances to obtain references to compare with (Charters et al., 1993, 1995; S. N. Dudd, 1999). Laboratory experiments of controlled degradation and anthropic influence of heating actively took part in the understanding of the molecular alteration of the residues trapped in ceramic vessels (Charters et al., 1995, 1997; Stephanie N. Dudd et al., 1998; R. Evershed et al., 1995; Raven et al., 1997).

To get archaeometric analysis optimal, two fundamentals' steps are required after the molecules are extracted from their environment: the molecular separation first and their identification then. Since the organic mixtures usually contained a great extent of molecules, chromatographic devices are strongly recommended to ensure a good separation and mass-spectrometric (MS) technologies are necessary to improve molecular characterization (Richard P Evershed, 2008). Indeed, without MS analyzers, molecular characterization is undergone with retention and migration time instead of structural molecular patterns, which clearly suffer from a lack of specificity (Léa Drieu et al., 2020).

2.2.2. Chromatographic methods

Developed from the 1950s onwards, chromatography has established itself as the leading technique for compounds separation. Resolution at the molecular level has consequently had a strong influence on various sciences, including archaeometry. Pioneer studies conducted by Condamin established the premises for the ORA, notably by tracing back fat remains from amphorae (J. Condamin et al., 1976).

Being the universal technique of choice, numerous are the publications calling on GC-MS to reveal organic hints. (Colombini and Modugno, 2009; Garnier and Valamoti, 2016; Mezzatesta *et al.*, 2021; Modugno *et al.*, 2006; Pecci *et al.*, 2020, 2013; Ribechini *et al.*, 2008). Indeed, Brassicaceae seed oil was characterized in Egyptian lamps using GC-MS thanks to the identification of dihydroxy and dicarboxylic erucic and gondoic acids. Archaeological grape-based derivatives were successfully identified from potsherds dating back to the Early Medieval Islamic Sicily, the Early Neolithic in the South Caucasus, the Prehistoric Northern Greece or the Late Chalcolithic Near Eastern (Barnard et al., 2011; Léa Drieu et al., 2021; Garnier & Valamoti, 2016; P. McGovern et al., 2017).

Being a referential method, we strongly considered GC-MS as the method of choice for this thesis research. The analytical set-up is described below.

2.2.2.1. Gas Chromatography-based method

The GC device is composed of 4 units: the injector, the chromatographic column, the oven and the detector (Fig. 22, adapted from ©CHROMacademy and (Cajka et al., 2016)). The solution is injected into the apparatus by vaporization and carried to the stationary phase (SP) by the mobile phase (usually an inert carrier gas, which also guarantees the transport of the component all along the chromatographic column). The routine mobile phase is helium. The injection can be manual or automatic. Both the split and splitless mode exist, allowing respectively partial or total injection of the solution into the column. In archaeological context, splitless is favored since concentrations are usually low.

The stationary phase corresponds to the inner coating of the open tubular column. The separation is achieved all along the chromatographic column thanks to affinity difference towards SP composition and polarity. The column is placed into an oven whose temperature increases gradually in order to slowly volatilize and eluate the compounds of the mixture, starting from the lowest volatilization temperature molecules to the highest.

When the carrier gas finally emerges from the column, the detector records a signal according to the difference in composition. Within the various types of detectors, the most powerful for organic traces remains the mass spectrometer which allows component identification through characteristic mass fragmentation. Its high sensitivity and selectivity perfectly allow residues analysis in archaeometrical fields of research.

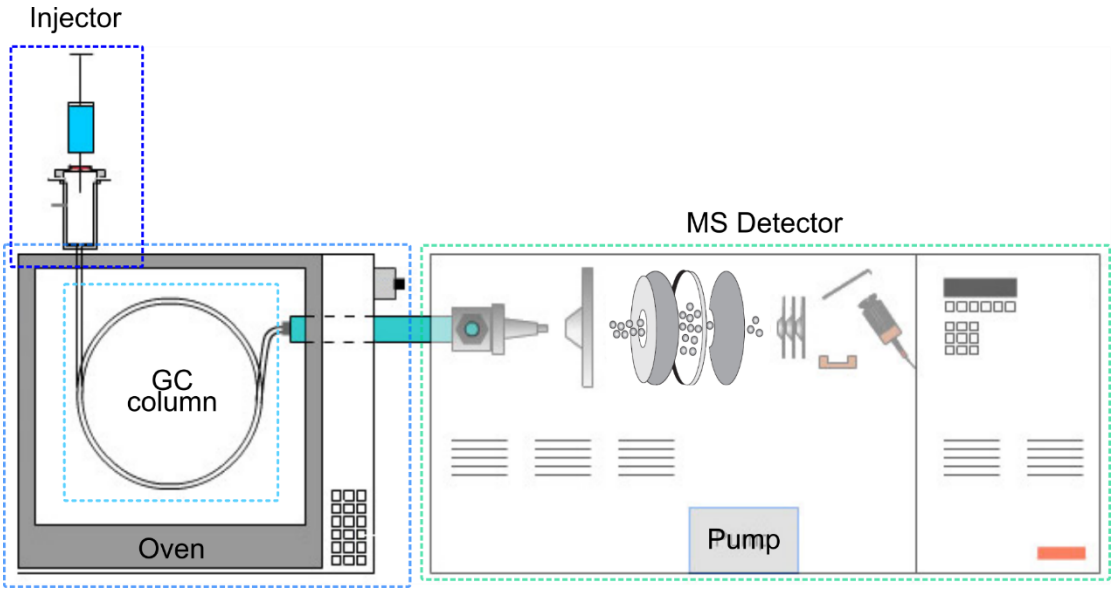

Figure 22. GC-MS schema equipped with a quadrupole ion trap

Samples are derivatized with N, O-Bis(trimethylsilyl) trifluoroacetamide (BSTFA): trimethylchlorosilane (TMCS) to improve transform hydroxyl and carboxylic acid polar groups into trimethylsilylated groups that are more suitable for volatilization. Different chemical options exist for derivatization, but trimethylsilylation was favored over methylation due to some naturally methylated compounds whose presence is archaeologically characteristic.

2.2.2.2. Liquid Chromatography-based method

High Performance Liquid chromatography (HPLC) is aimed to target high molecular weight molecules, considered to be of low volatility or with high polarity. Thus, the analysis of organic dyes or even proteins has been possible by LC (Buckley et al., 2013; Dallongeville et al., 2011; Mazzitelli et al., 2019; Pronti et al., 2018). Applied to the archaeometric field, LC is not the method of choice because of the detectors, since it requires the use of detectors that are specific to each family of compounds and does not allow for a global screening of the molecular composition but rather a specific search.

Among the rare exception, organic residues alterations have been followed by HPLC coupled with Electrospray Ionization - Quadrupole Time-of-Flight MS (HPLC-ESI-QToF) in order to target potential diagnostic biomarkers of fresh whale oil (Blanco-Zubiaguirre et al., 2018). Likewise, triglyceride profiles from drying oil, characterized with HPLC-ESI-Q-ToF, allowed to trace back the composition of plant oils (La Nasa *et al.*, 2013; Saliu, Degano and Colombini, 2014) or from the binding paint medium (La Nasa *et al.*, 2015) and helped identifying the pigments in the polymeric paint network (La Nasa *et al.*, 2019). Such advanced method has also been mentioned for the analysis of TAGs in archaeological vessels (Lucejko *et al*., 2018) and high molecular weight esters in archaeological beeswax and resinous substances (La Nasa *et al*., 2020).

Finally, the coupling of LC with a MS^2 detector allowed the detection of wine molecules in Egyptian and Nordic potsherds (Guasch-Jané *et al.*, 2004; Guasch-Jané, Andrés-Lacueva, Járegui, *et al.*, 2006; Guasch-Jané, Andrés-Lacueva, Jáuregui, *et al.*, 2006; McGovern, Hall and Mirzoian, 2013).

2.2.2.3. Pyrolysis-GC-based method

Since the beginning of the 21st century, new techniques for the analysis of lipids, proteins or polysaccharides, namely as pyrolysis GC-MS or Direct Temperature-resolved MS DTMS have emerged (Oudemans and Boon, 1991; Oudemans, Eijkel and Boon, 2007; La Nasa *et al.*, 2019). Allowing the screening of the overall composition by the analysis of different compounds families at the same time, they have grandly improved the overall characterization (Bonaduce *et al.*, 2017). Intense heating makes it possible to obtain the chemical fingerprint of the sample by showing off the different chemical families present. Direct exposure MS and electrospray–MS^2 indeed overcome the non-volatile compound barrier constrained by GC-MS to give insight on the composition of triterpenoid resins and beeswax (Garnier *et al.*, 2002; Modugno, Ribechini and Colombini, 2006; Kaal *et al.*, 2014; Kaal, López-Costas and Martínez Cortizas, 2016).

2.2.3. Mass Spectrometry

The MS apparatus is following 3 steps: ionization in the ion source, mass filtration in the mass analyzer and electromagnetic detection (Fig. 22). An inlet system guarantees the release of the gas stream from the mobile phase into the ion source at a controlled rate. 70 eV electronic impact (EI) is the prevalent method to produce positive ions. The incoming stream of gas phase molecules is ionized by interactions between an electron beam coming from a hot filament with the analytes (Fig. 23). Once ionized, resulting unstable radical cations are usually fragmented. Indeed, the electronic energy is largely sufficient to enhance the fragmentation of organic molecules. A positive voltage from a repelled electrode electrically pushes them out, generating a stream of positive ions and fragments to move out from the ion source to the mass analyzer through the hole located the superior electrode.

Ion trap analyzer looks like a “doughnut-shaped ring” with an annular electrode equipped with radiofrequency (R_f) voltage and two end caps supplied with alternative or direct voltages (AC or DC) (Fig. 22). It is commonly named “quadrupole” due to the shape of the magnetic field generated by the superposition of voltages applied from the electrodes. Submitted to a tridimensional electric field in the analyzer, ions start oscillating until reaching a so-called “infinite” stable shape whose oscillation amplitude and frequencies strictly depend on the ionic *m/z* ratio and Rf. In order to gradually eject the ions, present in the analyzer, R_f increases, therefore generating higher axial oscillation amplitude. Ions with lower *m/z* are destabilized first. They move out the analyzer to the MS detector which monitors the ion counting and converts this electric signal into a digital one, giving rise to the mass spectrum (Varella, 2012).

MS analyzers pave the way to isotopic techniques, such as stable isotope ratio MS analysis. Indeed, Gas Chromatography – Combustion - Isotope Ratio MS (GC–C–IRMS) help in identifying ruminant fats from non-ruminant dairies. (Mottram *et al.*, 1999; Mottram and Evershed, 2003; Romanus *et al.*, 2007). From

palmitic and stearic acids in archaeological ceramic vessels, Copley *et al.* demonstrated the presence of domesticated ruminants dated back to Neolithic, therefore early on exploited for dairy productions (Copley *et al.*, 2003).

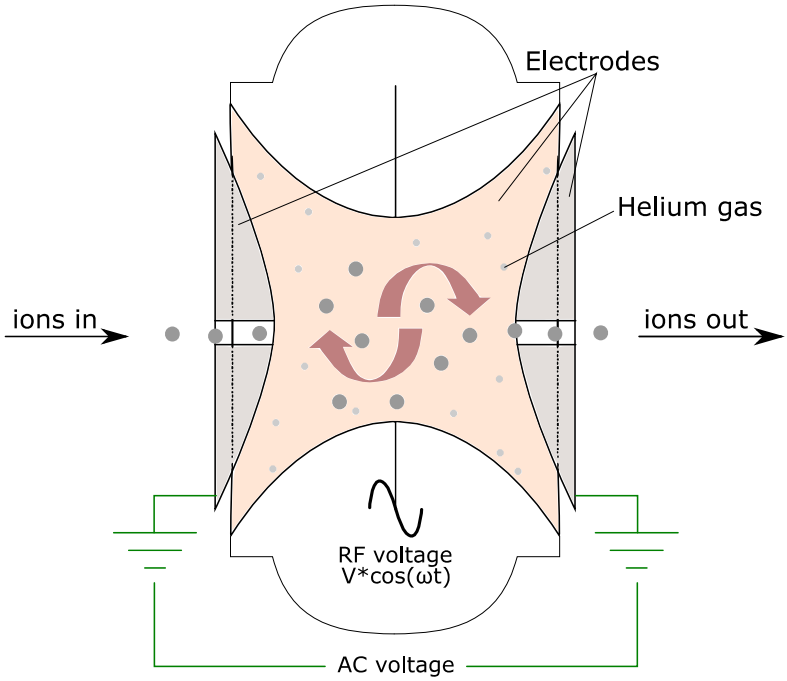

Figure 23. Ion trap set-up

MS gives the option to operate in different modes. Full scan is routinely used since it generates a qualitative picture of the different separated compounds, scanning from the smallest mass to the highest. It allows mass scouting and fragmentation pattern analysis. Selected ion monitoring (SIM) and multiple reaction monitoring (MRM) modes rather target quantitative focus on specific ions of interest.

2.2.3.1. SIM

Selected ion monitoring (SIM) is an analytical option provided by the detector to record ion currents only for preselected *m/z* ratio. Since the mass spectrometer does not record the entire mass range, SIM guarantees higher sensitivity and selectivity. Interferences are minimized increasing the detection limit to nanograms or picograms when the matrix permits it (Sparkman, Penton and Kitson, 2013). Ionization remains the same but only selected ion mass can reach the detector, which accounts for a better sensitivity compared to TIC chromatograms because the background is not acquired (Clench and Tetler, 2000). Although SIM analysis are not frequent in ORA, some researches have led to great conclusions regarding the presence of fermented beverages or beeswax in Iberian and Etruscan potsherds (Garnier *et al.*, 2002; Manzano *et al.*, 2016). For instance, SIM mode was employed to target bacteriohopanoids, revealing the archaeological presence of alcoholic beverages made out of fermented sap from agave plant in pottery vessels (Correa-Ascencio *et al.*, 2014).

2.2.3.2. MS^n

Tandem MS, also known as MS/MS or MS^2 involves two or more MS analyzers coupling which allows additional fragmentation steps. The main purpose is the great increase of specificity and sensitivity since many fragmented ions get ignored, which allows molecular compound focus within complex mixtures.

Coupled to MS/MS, multiple reaction monitoring (MRM) targets a singular *m/z* ion for fragmentation to generate a series of daughter ions and selects one (or more) of these daughter ions for the second MS detection. Guasch-Jané *et al.* reported the identification of wine markers from Egyptian vessels thanks to traces of tartaric and syringic acids, identified in tandem mode LC/MS/MS in MRM mode (Guasch-Jané *et al.*, 2004), with a limit of detection established to 0.01µg/mL (Stern *et al.*, 2008). Indeed, the second fragmentation gave great support for diagnosing specific wine markers.

Chapter 3

Pollen in archaeology:

A State of the Art

3.1. Once upon a time: the pollen

3.1.1. Pollen definition

Pollen grains are single-cell gametophyte produced by the reproductive systems of flowering plants. They carry the genetic information and account for the genetic variability of the. Pollen grains are small, usually ranging from 10 µm to 200 µm. During meiosis, pollen grains are initially formed as a tetrad. There exists as many pollen morphologies as plants, the impressive variety arises from the varying chemical composition and the structure of the exine, the outer wall of the grains. Among the main constituents of the exine (i.e., cellulose, hemicellulose, lignin, pectic compounds that compose the exine), sporopollenin is the one that confers indestructible properties to pollen grains. Being highly resistant to biological decay and non-oxidative chemical attacks, exine ensures pollen survival over geological times in a variety of sedimentary deposits. Indeed, pollen grains dating back to 15.7 Myr ago have recently been recovered from Antarctica ice cores (Feakins, Warny and Lee, 2012). Although the protecting effect of sporopollenin is established, little is known on its chemical composition. Oxidative copolymers of carotenoids and carotenoid esters have been identified though (Shaw, 1971; Brooks and Shaw, 1978). Recent investigations relying on solid-state NMR techniques highlighted the presence of aliphatic-polyketide-derived polyvinyl alcohol and 7-*O*-*p*-coumaroylated C16 aliphatic units within the very dense crosslinked biopolymer (F.-S. Li *et al.*, 2019).

3.1.2. Pollen dispersion

Pollen grains can be classified through their different dispersion mechanisms. Anemophilous pollen species are dominant in archaeological record. Being wind-pollinated, they can easily scatter over few kilometers from the generative plant (Bajpai and Kar, 2018; D. Li *et al.*, 2019). *Pinus* grains are a good example of anemophily that greatly scatter thanks to its saccate morphologies that favor lift capacities by increasing its surface area (Schwendemann *et al.*, 2007). Pollen get released when wind constrains balance the adhesives electrostatic and Van der Waals forces exerted from the anthers (Timerman and Barrett, 2021). Zoophilous pollen are spread thanks to animals and insects that promote plant pollination, carrying pollen grains from the anther to the stigma of another plant. In addition of being less represented in the plant kingdom, pollen is naturally covered by lipids to ensure pollen stickiness, which explains why zoophilous pollen are less likely to be recovered in fossil samples (Shillito *et al.*, 2020). Water-pollination from hydrophilous plants and self-pollination from cleistogamous plants are also barely represented in fossil records because of their little representation in plants (Bryant and Holloway, 1983).

3.1.3. Pollen preservation

Preserved by the chemically inert biopolymers of sporopollenin, pollen keep its structural integrity over extended times. An interesting study conducted over 20 years by Havinga showed the positive impact of the amount of sporopollenin on the longevity of pollen grains. The richer in sporopollenin the exine is, the better the resistance was observed (Havinga, 1964, 1984). As such, pollen taxa do not survive equally.

Indeed, thick-exine pollen transported by insects, together with pine, oak, grass or goosefoot grains are more preserved in fossil assemblages (Bryant, 1978; Bryant and Schoenwetter, 1987; Holloway, 1989; Bryant and Hall, 1993; Campbell and Campbell, 1994; Gorham and Bryant, 2001; Phuphumirat, Mildenhall and Purintavaragul, 2009).

In archaeological contexts, pollen deterioration can arise from different mechanisms. First, mechanical degradation induced by transportation and sedimentation phenomena can damage pollen grains (Campbell, 2010; Hunt and Fiacconi, 2018). To not misinterpret pollen representation, the taphonomy of the grains needs to be carefully evaluated to address any redeposition or differential preservation of more ancient sediments. Increasing the sediment compaction also alters the assemblage taphonomy (Ellison, 2008). Moreover, experimental wet-dry cycles demonstrate the rapid breakage of *Picea* and *Artemisia* pollen when recovered from desalinated sediments (Campbell and Campbell, 1994). In comparison, salinity promotes pollen structures preservation (or damage in lesser extent). Salt crystals are thought to absorb the mechanical constraints of water evaporation. Pollen grains trapped in the metal crystal network are also more protected (Rösch, 2005). Secondly, pollen grains are subject to chemical oxidation. Indeed, great evidence of pollen integrity over time has been reported in anaerobic sediments since the hydrogen released by the bacteria ensures reducing environment (Tschudy, 1969). Pollen has also been used to target the oxidation of organic matter in marine sediments. Keil *et al.* pointed out the great preservation of pollen for 100 000 years in anoxic environment from marine sediment cores while 10 000 years were sufficient for oxygen to degrade pollen in sediment where the gas was continuously diffused (Keil *et al.*, 1994). Regarding the degradation timescale, pollen is thus able to trace the oxygen content of the sediments. A recent study conducted on seven different pollen taxa from trees, shrubs and herbaceous plants aimed at outlining the factors that increase or delay pollen oxidative damage (Lebreton *et al.*, 2010). Although oxidation is intractable, both the morphology and the exine thickness have been shown to play an important slowing-down role. To a lesser extent, low pH seems to account for pollen preservation, while soil alkalinity is unfavorable (Dimbleby, 1957; Bryant *et al.*, 1994; Phuphumirat, Mildenhall and Purintavaragul, 2009). Finally, biological agents, namely fungi and bacteria, are pollinivores. Although the exact mechanisms remain unclear, may use the internal protoplast of pollen grains as nutrient, hence perforating the exine and triggering pollen degradation (Elsik, 1971; Bryant and Holloway, 1983; Twiddle and Bunting, 2010).

3.2. Palynology: a state of the art on the growing interest field

3.2.1. Historical point of view

The first record of pollen-based analysis was speculated by Iversen (1941) to corroborate the prehistoric introduction of agriculture with the disappearance of forest in Denmark (Iversen, 1941). He hereby interpreted the decline of elm pollen together with the great appearance of herbs and weedy plants as the human direct impact on the vegetational land through human occupation. Relying on the pollen diagram he made, he was also able to overview the prehistorical farmed plants. Although pollen diagrams were slowly showing up to clarify changing land assessment, pollen was nevertheless sparsely used and few are the evidence of pollen-based interpretation before the 1970 (Greig and Turner, 1974). While the discipline was still in its beginning stages, fundamental studies accounted for its emergence, notably

clarifying pollen morphologies and analysis methodologies (Erdtman, 1969; Moore and Webb, 1978; Faegri, Kaland and Krzywinski, 1989). In this way, pollen turned to be a reliable tool to assess the relative chronologies of past events, among which the beginning of animal domestication in Denmark (Troels-Smith, 1959); the buffalo grasslands expansion in Texas (Bryant, 1969), the inference of human activities on the Mesolithic vegetational land in England (Dimbleby, 1963) as well as the introduction of maize agriculture in Canada (McAndrews, 1976). Finally, Dimbleby and Evans (Dimbleby and Evans, 1974) unraveled the occurrence of two successive ecological environments from the same Neolithic chalk land. Although molluscan snail analysis gave insights into woodland, pollen surprisingly highlighted farmland. The authors marked a turning point in the discipline while publishing in the *Journal of Archaeological Science*, hence making clear the impressive benefits of pollen analysis and its concerning in anthropology, archaeology, palaeoecology and more broadly in the Quaternary science (Yeung *et al.*, 2015). As shown in Fig. 24, the number of publications of pollen-based investigations started raising from the 1970s (Edwards, Fyfe and Jackson, 2017). Data were extracted from https://app.dimensions.ai, with occurrence selected in titles and abstracts (n = 17930) from 1972-2020, all journals confounded.

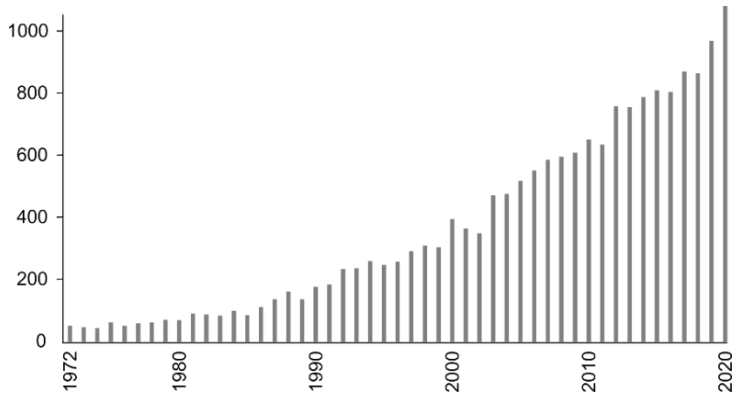

Figure 24. "Pollen analysis" occurrence in scientific publications.

3.2.2. From pollen analysis to archaeological meaning

Pollen analyses show a great potential for landscape reconstruction. Initially performed on single cores, the approach has been extended to multiple site sediment assemblages in order to provide comparative data (Magri, 1997; Tipping, 1998; Ledger, Edwards and Schofield, 2014). The comparison of pollen taxa also outlines species correlation, especially regarding their appearance or disappearance (Mercuri *et al.*, 2013). Thus, ecological, vegetational and spatial reconstructions are enhanced. The powerfulness of pollen markers have been enforced by the great development of pollen datasets such as the European Pollen Database (http://www.europeanpollendatabase.net/), the Neotoma Paleoecology Database (https://www.neotomadb.org/), the Global Pollen Project (https://globalpollenproject.org/), PalDat which is specific to fossil pollen (https://www.paldat.org/) and pollen atlases (Reille, 1992b, 1992a, 1995, 1998).

Human activities having a direct effect on the surrounding vegetation, pollen analysis has further supported the identification of past environmental variations, as well as climate changes. Enhanced by other research fields of archaeobotany, pollen demonstrated its great reliability as a reconstructive marker. Integrated archaeobotanical studies, including microproxies such as NPP (Non Pollen Palynomorphs), spores, diatoms, phytoliths, plants macrofossils and charcoals, have emerged (Di Rita, Celant and Magri, 2010; Burjachs and Expósito, 2015; Celant, Magri and Stasolla, 2015; Di Rita *et al.*, 2015; Mariotti Lippi *et al.*, 2018). Whereas pollen grains mirror the local landscape, charred plant macrofossils reveal the direct impact of human culture, sedentarization and agriculture (Lechterbeck and Jensen, 2020). When coupled to geochemistry or zooarchaeology, they can draw out a complete ecological reconstruction over time (Schofield *et al.*, 2010; Williams, 2012; Gismondi *et al.*, 2013; Tolksdorf *et al.*, 2020). For instance, palynological, anthracological, stratigraphical, geochemical and radiocarbon analysis of soil sediments, supported by historical evidence, helped in disclosing the negative impact of mining activities in archaeo-ecological systems in the Middle Bronze Age in France and Austria (Jouffroy-Bapicot *et al.*, 2006; Breitenlechner *et al.*, 2010). Multidisciplinary approaches are all the more favored to address heterogeneity in archaeological assembles, that might arise from time or spatial instabilities or even from the nature of the materials themselves. In contrast, lakes and mires are often considered to be stable enough to provide valuable insights on their own (Tweddle and Edwards, 2010; Edwards *et al.*, 2015). Nowadays, pollen analysis is additionally included in models and simulations, notably thanks to the incorporation of statistical tools to address transport or dispersal queries (Sugita, 2007a, 2007b; Edwards *et al.*, 2015). Recent interdisciplinary investigations have integrated ancient DNA data into pollen analysis (Jones *et al.*, 2017; Ekram *et al.*, 2021), notably for fossil coprolites (Beck, Bryant and McDonough, 2019; Petrigh *et al.*, 2021), with the specific aim of better understanding diet, disease, cultural and daily-life past practices. More broadly speaking, pollen analysis has supported animal and plant domestication and geographical distribution (Argant, López-Sáez and Bintz, 2006; Zarrillo *et al.*, 2008; Nott, 2010; Lipe *et al.*, 2016; Mercuri *et al.*, 2021); burial rituals (Mora *et al.*, 2018; Amerongen, 2019); on and off-site dating (Scott and Woodborne, 2007); archaeological technologies, manufactures and savoir-faire (Muller, 2004; Willis, 2017).

3.3. Methodological approaches and data analysis

3.3.1. Sampling issues

The highly preserved nature of pollen that makes it so powerful in palaeocology also confers it certain limitations. Being the primary tool for past plant reconstructions, any contamination by pollen that do not belong to the targeted time scale or any discriminatory degradation of certain pollen due to a lower resistance of its sporopollenin (Lebreton *et al.*, 2010) can lead to misinterpretation. Indeed, pollen progressive degradation or partial preservation biases pollen counts (Bottema, 1975; Hall, 1981). To avoid such pollen distorted representation, different rules have been set up. Sampling must be performed after considering the potential surrounding degradation to prevent the inclusion of reworked grains in the analysis. Each site is different and must be carefully studied to limit any contamination. Undisturbed sediments are fundamental. When dealing with archaeological artefacts such as potteries, sealed jars are obviously favored. If not, the archaeological site needs to be carefully studied before any sampling to assess any contamination from erosion, suffusion, post sedimentation or sediments relocation due to

mechanical displacement of unstable sites, bioturbation, recycling soil sediments, *etc*. The position of the objects regarding the site is extremely relevant. Then, the reliability of the archaeological interpretation requires multiple-sample analysis. A certain pollen occurrence must be established in order to draw stable conclusions and limited total pollen concentration are to interpret with caution (Hall, 1981). A sole sediment sample cannot induce valuable palaeoecological reconstruction. The trends it may raise will necessarily have to be corroborated with other sediment analysis or integrated into multidisciplinary studies. Indeed, the impact of human activities on the land environment cannot be translated by a single analysis and must be part of a larger amount of analysis. The use of control samples is mandatory. External samples from the surrounding near-environment can address "background" contamination from soil or water currents when studying potteries content (Gorham and Bryant, 2001; Torrence, 2016; Pecci *et al.*, 2020). Off-site and non-site control comparison can sort post depositional contamination out (Loy and Barton, 2006). Stratigraphic and flotation controls may evaluate chronology and sediment fill deposits. For too long, sampling has suffered from the absence of assistance from experienced researchers (Bryant and Holloway, 1983), who then established various pre- and post-sampling protocols (Pearsall, 1990; Hunt, 1994; Weinstein-Evron, 1994; Bunting and Tipping, 2000; Tipping, 2000). A close collaboration between archaeobotanists and archaeologists is more than recommended before undertaking excavations, in order to organize the field study and plan sediment sampling and its frequency (Bryant and Hall, 1993). Only this way anthropogenic repercussion and post depositional contamination can be estimated, and the original pollen content can be accurately interpreted.

Recently, UV-fluorescence microscopy has been mentioned to outline pollen contamination from younger specimen intrusion and recycled pollen (Hunt, 1998; Yeloff and Hunt, 2005). With time, pollen grains age and the relative fluorescent intensity of the outer wall diminishes. This gives a valuable key for fresh and fossil pollen distinction. The technique also provides meaningful insights on the thermal maturity of pollen, outlining burnt material (Hunt, Rushworth and Dykes, 2007).

3.3.2. Pollen extraction

After sediments are sampled and contamination issues sorted out, pollen grains need to be extracted from their matrix to be observed under a microscope. The extraction being destructive, specific care must be taken regarding the protocol and sample amounts. It consists of chemical and/or physical processes. Since pollen can be lost at any step if precaution care is not sufficient, it remains fundamental to avoid useless treatments (Horowitz, 1992).

From the beginning of the palynological research, many extractive approaches have been published to remove organic and inorganic materials microfossils are trapped in (Erdtman, 1960; Faegri, Kaland and Krzywinski, 1989; Pearsall, 1990; Wood, Gabriel and Lawson, 1996; Magri and Di Rita, 2015; Riding, 2021). Protocols usually consist of: (i) chemical breakdown of the matrix, (ii) concentration of pollen grains and (iii) mounting on slides to preserve pollen from oxidation and make them observable under the microscope. The chemical treatment chosen is directly dictated by the sample composition (Erdtman, 1960; Magri and Di Rita, 2015). In presence of carbonate minerals, recurrent in sediments, undiluted hydrochloric acid (HCL) is utilized, sometimes with glacial acetic acid (Bryant and Holloway, 1983). Sand and silicates are digested with highly concentrated hydrofluoric acid (HF). Produced fluoride crystals need then to be removed before continuing. Then, palynomorphs are concentrated as follows: deflocculation,

assisted by sonication, can sort out clay persistence in the samples. Ultrasound will help in fragmenting the amorphous organic phase, then removed by sieving. Oxidation and alkali treatments can be performed if extraneous organic matter is still present. When the ratio of pollen over mineral sediments is extremely low, gravity separation may be an option. The last step specifically targets coarse particles (> 200 µm) that can be drawn away with sieving. When the samples still exhibit organic matter coming from insufficient degradation of plant remains, such as resins, lignin, waxes, carbohydrates and its relative biopolymers of cellulose and hemicellulose, acetolysis becomes inevitable to dissolve the organic fraction. Although it removes the remains from uncomplete degradation of microfossils by disintegrating residual fragments of sporopollenin (Erdtman, 1960), it can additionally distort pollen counting since acetolysis breaks down pollen protoplasms. Fresh pollen usually identified by their "filled" aspect are thus emptied, making them indistinguishable from fossil pollen. A staining step can be added to dark pale specimen and make them more contrasted. Different mounting media have been proposed. Glycerol is frequently used since it facilitates grain motions under slight pressure during microscope observation, although it does not ensure long-term preservation. Cellosize and polyvinyl alcohol are also widely named (Riding, 2021). In any case, the mounting medium must be selected based on its durability, contrast effect and its optical properties to enhance pollen morphological observation.

3.3.3. Pollen identification

Pollen morphologies being different from each other, the characterization is performed through successive key point identification. The pollen unit, the size, the shape, the aperture numbers and types and the exine ornamentation do matter. The taxonomic level, up to which pollen grains can be identified, strongly depend on the taxa. Indeed, the ornamentation visible on the exine does not strongly vary within the same genus. For instance, *Pinus* species are hardly distinguishable from each other although some attempts tried to discriminate them (Desprat *et al.*, 2015). From fresh pollen records, they proposed to cluster *P. halepensis*, *P. pinea* and *P. pinaster* together as Mediterranean while *P. nigra* and *P. sylvestris* belong to the highland group. Additionally, it is important to notice that fresh, hydrated and fossil pollen differ in size. In fresh pollen, the protoplasmic material prevents detailed observation of the ornamentation.

3.3.4. Pollen interpretation

After identification and counting, pollen is evaluated upon its concentration regarding the initial amount of analyzed material. Calculations are based on the known quantity of exotic markers (usually *Lycopodium* spores (Stockmarr, 1971)) and pollen representation are expressed in numbers of fossil grains per weight or volume as follows (Benninghoff, 1962; Magri and Di Rita, 2015):

$$Pollen\ concentration = \frac{\frac{exotic\ spore\ added * \ pollen\ counted}{exotic\ spore\ counted}}{grams\ of\ sediment\ treated}$$

The total pollen content is chronologically plot in pollen diagrams of core sediment analysis. Statistical tools integrating principal components analysis and cluster analysis help generating transfer functions with relative pollen frequencies or radiocarbon dating (Horbe *et al.*, 2011; Michczyński *et al.*, 2013).

Recovered from fossil sediments, pollen is classified into background or economic entities. While background refers to usual environmental taxa commonly found on the ground, in water, on food, economic pollen targets the taxa related to anthropic activities. For instance, in the analysis of amphorae, background pollen would revert to the atmospheric conditions where the amphora has been produced, located, transported, *etc*. and economic pollen would regard the taxa indicative of the content. Setting aside landscape reconstruction and focusing more on the analysis of ceramic vessels, microfossils have provided valuable insights to shed light on vessels functionalities and civilizations diets (Zarrillo *et al.*, 2008; Torrence, 2016). Information on the provenance, the vessel function or the manufacture technologies employed have been drawn from palynomorph analysis from baskets (Bohrer, 1968), stone tools such as mortars or milling stones (Hevly, 1964; Bryant and Morris, 1986; Fullagar, Furby and Hardy, 1996), as well as from the surface of potsherds (Magid and Krzywinski, 1988; Jones *et al.*, 1998; Ghosh, D'Rozario and Bera, 2006; Peto *et al.*, 2013; Dumpe and Stivrins, 2015). Since such artefacts were originally used for food storing, cooking and eating, pollen can be recovered nowadays thanks to their trapping into the porous ceramic. This way, pollen has greatly supported fermented beverages tracing (Guerra-Doce, 2014). Archaeological beers and meads have been evidenced through the presence of cereal pollen and plant macroremains (Barclay *et al.*, 1994; Dineley and Dineley, 2000; Lageras, 200; Bartel *et al.*, 1997; Bueno Ramírez *et al.*, 2005; Rösch, 2005, 1999), sometimes corroborated with organic residue analysis. For instance, Moe and Oeggl (Moe and Oeggl, 2014) understood the important presence of *Filipendula ulmaria* together with Cerealia pollen as a sign of mead consumption since the beverage was usually prepared with meadowsweet to enhance flavour. Henbane-based preparations have been evidenced by the presence of *Hyoscyamus niger in* Late Neolithic British vessels (Barclay *et al.*, 1994). The flower was currently used for ritual and medicinal purposes. Furthermore, the important diversity in pollen recovered from a bronze vessel dated to the German Iron Age was interpreted as a sign of archaeological honey (Rösch, 1999; Kvavadze, 2006). Grape beverages have been mentioned in the understanding of amphorae content with findings of *Vitis vinifera* pollen (Gorham and Bryant, 2001; Arobba *et al.*, 2014). Moreover, the ceramic clays themselves can trace the provenance. For instance, Ghosh *et al.* assessed the socioeconomic development through the analysis of pollen trapped in Gangetic West Bengal potsherds. Following his minds, micro and macro vegetal remains (i.e., pollen, spores, phytoliths, fruits and seeds) from local consumption get deposited on the clay. Since surrounding clays were used for vessels manufacture, dissolving the ceramic matrix can indicate ancient anthropological activities (Ghosh, D'Rozario and Bera, 2006). They demonstrated that pollen contained in the ceramic clay can survive to high temperature firing, up to 1000°C when anoxic conditions are ensured. Such conditions were in fact originally used to decorate the vessels (Shaw *et al.*, 2001).

Like any spectacularly powerful marker, pollen also exhibit limitations and a few words must be said about this. As often stated by Bryant *et al.* (Bryant, 1978; Bryant and Holloway, 1983), it is important to remember that the only information pollen give access to is about palaeovegetation. Any further settlement regarding climate, animal life, past cultures and anthropic activities derive from palaeovegetation interpretation. First, it requires a high degree of precaution during all steps, from sampling to identification. Individual responsibility is then engaged in each step, in order to provide the most reliable possible data. The reconciliation of data with similar spatial, geographical, temporal or

anthropic characteristics can only be done when the rigor of analysis is comparable. Aside from the individual impact on the analysis, the phenomena inherent to pollen (dispersion) and external to it (contamination by post deposition, redistribution of sediments, partial and/or selective degradation) bias the analysis. Statistical treatments, although powerful regarding the interpretations they suggest, cannot deviate from this rigor (Mosimann and Greenstreet, 1971; Ogden, 2010).

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Chapter 4

Protocol comparison for organic residue analyses from waterproofing materials and shards of Roman archaeological amphorae

This chapter has taken shape following a PhD thesis (Fujii, 2018), previously carried out in the IRPNC research unit, which aimed to develop analytical protocols for the characterization of molecular markers extracted from archaeological amphorae. The lab indeed enjoying a long experience in the analysis of organic residues from archaeological materials, the research was conducted in light of this expertise. In particular, it allowed the use of the ultrasound probe to be optimized to improve extraction. As the analysis of residues paved the way in deciphering the history beyond the archaeological container, innovative protocols for the extraction of biomarkers are frequently appearing in the field. In this regard, the following chapter considers a global comparison of the extractive capacities of conventional protocols, regularly employed or recently developed for the analysis of organic residues in ceramic vessels and waterproofing coatings, as detailed in Figure 25.

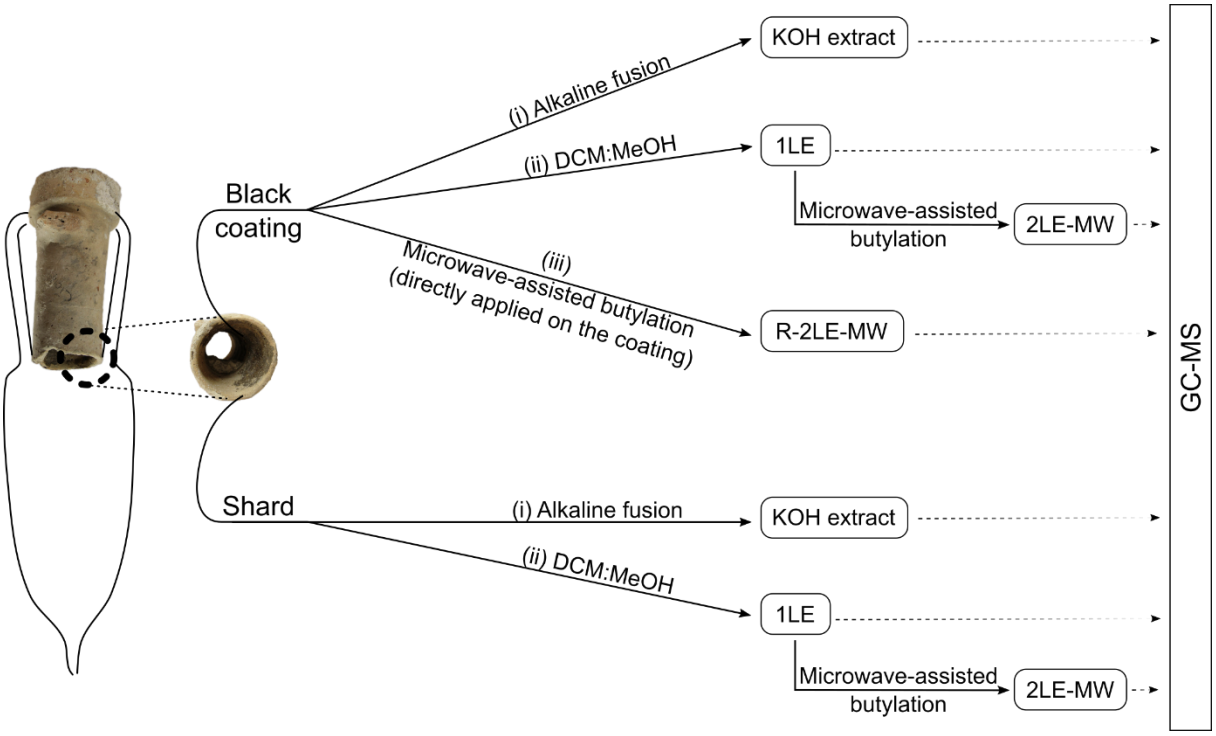

Figure 25. Protocol comparison for the extraction of grape derivatives

The comparison of protocols is applied to 16 artefacts of waterproofing organic linings made out of resinous materials and inorganic shards of ceramics that were supposed to have contained grape derivatives. The objects come from two marine contexts: the shipwreck of Planier 3 (France) and the ancient anchorage of San Felice Circeo (Italy).

In the next chapter, I took charge of the analysis of organic residues, from molecular characterization to archaeological interpretations shaping after having participated in the material sampling. While the project had initially been conceptualized by my supervisors, I partaked in adapting and advancing the research outlines. The expertise of the lab in terms of organic material investigation and analytical competences and equipment possibilities provided a considerable support. This way, I acquired practical skills, mainly related to the sampling, the handling and the analysis of archaeological material in the lab.

The protocol never being handled in the lab since it was published in 2016 and not included in the optimization performed during the previous thesis, I matured methodological competences through the development of the microwave-assisted butylation protocol, initially meant for ceramics and through the adaptation to the internal waterproofing lining. I improved technical skills thanks to the use of gas chromatography, mass spectrometry and the associated analytical modules. The archaeological samples being analyzed with several protocols to get the more information out, I was trained to chemometric tools with the aim of synthesizing results since I had to deal with important data quantities obtained from molecular identifications. Finally, I also gained experience in by generating huge scientific writing and by setting up the original draft and edition of the following publication.

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Protocol comparison for organic residue analyses from waterproofing materials and shards of Roman archaeological amphorae

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Highlights

- Microwave assisted butylation is proposed for the identification of grape markers
- BF_3-catalyzed esterification is developed for the analyses of pitch coatings
- Molecular signatures of grape-derivatives, *pine* pitch and wood tar are formulated
- Extractive protocols are compared based on their extractive capacities
- Archaeological fermented grape beverages are detected in Dressel 5 and 18

4.1. 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 BF3-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 provides 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

4.2. 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 (Heron and Evershed, 1993). 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 (Evershed, 2008). For instance, the earliest consumption of wine could be dated back to the Neolithic in the South Caucasus through molecular markers (McGovern *et al.*, 2017).

Although pioneering studies laid the foundation using Feigl spots, infrared spectroscopy or HPLC to identify tartaric acid (Michel, McGovern and Badler, 1993; McGovern and Michel, 1996; McGovern *et al.*, 2004), the emergence of chromatographic tools offered great substitutes to traditional methods to prevent from false-positives (Garnier *et al.*, 2003; Jerković *et al.*, 2011; Pecci *et al.*, 2013). As rightly criticized in literature, neither molecular identification through sole retention or migration time, nor the precision of UV detection are reliable (Stern *et al.*, 2008; Drieu *et al.*, 2020). 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 (Garnier and Valamoti, 2016; Fujii *et al.*, 2019, 2021; Pecci *et al.*, 2020). 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 (Guasch-Jané *et al.*, 2004, 2006; Barnard *et al.*, 2011; Manzano *et al.*, 2016; McGovern *et al.*, 2017), 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 (Michel, McGovern and Badler, 1993; Rageot *et al.*, 2019). Although the molecule is not exclusively produced by grapes, its concentration remains higher than in other exotic plant sources such as tamarind, yellow plums (Barnard *et al.*, 2011) or pomegranates (Hasnaoui *et al.*, 2011). Moreover, it better survives upon archaeological time compared to other grape acids (Pecci *et al.*, 2013). For this reason, it is usually considered as a grape biomarker when supported by archaeological contexts and/or archaeobotanical grape evidence (Garnier and Valamoti, 2016; McGovern *et al.*, 2017; Pecci *et al.*, 2018; Drieu *et al.*, 2020). Although synthesized by numerous plants and fungi, pyruvic, fumaric and malic acids are additionally produced during the fermentation process (Ribéreau-Gayon *et al.*, 2006). Syringic acid highlights red wine since it arises from malvidin, the pigment responsible for dark grape coloration (Guasch-Jané *et al.*, 2004; Barnard *et al.*, 2011).

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 (Blanco-Zubiaguirre *et al.*, 2019). 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 (Pecci *et al.*, 2013; Correa-Ascencio and Evershed, 2014; Garnier and Valamoti, 2016). Alkaline treatment is followed by acidification to make tartaric acid more soluble in ethyl acetate before extraction (Pecci *et al.*, 2017, 2020). 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 (Garnier and Valamoti, 2016; Frère and Garnier, 2017; Drieu *et al.*, 2021). 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 coatings 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 coatings 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. 10 shards and 8 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.

4.3. Material

4.3.1. Archaeological samples

This comparative study integrated 16 archaeological amphorae coming from two different contexts. 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 5). 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 (Tchernia, 1968). 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 (Tchernia, 1969). Such discoveries outlined the luxurious assumptions of traded products transported on the ship.

Sample	Archaeological Site	Typology	Coating	Shard	Grape derivatives	Pinaceae products
1014	Planier 3	Dressel 1	X	X	Fermentated	Wood tar
749	Planier 3	Lamboglia 2	X	X	Fermentated	Wood tar
6570a	Planier 3	Dressel 1	X		Fermentated	Wood tar
SFC1	San Felic Circeo	Dressel 1	X		Fermentated	Wood tar
SFC2	San Felic Circeo	Dressel 1	X		Fermentated	Wood tar
SFC3	San Felic Circeo	Mañà C2	X		Fermentated	Wood tar
SFC4	San Felic Circeo	Greek-Italian	X		Fermentated	Wood tar
SFC5	San Felic Circeo	Lamboglia 2	X		Fermentated	Wood tar
6904	Planier 3	Chios amphora		X	Fermentated	Pitch
6828A	Planier 3	Lamboglia 2		X	Fermentated	Wood tar
6828B	Planier 3	Lamboglia 2		X	Fermentated	Wood tar
6565	Planier 3	Lamboglia 2		X	Fermentated	Wood tar
6793	Planier 3	Lamboglia 2		X	Fermentated	Wood tar
6545	Planier 3	Dressel 5		X	Fermentated	Wood tar
6566	Planier 3	Lamboglia 2		X	Fermentated	Wood tar
6828C	Planier 3	Lamboglia 2		X	Fermentated	Wood tar

Table 5. Archaeological amphorae investigated

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 7m 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 (Olmer, 2012). 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 (Cipriano and Carre, 1989; Panella, 1998). They have largely contributed to the trade of wine in the western Mediterranean (Lamboglia, 1952). Chios amphora were surely meant for wine trading since the island was prized for the great quality of wine they produced (Okan, Atila and Akyol, 2015). 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 (Bonifay, 2016). Dressel 5, also referenced as Rhodian amphorae, usually contained wine from the Aegean coast (Peña, 2007). The present study focuses on 8 samples from waterproofing coatings and 10 samples of ceramic shards (see Table 5). Samples no. 749 and 1014 were analyzed with both the coating and the ceramic clay.

4.3.2. 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, Germany) and BF_3 diethyl etherate from Alfa Aesar (Kanderl, Germany). The fresh colophony resin standard was purchased by Kremer Pigmente GmbH & Co KG (Aichstetten, Germany).

4.4. Methods

4.4.1. Optimization of the acid-catalyzed esterification: microwave-assisted butylation

The protocol aiming at butylating wine acid markers was adapted from Garnier and Valamoti (Garnier and Valamoti, 2016). It was firstly developed for tartaric acid before being extended to other standard molecules of maleic, succinic, fumaric, pyruvic, malic, syringic acids. 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 (Kappe, Dallinger and Murphree, 2008). 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 (version 0.9). 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).

4.4.2. 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.* (Pecci *et al.*, 2013) 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 (1M; 2 mL) using an ultrasound probe (VCX 130 Vibra-Cell Sonics, Sonics and Materials, Newtown, U.S.A.) 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_20 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.

4.4.3. 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 4.4.2.). The second was the adapted two-step lipid extraction with MW optimization for the butylation as previously described (see section 4.4.2.). 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. 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.

4.4.4. 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 (ThermoFischer Scientific, Illkirch-Graffenstaden, France). A ThermoGOLD™ TG-5MS fused silica capillary column (5% diphenyl; 95% dimethyl polysiloxane) of 30 m length x 0.25 mm i.d. x 0.25 µm thickness ensured the separation of the mixture carried with helium at a constant flow rate of 1 mL min^{-1}. 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 (version 4.3). 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).

4.4.5. Radar plot construction

The building of the radar plots (Figs. 26 and 27) 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).

4.5. Results and discussion

4.5.1. Optimization of the acid-catalyzed butylation

The esterification advancement, followed by thin-layer chromatography, ensured the effective butylation of standard molecules after 3 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.

4.5.2. 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 (Fig. 26). 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).

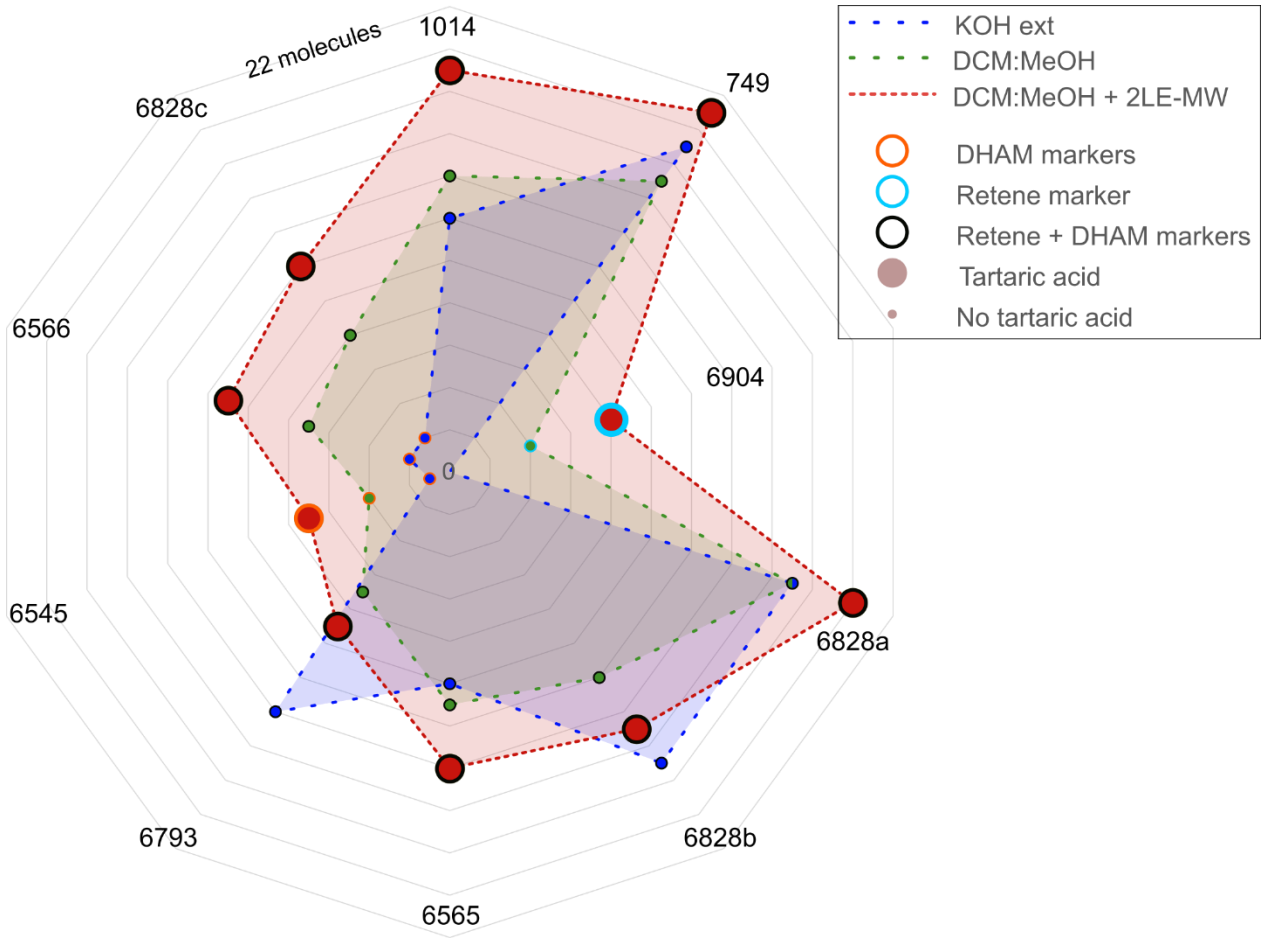

Figure 26. Radar plot of the shards

For all the 10 shards, only the BF_3-catalyzed butylation allowed DBT, highlighting the presence of tartaric acid (Fig. 26). Partially dissolving the ceramic clay, BF_3, optimized the release of the organic compounds strongly bonded, or even polymerized (Correa-Ascencio and Evershed, 2014; Garnier *et al.*, 2018). 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 (Garnier and Valamoti, 2016). 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 (Pecci *et al.*, 2013, 2017, 2020), no tartaric acid could be identified with this protocol (Fig. 26). 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 (Guasch-Jané *et al.*, 2004; Pecci *et al.*, 2013). In fact, tartaric acid resists archaeological time thanks to the formation of salts that strongly interact with the clay matrix (Michel, McGovern and Badler, 1993). 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 (Guasch-Jané *et al.*, 2004; Drieu *et al.*, 2020). The hypothesis of tartrate salt that would be formed once the alkaline fusion released tartaric acid from the ceramic cannot be ruled out (Drieu *et al.*, 2020). 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 (Drieu *et al.*, 2020). Additionally, Garnier and Valamoti reported the detection of tartaric acid up to 10 ng/g shard with the acido-catalyzed protocol (Garnier and Valamoti, 2016). 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 6). 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 3 fermentation acids (over the 5 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 9 shards. Neither KOH nor butylation reached such great extent (Table 6) and the molecule that originates from malolactic fermentation (Ribéreau-Gayon *et al.*, 2006) was only identified in 2 shards with alkaline fusion and never recovered as dibutylcetal. To the contrary, fumaric acid which is considered as marker of alcoholic fermentation (Garnier *et al.*, 2018), was preferentially extracted with alkaline fusion and never identified with butylation (Table 6). 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 ex-tractive capacities.

Although syringic acid is naturally present in many plants and in wood lignin (Barnard *et al.*, 2011; Pecci *et al.*, 2020), malvidin origin has been clearly evidenced (Guasch-Jané *et al.*, 2004). 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 (Garnier and Valamoti, 2016; Pecci *et al.*, 2017). 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 ac-id-catalyzed extraction hence promotes the tracing of malvidin origin. Following Table 6, 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 2 to 10 molecules per sample over the 22 molecules targeted (Fig. 26). 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 6). 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 (Fig. 26). 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 (Mezzatesta *et al.*, 2021).

Table 6. 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-15hydroxy-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.

Sample	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	-	+	-	-	+	+	+											

Almost all the protocols extracted DHAM markers, traducing an archaeological use of Pinaceae wood tar (Colombini and Modugno, 2009) (Fig. 26). 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. (Modugno, Ribechini and Colombini, 2006). 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 (Proestos *et al.*, 2005) which can be related to the insufficient. No retene was observed for the amphorae nos. 6566 and 6828c with alkaline fusion whereas it did with DCM:MeOH (Fig. 26). Even though no chemical explanation can afford it, the archaeological meaning refers to the heating treatment of the resin (Mezzatesta *et al.*, 2021). 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.

4.5.3. 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 (Fig. 27) overviews the extractive molecular capacities of the different protocols for each of the 8 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).

Tartaric acid was successfully identified in all of the 8 coatings when the butylation was directly applied on the pitch (R-2LE-MW) (Fig. 27). In comparison, when it was applied after organic extraction (2LE-MW), the esterification allowed DBT to be identified from only 7 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 (Fig. 27), 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) (Kawamura, 1993). 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 7). Moreover, KOH fusion allowed 8 additional grape-acids (i.e., succinic, fumaric and malic acids) to be extracted from 5 amphorae (nos. 6570a, SFC1, SFC3, SFC4 and SFC5) that traditional solvents did not provide.

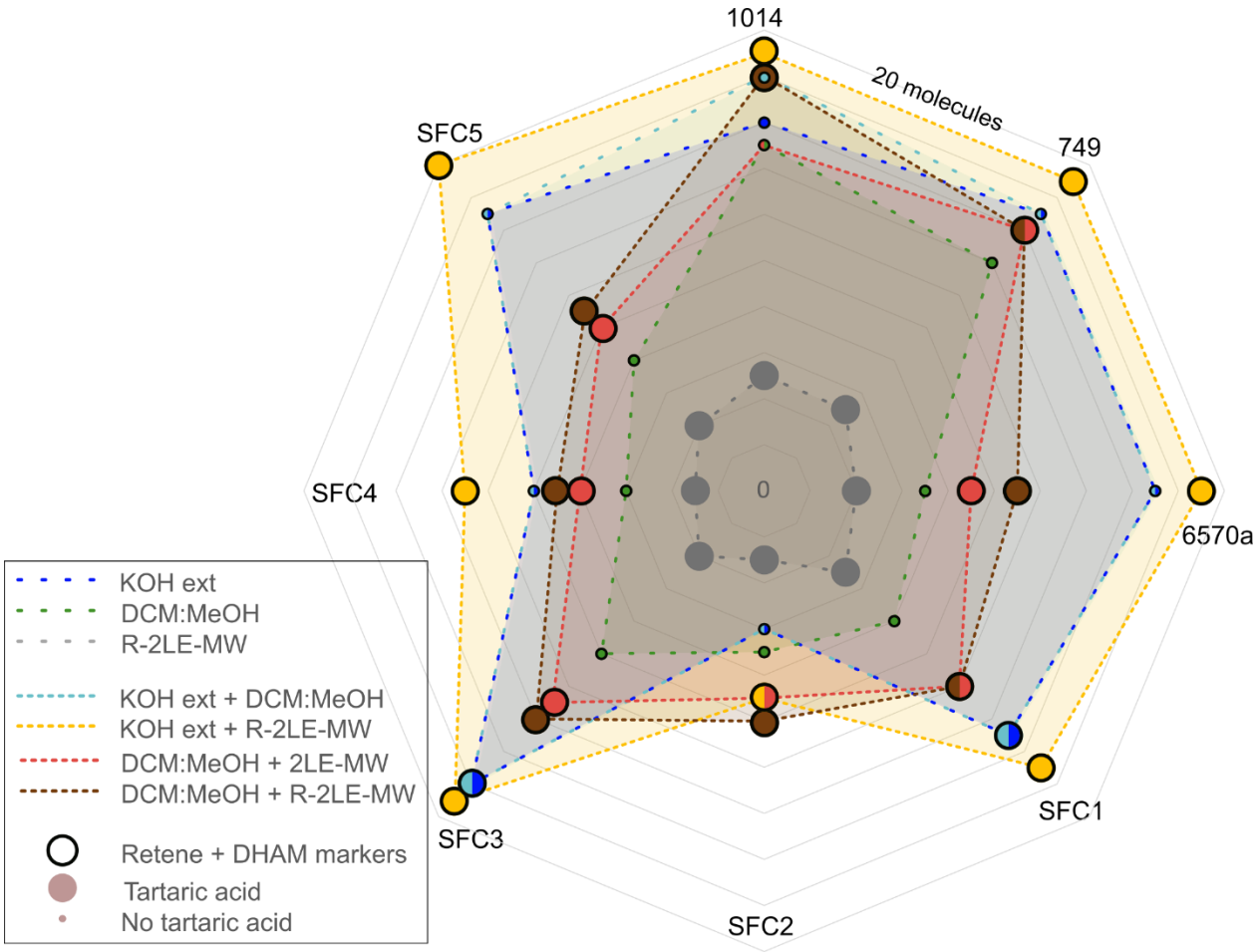

Figure 27. Radar plot of the coating materials

Syringic acid was not detected in any of the amphorae SFC2, SFC4 and SFC5 (Table 7), 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).

Table 7. 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

Sample	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	-	+	+	-	+	+	-											

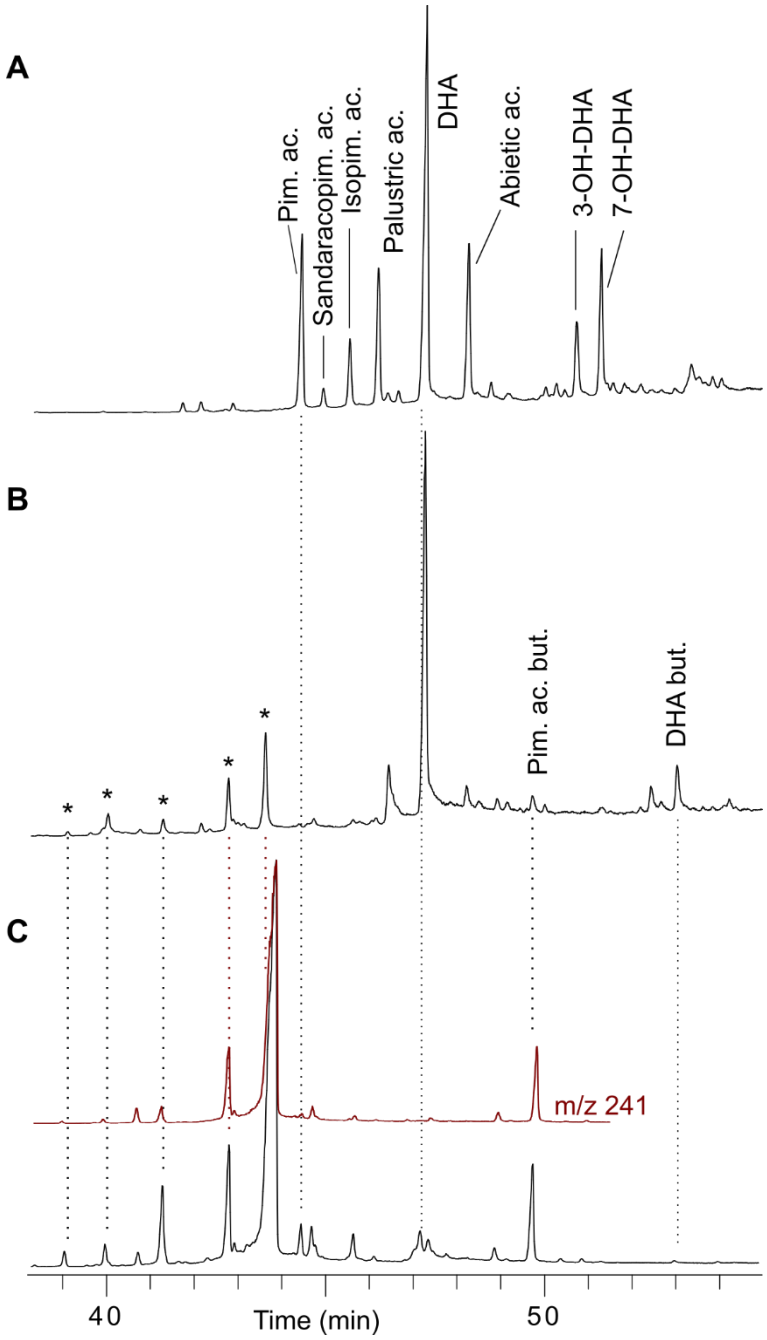

Figure 28. TIC Chromatograms (A) Standard colophony extracted with DCM:MeOH; (B) Standard colophony extracted with MW-assisted butylation and (C) Standard pimaric acid extracted with MW-assisted butylation with m/z 241 chromatogram. ac.: acid; but.: but

Interestingly, diterpenic markers could not been 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 (Fig. 28C), 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

(Fig. 28B-C). Induced by the harsh Lewis-acid conditions, diterpenic skeletons were reported to undergo isomerization, skeletal transposition, isomerization, rearrangement and proton migration (Delmond, Taran and Valade, 1978; Fujita *et al.*, 1979; Taran and Delmond, 1986). Moreover, the butylation of standard colophony selectively esterified the diterpenoids present in the resin (Fig. 28A-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 (Fig. 27). 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 6 of them, dihydroxy-DHA for 4 of them and hydroxy-oxo-DHA for 3 pitches over 8). 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 (Fig. 27), attested of Pinaceae wood tar produced under high temperature pyrolytic treatment.

4.6. Conclusion

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 (Kappe, Dallinger and Murphree, 2008), 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 (McGovern *et al.*, 2017). 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.

Glossary

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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Author contribution

Conceptualization, L.C., C.V., C.M.; **methodology**, L.C.; **formal analysis**, L.C.; **investigation**, L.C., C.M.; **resources**, F.O., C.D., A.C.; **visualization**, L.C.; **supervision**, C.M.; **project administration**, C.V., D.M., C.M.; **funding acquisition**, A.C., C.V., D.M., C.M.; **writing—original draft preparation**, L.C., C.M.; **writing—review and editing**, L.C., F.O., C.D., A.C., C.V., D.M., C.M. All authors have read and agreed to the published version of the manuscript.

Conflict of interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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Chapter 5

Archaeobotanical and chemical investigations on wine amphorae from San Felice Circeo (Italy) shed light on grape beverages at the Roman time

After the successful adaptation of the microwave-assisted butylation to organic coatings and the parameters optimization to promote the extraction of very meaning grape and fermentation markers, this fifth chapter focuses on both the archaeobotanical content and the organic residues of the waterproofing coatings of amphorae. The initial purpose aimed at verifying the pollen presence and availability in the resinous coatings in order to provide a multi-analytical diagnosis of the object. Palaeobotanical analyses were carried out at the Department of Environmental Biology of Sapienza University, as initially foreseen by the co-supervision agreement of the Joint Doctorate ARCHMAT program. As the laboratory has significant expertise in the analysis of micro- and macroproxies from distinct sediments or archaeological contexts, the existing methodology was adapted, for the first time, to archaeological organic linings collected from the internal part of amphorae. Seven artefacts for which the black coating was visible at naked eyes, all recovered in a maritime context, were included in this explorative study, the objects exhibiting different physical shape integrities, typologies and states of conservation though. Five amphorae come from the ancient anchorage of San Felice Circeo and were initially employed to develop the diagnostic methodology. Two additional artefacts from the Planier 3 wreck were included to further detail the conclusions initially drawn from the analysis of organic residues. Not all amphorae are directly discussed in the following chapter as we decided to focus on the means of *Vitis vinifera* pollen in the article once the methodological feasibility was verified. Results being dictated by the objects themselves; we used the archaeobotanical aspect as a major asset in the following chapter while organic residue analyses played a collaborative secondary role. Nonetheless, the non-detailed results are presented in the discussion part of the thesis.

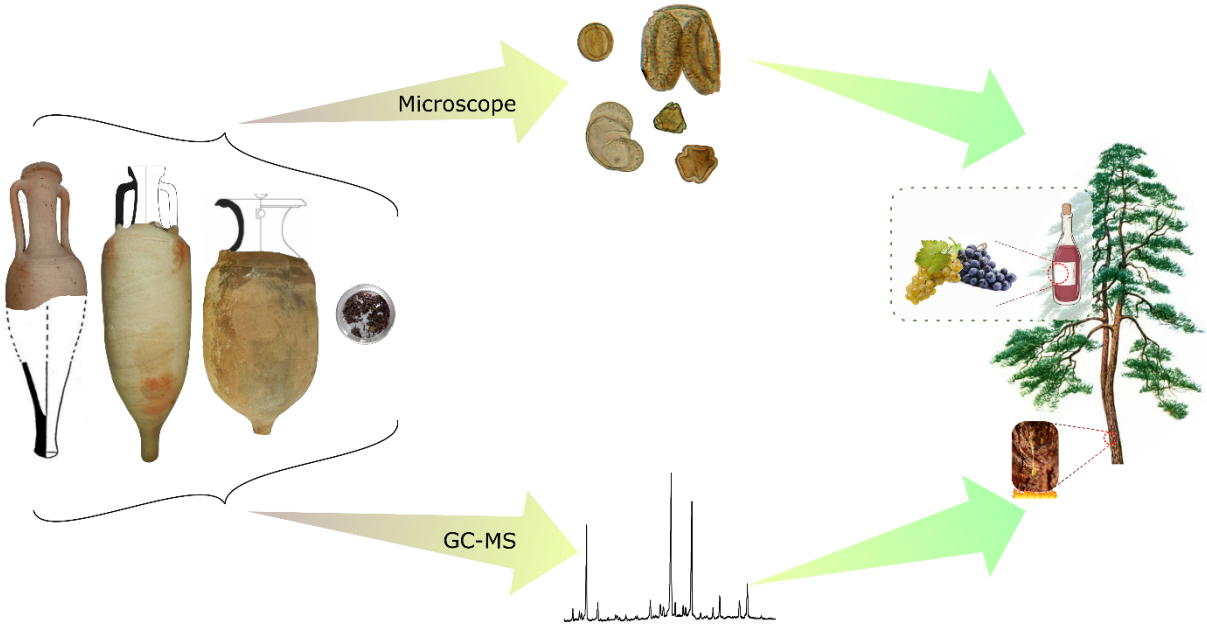

Figure 29. Archaeobotanical and chemical analysis of the San Felice Circeo amphorae

In this chapter I suggested the analytical methodology to study the archaeobotanical content and conducted the formal analysis and identification survey. Federico Di Rita and Fabrizio Michelangeli provided my training in terms of pollen characterization and remarkably helped me in this laborious task. Besides the conceptualisation of the article, I wrote the original version and took care of the editing under the supervision of my supervisors, Donatella Magri and Alessandra Celant.

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Archaeobotanical and chemical investigations on wine amphorae from San Felice Circeo (Italy) shed light on grape beverages at the Roman time

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5.1. Abstract

We hereby investigate the pitch used for coating three Roman amphorae from San Felice Circeo (Italy) through a multidisciplinary study. The identification of molecular biomarkers by gas chromatography - mass spectrometry is combined with archaeobotanical evidence of pollen and plant tissues of *Vitis* flowers. Diterpenic chemical markers together with *Pinus* pollen and wood revealed Pinaceae tar coating. Aporate 3-zonocolpate pollen, identified as *Vitis*, together with tartaric, malic and pyruvic acids elucidate the grape-fermented nature of the content. Our conclusions open new consideration on the use of grape derivatives that cannot be supported by traditional analytical methods. Based on the finds of aporate *Vitis* pollen, found also in local modern and Middle Pleistocene samples, we hypothesize the use of autochthonous vines. The presence of a medicinal wine (historically reported as *oenanthium*) is also considered. We interrogate *Vitis* pollen capacity to target grapevine domestication, thereby providing innovative tools to understand such an important process. We anticipate our study to encourage a more systematic multidisciplinary approach regarding the analyses of wine amphorae.

5.2. Introduction

Pilot experimental protocols with the specific aim of accessing pollen trapped in organic resins of archaeological artefacts were advanced by Pons (Pons, 1961), Arobba (Arobba, 1976), Jones *et al.* (Jones *et al.*, 1998), and Jacobsen and Bryant (Jacobsen and Bryant, 1998). From there, only a limited number of pollen studies have been conducted on amphorae. They mainly focus on the liquid recovered from sealed jars (Gorham, 2000; Arobba *et al.*, 2014) and on sediments contained in the ceramics from cargo containers from marine contexts, with the objective of identifying pollen or phytoliths (Gorham and Bryant, 2001). Pollen analyses from resins of archaeological artefacts has been little used with the purpose of better understanding the history beyond the object. Significant *Pinus* and *Vitis* pollen representation highlighted pine pitch coating used for wine jars (Arobba, 1976; Arobba

et al., 1983; Vogt *et al.*, 2002; Rösch, 2005; Kvavadze *et al.*, 2019). Coupling palaeobotanical to isotopic and chemical characterization, Arobba *et al.* (Arobba *et al.*, 2014) were able to trace back the oenological content of sealed amphorae from a Roman shipwreck, as well as the central-southern Italian provenance of the cargo. Although they demonstrated the effectiveness of pollen analyses in the identification and characterization of the nature and geographical origin of the transported wine, their methodologies have barely been followed and similar investigations are still rare. Even other types of organic materials, e.g., rope, caulking material, laces watercrafts, and organic coffin have been seldom investigated through pollen (Mariotti Lippi and Mercuri, 1992; Ciuffarella, 1998; Muller, 2004; Deforce *et al.*, 2014; Willis, 2017). Archaeobotany has been often combined with other analytical disciplines to promote interdisciplinary approaches (Cappellini *et al.*, 2010; Figueiral *et al.*, 2010; Bouby *et al.*, 2013; Brown *et al.*, 2015; Garnier and Valamoti, 2016; Mariotti Lippi *et al.*, 2018, 2020) but palynology is still barely associated to chemical analyses (Maghradze *et al.*, 2016; McGovern *et al.*, 2017; Kvavadze *et al.*, 2019; Pecci *et al.*, 2020).

At the same time, analytical methods are increasingly interested in using cutting edge techniques applied to archaeological materials. Among them, liquid or gas chromatography coupled with mass spectrometry (GC-MS) dominate the field, due to highly sensitive and selective capacities to target molecules (Guasch-Jané *et al.*, 2004; Pecci *et al.*, 2013; Garnier and Valamoti, 2016; McGovern *et al.*, 2017). Retention time and molecular fragmentation account for trustworthy molecular identifications (Stern *et al.*, 2008). However, specific care should be taken to not overinterpret the results. Scientific and archaeological consensus are prevailing, herewith stating that independent evidence must be sustained to assess with certainty the history of the containers (Drieu *et al.*, 2020). Only this way false positive can be tackled and contamination averted. Indeed, organic analyses residues aims at extracting and interpreting molecular markers either trapped in the organic coating or in the potsherd matrix of vessels (Evershed, 1993). Archaeological interpretation naturally derives from the absence and/or presence of such biomolecular indicators (Evershed, 2008). However, caution is needed when interpreting chemical analyses in archaeological terms. For example, regarding the possible overinterpretation due to the presence of the tartaric acid in chemical analyses, up to now considered as a grape marker (McGovern *et al.*, 2004). The resort of control becomes indispensable since chemical analyses cannot support archaeological interpretation on its own. Indeed, tartaric acid can be released from phthalates contained in plastic bags under acidic treatment (Drieu *et al.*, 2020), and it can also migrate from surrounding soils (Barnard *et al.*, 2011). Systematic sampling and analyses of sediments associated to the studied materials are highly recommended to prevent false positives (McGovern *et al.*, 2017). However, the awareness of this problem is recent, and the question remains open for artefacts excavated long time ago, washed, restored and preserved in deposits and museums, for which no associated sediment is available.

In the present work, three marine amphorae, retrieved in 2018 from the ancient anchorage of San Felice Circeo (Italy), offered a rare opportunity to develop interdisciplinary research through archaeobotanical and chemical analyses. The aim of this article is to discuss the effectiveness of a multidisciplinary approach, initially developed to identify the nature of the organic content of the amphorae, to trace back the history beyond the artefacts.

5.3. Materials and methods

5.3.1. Archaeological materials

5.3.1.1. Archaeological context

In 2018 notable winter storm tides have allowed to identify a huge scattering of different archaeological finds on a seabed close to the modern harbor of San Felice Circeo (Latina – Italy), ca. 90 km SE of Rome (41°13'49.0"N, 13°06'30.1"E). The area is located at a distance of about 500 m from the present coastline; the depth of the seabed varies from 5 to 7 m under the sea level.

Since then, regular underwater archaeological surveys have been conducted by the Soprintendenza Archeologia Belle Arti e Paesaggio per le province di Frosinone e Latina (the local Office of the Italian Ministry of Culture) in order to elaborate a seabed mapping of the archaeological area, to delimit the zones of sherd scattering, and to obtain a clearer framework of underwater record. These surveys, which are still ongoing, revealed a broadly consistent chronological representation, with ceramic finds spanning from the Republican period through the Late Roman period up to the post medieval period. The limited amount of morphologically and chronologically similar ceramic containers, the fragmentary state of most of the recovered pots, and the pattern of dispersion may be interpreted as an evidence of an ancient anchorage area (Delpino and Melandri 2018, unpublished data). In previous topographic studies, the existence of a Roman port close to the finding area was supposed mainly because of the presence of an ancient mouth of the Ufente river (Cancellieri, 1986). As a working hypothesis, the recent discovery of various late Greco-Italic/transitional Dressel 1A amphorae also suggests the possible presence of a small shipwreck, which needs to be confirmed by underwater surveys. The majority of the recovered Roman amphorae belongs to late Greco-Italic (referred to as Lyding Will e) and Dressel 1A type, dating from the second half of 2nd century BC to the middle of the 1st century BC. The late Greco-Italic type is a wine amphora with a wide distribution in the Mediterranean from the second quarter of 2nd century up to around 140-130 BC. The latter Italic Dressel 1A amphora is an evolution of the late Greco-Italic type (Lyding Will, 1982). The transition to the new container is not sudden and does not involve a clear break with the previous production; the transitional type is known as "Lyding Will e". Dressel 1A, the most common among late Republican Roman amphorae, were mostly filled with wine (Peacock and Williams, 1986; Panella, 1998). Mainly produced in southern-central Italy, from Campania to Etruria where a number of kiln sites along the coastal area are known, these amphorae have widely circulated in Gaul, Britain, Spain and central Europe (Peacock and Williams, 1986; Tchernia, 1986; Panella, 1998). The manufacture area does not necessarily coincide with the loading site. However, considering the possibility of San Felice to be a center of redistribution and assuming the presence of a manufacture site nearby (Hesnard *et al.*, 1989), we can hypothesize that the loading site was San Felice itself, highlighting a production site in Latium for the studied amphorae SFC1 and SFC2 (Fig. 30).

The third investigated amphora SFC 5 belongs to Lamboglia 2 amphorae (Fig. 30). Coming from the Adriatic coast (Cipriano and Carre, 1989; Panella, 1998), Lamboglia 2 were widely distributed throughout the western Mediterranean but a production in western Italy alongside the Dressel 1 amphorae has also been suggested (Cipriano and Carre, 1989). This typology was meant for the maritime transport of wine or olive oil (Buchi, 1971; Lyding Will, 1989). The analyses of the vessels from the Madrague de Giens shipwreck suggested wine content (Formenti, Hesnard and Tchernia, 1978). Wine is strongly suggested regarding the remaining presence of internal resin coatings observable in numerous Lamboglia 2 (Panella, 1998) found in different shipwrecks such as Cavaliére A

(n° 282), Cap Roux n° 197), Punta de Algas (n° 9191), Ponza (n° 1060) (Parker, 1992). The olive oil hypothesis is disfavored as it would have reacted with the pitch, degrading the oil quality and taste (Garnier, Silvino and Bernal Casasola, 2011).

Sample label	Description	Amphorae
SFC1 (20.S321-31.884)	Late Greco-Italic amphora / Dressel 1A amphora. Painted inscription (*titulus pictus*). Pronounced triangular rim, high cylindrical neck, lightly narrowed at the bottom and thin, s-shaped handles, rounded shoulder. The *titulus pictus* L. M. is difficult to interpret. Height: 85 cm. Second half of the 2nd century BC.	
SFC2 (20.S321-31.858)	Dressel 1A amphora. Cylindrical body shape with an angular shoulder. The bottom of the neck is cylindrical; the upper part of the neck and handles are not preserved. Height: 80 cm. Last quarter of the 2nd century - first half of the 1st century BC.	
SFC5 (20.S321-31.875)	Lamboglia 2 amphora. Thick-walled bag-shaped body; neck and handles are not preserved; the spike is broken. Height: 62 cm. Second half of the 2nd century - 1st century BC.	

Figure 30. Investigated archaeological amphorae

5.3.2. Chemical analyses

5.3.3.1. Solvents and reagents

Solvents and reagents used to analyze the pitch of the three amphorae were of analytical grade. N, *O*-Bis(trimethylsilyl)trifluoroacetamide/trimethylchlorosilane (BSTFA/TMCS) and hexane were supplied by Sigma-Aldrich. Dichloromethane (DCM), methanol (MeOH), diethyl ether, Na_2CO_3 and Na_2SO_4 were purchased by Merck; anhydrous butanol and cyclohexane by Acros Organics. BF_3 diethyl etherate was supplied by Alfa Aesar.

5.3.3.2. Sample preparation for chromatographic analyses

Samples of the archaeological coatings were recovered from the internal body and bottom of the amphorae by scraping the organic layer with a scalpel and were treated following a two-step protocol adapted from Garnier and Valamoti 2016 to analyze organic matrices [Chassouant *et al.* unpublished].

Briefly, the first extraction corresponds to an organic lipid extraction (1LE) while the second step is a microwave-assisted transesterification catalyzed by a Lewis acid (2LE-MW). 20 mg of crushed sample were extracted 3 times with DCM:MeOH (1:1, v/v) under VCX 130 Vibra-Cell Sonics probe ultrasonication (20 kHz, 130 W). After centrifugation, the supernatant was recovered, evaporated to dryness before being trimethylsilylated with BSTFA/TMCS (99/1, v/v) (70°C, 30 min). The first extract was dissolved in 1-2 mL of hexane/DCM (1/1, v/v) and filtered on a PTFE cartridge (0.45µm, VWR) before injection in GC-MS. After the first extraction completed, the solid powder remaining was treated with a mixture of BF_3-etherate complex, butanol and cyclohexane (1:2:4, v/v/v). The butylation was achieved through 3 dynamic cycles of microwaves of 5 min each, with automatically regulated pulse to maintain the temperature at 80°C using a single-mode CEM Discover Lab-mate microwave operating at 50 Hz with a maximum power of 300 W. The reaction mixture was then neutralized with 1 mL of saturated Na_2CO_3. The organic phase was extracted 2 times with diethyl ether. After washing 2 times with Milli-Q water, the mixture is dried with anhydrous Na_2SO_4. Trimethylsilylation is completed following the same step than described earlier and the 2LE-MW fraction was dissolved in 0.2-0.6 mL of hexane/DCM (1/1, v/v) before injection in GC-MS.

5.3.3.3. Gas chromatography – Mass Spectrometry

GC–MS analyses were performed on a Thermo Scientific™ Focus system equipped with a Thermo Scientific Al 3000 autosampler and coupled to a Thermo Fisher Scientific™ ITQ™ 700 Series Ion Trap Mass Spectrometer. The separation was achieved on a 30 m x 0.25 mm internal diameter x 0.25 µm film thickness fused silica capillary column ThermoGOLD™ TG-5MS (5% diphenyl; 95% dimethyl polysiloxane). 1 µL solution was injected in splitless mode at 250°C. The transfer line and the ion trap were respectively maintained at 300 and 200°C. Molecular components were carried by a constant 1 mL/min helium flow. Data treatments were carried out on Xcalibur software. Molecular compounds were identified by retention time, comparison with mass spectrum of commercial molecular standards, with the internal molecular library of the laboratory and with NIST MS Search 2.0 database recorded with an electronic ionization of 70 eV. The oven temperature was held at 50°C for 2 min, increased to 140°C at 8°C/min held for 2 min before reaching 160°C at 2.5°C/min and finally 330°C at 15°C/min and held for 3 min. Spectra were recorded in the 50-650 *m/z* mass range.

5.3.3. Archaeobotany

Reference modern grapevine flowers, both male and female, as well as fruits, used in this study were collected near Rome, in the municipality of Morlupo (42°09'19"N; 12°30'26"E). Reference pollen grains were also collected from the surface of the fruits of a wild grape from Tivoli (41°57’09"N; 12°49’04"E). All grapes were sampled in wooded rims of river valleys, within a riparian vegetation characterized by *Quercus cerris*, *Q. pubescens*, *Fraxinus ornus*, *Ulmus minor*, *Populus nigra*, and *Alnus glutinosa*.

As a reference for pre-domestication *Vitis* pollen, the Middle Pleistocene diatomite sediments from Fosso di San Martino (Di Rita and Sottili, 2019), located in the municipality of Rignano Flaminio (42°11‘26''N, 12°31'13''E), near Rome, were re-analysed to observe the morphological characters of wild pollen grains in the region.

Adapted from (Muller, 2004), pitch samples of ca. 0.5 g were systematically dissolved in tetrahydrofuran and ethanol before the addition of a tablet with a known number of exotic

Lycopodium spores to estimate the pollen concentration. To limit contamination from modern pollen grains, whole pieces of pitch were treated. Acetolysis was not needed.

Modern pollen was acetolyzed following the standard procedure (Magri and Di Rita, 2015). Modern fruits from *Vitis vinifera* subsp. *sylvestris* were hydrated in water for 12 hours before being heated for 10 min in NaOH (10%) and acetolyzed.

Pollen was observed under a Zeiss Axioscope light microscope at 400x and 630x magnifications. Identifications were supported by pollen morphology atlases (Reille, 1992; Punt, Marks and Hoen, 2003; Beug, 2004); websites https://www.paldat.org; https://globalpollenproject.org, and the reference collection of the Laboratory of Palaeobotany and Palynology of Sapienza University of Rome. Morphological pollen and wood observations were also performed by Environmental Scanning Electron Microscope (ESEM) Hitachi TM-3000 Tabletop operating at 15Kv without previous coating. The images were recorded at magnifications varying from 150x to 700x.

5.4. Results

5.4.1. Chemical analyses

For all the amphorae SFC1, SFC2 and SFC5, the first extraction (1LE) with polar organic solvents (DCM:MeOH) interestingly revealed a conifer resin made out of Pinaceae wood tar (dehydroabietic acid (DHA); methyldehydroabietate (DHAM)) (Fig. 31A). Aromatized (retene, norabietatrienes) and oxidized (hydroxy- and oxo-DHAM derivatives) abietanes highlighted a high temperature formulation and the ageing of the resin (Mills and White, 1994; Mezzatesta *et al.*, 2021). Oxidized abietanes were identified through their characteristic fragment ions (*m/z* 191; 253) (van Den Berg *et al.*, 2000).

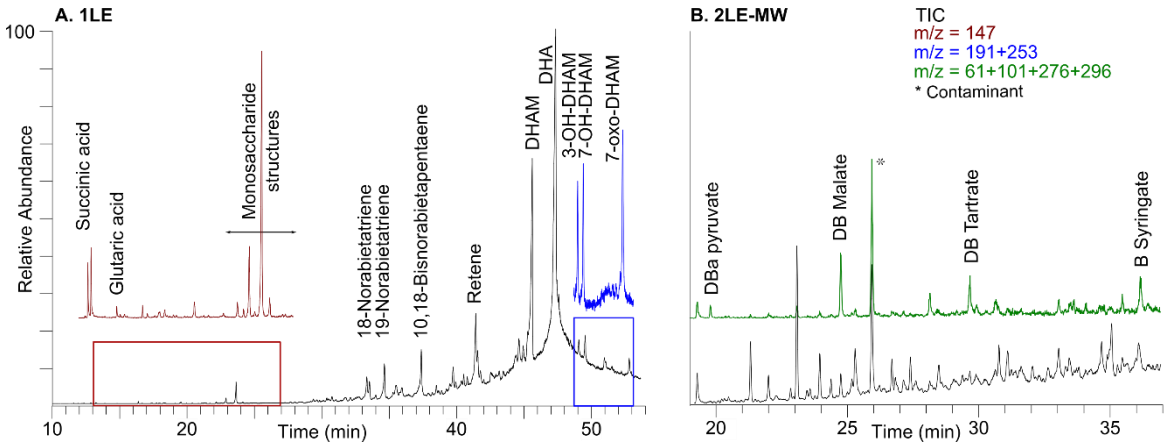

Figure 31. GC-MS chromatogram for the pitch of SFC1. A. represents the first extraction (1LE) and B. corresponds to 2LE-MW, the butylated second extraction. Total Ion Current (TIC) is black-colored. Red, blue and green colors refer to fragment ions searching (respectively m/z 147 (succinic and glutaric acids) and m/z 191; 253 (hydroxy- and oxo-abietanes) in 1LE and m/z 61; 101; 276; 296 (dibutylacetal pyruvate; dibutyl malate; dibutyl tartrate; butyl syringate in 2LE-MW)

All the archaeological coatings contained tartaric (*m/z* 276), malic (*m/z* 303) and pyruvic (*m/z* 61, 89, 117, 173) acids, greatly identified as butylated and butylacetal derivatives. Syringic acid was only identified in the amphora SFC1 and never present in both extractions for SFC2 and SFC5. For all the amphorae, succinic and glutaric acids were identified in 1LE through their characteristic fragment ion *m/z* 147 in the trimethylsilylated form while no traces could be identified in 2LE-MW.

Since acids were transesterified in the second step, molecules identification in 2LE-MW was restricted to grape derivatives markers (succinic, pyruvic, malic, tartaric and syringic acids). Considering the presence of diethyl or butyl ethyl grape acids reported by Garnier and Valamoti (Garnier and Valamoti, 2016) in a Neolithic jar, similar reactions were controlled in our samples but no esters were observed. Such compounds would indeed be produced by esterification with the ethanol contained in the fermented beverage.

5.4.2. Archaeobotanical analyses

5.4.2.1. Pollen

The pollen concentration of samples SFC1, SFC2 and SFC5 is noticeably low (1771, 175 and 213 pollen grains/g resin, respectively: Table 8). The number of identified pollen grains are 150, 61 and 48 respectively. SFC1 displayed the richest pollen content with a major presence of *Quercus* (46%), *Pinus* (28.7%) and minor contributions of *Olea* (6.7%), *Phillyrea* (6%), Brassicaceae (2%), *CarPinus* and *Erica* (1.3%), *Myrtus*, *Plantago* and *Ranunculus* (0.66%). SFC2 showed a significant presence of *Pinus* (65.6%), followed by *Quercus* (16.4%), *Phillyrea* (3.3%), *Cedrus* (3.3%), and *Erica* (1.6%). SFC5 exhibited *Pinus* (35.4%) and *Quercus* (29.1%) followed by *Artemisia* and *Phillyrea* (8.3%), *Ranunculus* (6.3%), *Alnus*, *Erica* and *Ostrya* type (2.1%). *Vitis* represented 4.7%, 9.8% and 6.3% of total pollen, respectively (Table 8).

Assumption of highland pine species is suggested regarding the small pollen size (55 to 80 µm). Following the classification by Desprat *et al.* (Desprat *et al.*, 2015) and keeping in mind that fossilization can alter the grain size, the identification was possible up to the subsection including *P. mugo*, *P. nigra* and *P. sylvestris*. Unfortunately, it remained unreliable to identify the pollen grains to the species level.

In all the three pitch samples, pollen observations featured the presence of aporate 3-zonocolpate grains, ranging 20-25 µm, with psilate to micro-scabrate ornamentation (Fig. 32). They displayed narrow and long slit-like colpi, making the pollen round to slightly oval in equatorial view (EV) and obtuse triangular to hexagonal outline in polar view (PV) with straight to softly concave sides. As illustrated in Fig. 32, the main feature remained the total absence of porus within the colpi and a thick exine (up to almost 5 µm for the pollen grain in Fig. 32I-J, with small residues stuck to the exine).

Table 8. Pollen grains recovered from the analyses of amphorae SFC1, SFC2 and SFC5

	Asteroideae	Betulaceae	Brassicaceae	Ericaceae	Fagaceae	Myrtaceae	Oleaceae	Pinaceae	Plantaginaceae	Ranunculaceae	Vitaceae	Total pollen grains	Pollen concentration (pollen/g)
SFC1		*Carpinus betulus* 1.3%	Brassicaceae 2.0%	*Erica* 1.3%	*Castanea* 0.66% *Quercus* 46%	*Myrtus* 0.66%	*Olea* 6.7% *Phillyrea* 6.0%	*Pinus* 28.7%	*Plantago* 0.66%	*Ranunculus* 0.66%	*Vitis* 4.7%	150	1771
SFC2				*Erica* 1.6%	*Quercus* 16.4%		*Phillyrea* 3.3%	*Pinus* 65.6% *Cedrus* 3.3%			*Vitis* 9.8%	61	175
SFC5	*Artemisia* 8.3%	*Alnus* 2.1% *Ostrya* type 2.1%		*Erica* 2.1%	*Quercus* 29.1%		*Phillyrea* 8,3%	*Pinus* 35.4%		*Ranunculus* 6.3%	*Vitis* 6.3%	48	212

Pollen grains from female flowers and from the surface of the fruits of modern wild grapevines from Morlupo and Tivoli are morphologically consistent with the fossil grains from the pitch, being tricolpate, aporate and with a relatively thick wall of up to 4 µm in polar view (Fig. 32C-D and S1 Fig. 34). Such thickness is consistent with pollen of Balkan indigenous Žilavka and Blatina cultivars described in the literature (Jovanovic-Cvetkovic, Micic and Djuric, 2016; Mićić *et al.*, 2018). Mercuri *et al.* (Mercuri *et al.*, 2021) also reported thicker exine dimension of 1.6 µm (± 0.70) in polar view for wild dioecious plants, while current "ancient cultivars" of Lambrusco Grasparossa or Bianca di Poviglio measured less than 1 µm. Likewise, although with a slightly smaller grain size (18-22 µm), the aporate morphology of *Vitis vinifera* was also found in pollen grains from sediments belonging to the Middle Pleistocene sediments of Rignano Flaminio (Fig. 32A-B and S1 Fig. 34B). Apart from differences in the apertures, these grains exhibit the same morphological features and micro-rugulate ornamentation with respect to *Vitis vinifera* displayed by standard palynological references (Reille, 1967, 1992; Punt, Marks and Hoen, 2003; Kvavadze, Chichinadze and Martkoplishvili, 2010; Arobba *et al.*, 2014; Kvavadze *et al.*, 2015, 2019; McGovern *et al.*, 2017). However, they differ from the typical morphology of the pollen grains from the non-functioning stamens of female flowers (Coito *et al.*, 2019), which are aporate and acolpate, but with the same ornamentation of pollen from male flowers (Lombardo *et al.*, 1978; Kevan, Longair and Gadawasi, 1985; Caporali *et al.*, 2003; Abreu *et al.*, 2006; Gallardo *et al.*, 2009; Jovanovic-Cvetkovic, Micic and Djuric, 2016; Mićić *et al.*, 2018; Mercuri *et al.*, 2021).

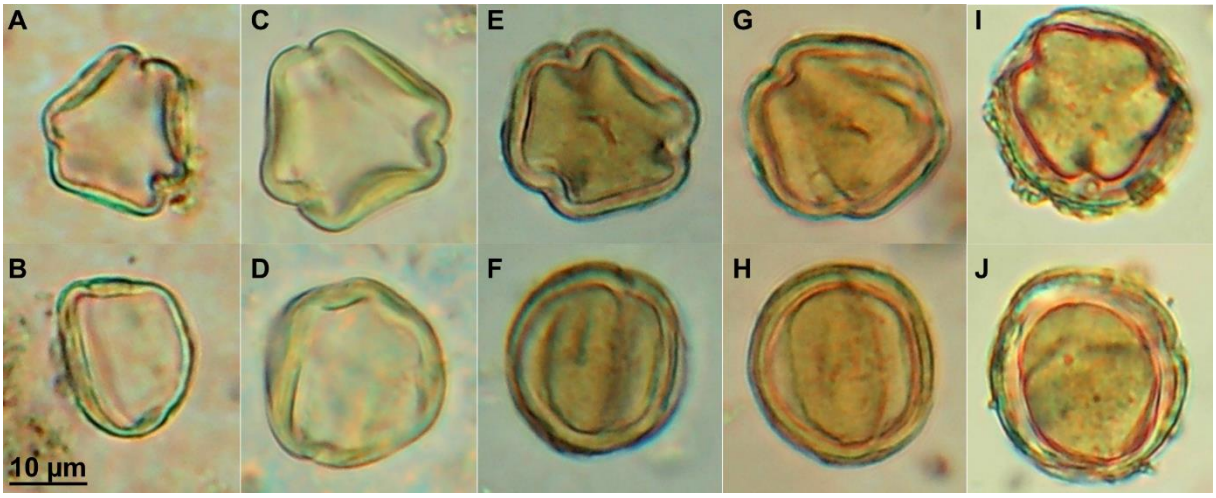

Figure 32. Vitis pollen in polar and equatorial view. Pollen grains recovered from: A-B. Fossil sediments from Rignano Flaminio (18-22 µm); C-D. Surface of modern wild fruits of Vitis from Tivoli (20-24 µm); E-F. Pitch of amphora SFC1; G-H. Pitch of amphora SFC5; I-J. Pitch of amphora SFC2.

5.4.2.2. Plant tissues

Remains of plant tissues trapped in or attached to the resin of SFC2 were found during microscopic observation (Fig. 33A). By comparison with modern *Vitis* flower observations after dissection of the stamen (Fig. 33B), we assigned them to the filament of *Vitis* stamen which connects the anther to the pedicel.

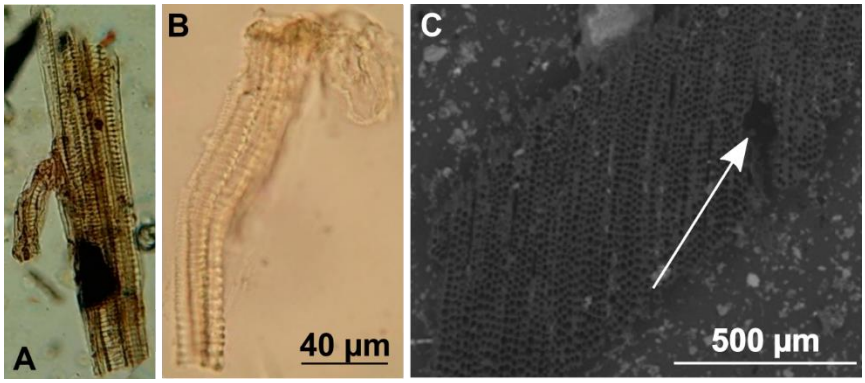

Figure 33. Microscopic observation of (A) archaeological plant tissues trapped in the resin of SFC1; (B) filament from the stamen of a modern wild Vitis vinifera flower, and (C) ESEM observation of a transverse section of charred Pinus wood trapped in SFC2 coating. The white arrow indicates the resin canal.

Radial and transversal sections of charred woods were recovered from the pitch of SFC2 and SFC5. Diagnostic features for the identification were: presence of resin canals (Fig. 33C), uniseriate rays, and large fenestriform pits in cross-fields. Based on these characters, the wood fragments were identified as *Pinus* group *sylvestris*, including *P. mugo*, *P. nigra* and *P. sylvestris*, whose wood anatomies are undistinguishable from each other with microscopic tools (Schweingruber, 1990; Schoch *et al.*, 2004).

5.5. Discussion

We hereby combined chromatographic tools with archaeobotanical approaches to reach a better understanding of the coating and content of the amphorae and of their use.

5.5.1. Coating of the amphorae

Chemical and archaeobotanical outcomes frame the use of Pinaceae wood tar to coat the amphorae, also frequently reported in the literature (Font *et al.*, 2007; Fujii *et al.*, 2019). Aromatic hydrocarbons such as norabietatrienes, retene and simonellite characterize an intensive heating under anaerobic environment (Connan *et al.*, 2002; Connan and Nissenbaum, 2003). Interestingly, resin is often used to flavor wines, additionally to its bactericide and waterproofing effects (Tonutti and Liddle, 2010). Hostetter *et al.* (Hostetter, Beck and Stewart, 1994) notably evidenced the resinated wine *vinum picatum* described by Pliny the Elder thanks to an accumulation of resin reported in Etruscan wine cauldrons.

Besides the prominent representation of *Pinus* pollen (sometimes in lumps) that accounts for almost one third (SFC 1 and SFC5) to two thirds (SFC2) of the total grains, the hypothesis of wood pyrolysis is substantiated by the presence of pine charcoal and DHAM markers. Indeed, the DHAM compound is obtained via methanolysis during the distillation of wood: the methanol contained in wood bark esterifies DHA molecules from the diterpenic resin when heated together at very high temperatures (Colombini and Modugno, 2009). Doubtlessly, wood was consumed during resin production and pollen grains were attached to the hot resin, since pollen resists high temperatures very well (Mascarenhas and Crone, 1996). The presence of both pollen and charcoal allowed a better understanding regarding the pitch origin, which is impossible to reach through organic residue analyses alone.

Despite the identification of *Pinus* to the species level is not possible, the botanical assignment to highland pine species (*P. mugo*, *nigra* or *sylvestris)* is strengthened by Pliny, whose *Naturalis Historia* stated that fire-extracted pitch from mountainous species logs of *P. mugo* (namely "taeda") is resin-richer, and notwithstanding its restricted spatial distribution, highland species were abundantly manufactured (Pliny, *N. H.* XIV, 9, 17, 21, 22) (Orengo *et al.*, 2013). The current distribution of *P. mugo* is very restricted in central Italy, while it is commonly found in alpine environments, where also *P. sylvestris* is widely distributed (Caudullo, Welk and San-Miguel-Ayanz, 2017). In contrast, *P. nigra* is uncommon on the Alps, and sparse in the central and northern Apennines, while the subspecies *P. nigra* subsp. *laricio* is present in Calabria, in Sicily (slopes of Mount Etna) and in Corsica. In Roman times the production of pitch from the mountains of Calabria and Sicily was renowned (Dionysius of Halicarnassus, XX, 6; Pliny, N. H. III; Cicero, *Brutus* XXII, 85-88). In any case, we can exclude a local origin of the pine used for the production of pitch of the studied amphorae.

The dominance of *Quercus* (46% in SFC1 and 29% in SFC5, Table 8) can be explained by environmental abundance in the region of wood tar production (Spada *et al.*, 2008).

5.5.2. Content of the amphorae

The amphora typologies of Dressel 1A (SFC1 and SFC2) and Lamboglia 2 (SFC5) have been frequently reported as grape-derivatives containers (Panella, 1998; Arobba *et al.*, 2014; Fujii *et al.*, 2019). Tartaric acid, together with malic acid (although less specific), identified by GC-MS, point out a grape-based content. Fermentation assumptions is enhanced by succinic and glutaric acids. Pyruvic acid resulting from spontaneous malolactic fermentation refers to wine content (Ribéreau-Gayon *et al.*, 2006). For SFC1, syringic acid present in the second extract, despite its absence in the first extract, originates from malvidin oxidation, thus ruling out potential contamination from free extractible origin (Guasch-Jané *et al.*, 2004; Garnier and Valamoti, 2016). Red (for SFC1) and white winemaking processes (for SFC2 and SFC5) are therefore brought to light. Although the amount of tartaric acid is remarkably higher in grape bunches than in other edible products, its use as a reliable grape biomarker must be confirmed by other evidence (McGovern *et al.*, 2017; Drieu *et al.*, 2020). Thus, macroremains of plant tissues recovered from the pitch and identified as part of the filament of *Vitis* flower in samples SFC1 and SFC5 bring this needed evidence of grape derivatives content.

Regarding microremains, although the tricolpate pollen type (Fig. 32) does not exhibit any apparent pore, attribution to *Vitis* is straightforwardly demonstrated by the identical aporate grains observed from wild vines (Fig. 32C-D) and from the Rignano Flaminio fossil sediments from the same Lazio region (Fig. 32A-B) (Di Rita and Sottili, 2019). The micro-rugulate ornamentation evidenced by SEM observation of Rignano Flaminio sediments and from the surface of wild fruits from Tivoli (S1 Fig. 34) is in accordance with the literature for *Vitaceae* pollen (Punt, Marks and Hoen, 2003). Nevertheless, attribution to *Vitis vinifera* subsp. *vinifera* is highly questionable since grapevines were surely not domesticated in the Middle Pleistocene (This, Lacombe and Thomas, 2006; Zhou *et al.*, 2017). Furthermore, the fossil impression of a grapevine leaf, identified as *V. sylvestris*, coupled with pollen grains, reported from another fossil site 16 km away from Rignano Flaminio, confirms the presence of wild grapevines in this area since at least the Middle Pleistocene (Follieri, 1958). *Vitis vinifera* subsp. s*ylvestris* is well represented in Italy, especially along the Tyrrhenian coast (Arnold, 2002; Biagini *et al.*, 2014). Although its survival is highly threatened (Magri, Celant and Di Rita, 2019), wild grapevines are still present also in southern Latium, close to San Felice Circeo (Arnold, Gillet and Gobat, 1998; Biagini *et al.*, 2014).

As far as we know, this is the first time tricolpate aporate *Vitis vinifera* is found in Roman amphorae, although inaperturate (aporate and acolpate) *Vitis* grains were recently retrieved from the Middle Bronze Age site of Terramara di Poviglio (Mercuri *et al.*, 2021). Interestingly, some morphological abnormalities of *Vitis* grains displaying one, two or four pores have also been published in modern cultivars (Pereira *et al.*, 2018).

To truly assume a grape derivative content in the amphorae, the presence of aporate grape pollen in the pitch shall be explained. Three hypotheses are discussed.

A first possible answer regards the sterility of the grapevine used. The presence of aporate pollen grains on *Vitis* fruits from wild plants near Tivoli demonstrates the permanence of *Vitis* pollen from female flowers on the fruit surface, despite its development (Fig. 32C-D). Pollen, subsisting upon time, weather and environmental circumstances, may be plucked with the fruits and remain even in the fermented beverage. Since beverages were not filtered at this time, pollen could remain in the amphorae attached to the pitch and bring evidence of the ancient content (Arobba, 1976; Vogt *et al.*, 2002; Rösch, 2005). The pollen we observed might be sterile as suggested by the strikingly thick exine observed, reaching up to 5 µm (Fig. 32I-J) whereas tricolporate pollen rather exhibits a "thin to fairly thin" wall (Punt, Marks and Hoen, 2003). Although cytogenetic characteristics are preserved during the grain development, Caporali *et al.* (Caporali *et al.*, 2003) explained *Vitis* pollen sterility by morphological germination inhibition caused by wall structure abnormalities: during the grain formation, the pollen surface is covered by structural nutrients contained in the exine (Caporali *et al.*, 2003). An excess of exine cover during pollen hydration may turn a mechanical hurdle into sterility (Mićić *et al.*, 2018). Physiological and cytological functions are nevertheless maintained, and pollen grains can disperse once released by the anthers. The absence of germinative pores causes grain sterility by avoiding pollen tube development even though pollen grains are viable (Lombardo *et al.*, 1978; Jovanovic-Cvetkovic, Micic and Djuric, 2016).

Pollen sterility is strictly related to wild features. At early stages, flowers of *Vitis vinifera* are all hermaphrodite, until they may become unisexual due to the abortion of one reproductive organ (Levadoux, 1946; Caporali *et al.*, 2003; Ramos *et al.*, 2014). Sex determining genes therefore divide flowers into functional "male" (or staminate) and "female" (or pistillate), showing up fertile pollen (and rudimentary pistil) or functional pistil (and sterile pollen), respectively (Negi and Olmo, 1970; Coito *et al.*, 2019). Pollination is achieved through the intermediary of a fertile pollen coming from either a functional male (*V. vinifera sylvestris*) or hermaphrodite plant (*V. vinifera vinifera*).

Pollen sterility also involves dioecy, which was lost during the domestication process (This, Lacombe and Thomas, 2006; Massonnet *et al.*, 2020). Grapevine domestication targets the ensemble of "genotypic, phenotypic, plastic and contextual impacts that can be used as markers of evolving domesticatory relationships" (Zeder, 2015). Asides the increase of sugar content in the fruits, berry sizes or changes in pips morphology, more factual definitions point to the shift from dioecy to hermaphrodism (This, Lacombe and Thomas, 2006). The reverting to hermaphrodism is assumed to have occurred through a rare event of male and female haplotypes recombination (Massonnet *et al.*, 2020; Mercuri *et al.*, 2021). Nonetheless, domestication and hermaphrodism have to be clearly separated from each other, since they differently relate to cultivation. Although cultivated *V. vinifera* are thought to have been domesticated from their wild *V. vinifera sylvestris* ancestors (Levadoux, 1956), not all the cultivated plants were necessarily hermaphrodite at the beginning of domestication, which was a long and multi-located process (Arroyo-Garcia *et al.*, 2006; Bouby *et al.*, 2013; Ucchesu *et al.*, 2015). As observed from wild cereals, grapevine cultivation for food consumption is presumed to have started long before its domestication (Mercuri *et al.*, 2015, 2018; McGovern *et al.*, 2017). One point remains certain: the switch to hermaphroditism grandly facilitates the fruit production, turning

grapevines into self-pollinating plants, with entomophilous and anemophilous cross-pollination (Turner and Brown, 2004; This, Lacombe and Thomas, 2006).

Additional evidence for cultivation is based on a considerable progress of statistic and modeling morphometric tools applied to pips and the emerging field of ancient DNA (Bouby *et al.*, 2013, 2020; Wales *et al.*, 2016; Aversano *et al.*, 2017; Zhou *et al.*, 2017; Mariotti Lippi *et al.*, 2020). SSR and SNP markers allowed genotype classification into cultivated and wild types (Grassi *et al.*, 2003; Butorac *et al.*, 2018). However, despite the important genetic dissimilarity reported between *V. vinifera* and *V. sylvestris*, a remaining presence of wild characters cannot be ruled out (Grassi *et al.*, 2003; Arroyo-Garcia *et al.*, 2006; Myles *et al.*, 2011).

Historical and archaeological evidence supports the use of wild grapes at the same time of cultivated grapes. In his *Naturalis Historia*, Pliny repeatedly reported the use of *V. sylvestris* grapes, wood and leaves in addition to cultivated grapevines (Pliny, *N. H.* XIV). In the Middle to Late Bronze Age site of Santa Rosa di Poviglio in the Po Plain, tricolporate *Vitis* pollen was abundantly recovered, up to 18% (Cremaschi *et al.*, 2016). The re-examination of the archaeological sediments highlighted 15 inaperturate *Vitis* pollen grains that demonstrate the use of *V. sylvestris* (Pecci *et al.*, 2020; Mercuri *et al.*, 2021). Although present in limited quantities (7.7% of the total grape pips), Mariotti Lippi *et al.* (Aranguren *et al.*, 200AD; Mariotti Lippi *et al.*, 2009, 2020) identified grape pips as wild morphotypes in Tuscany, belonging to Middle Bronze Age and Etruscan-Roman archaeological contexts. Castellano (Castellano *et al.*, 2017) pointed out the presence of *V. sylvestris* pollen in honey dated to the Iron Age in northern Italy, suggesting that bees fed on nectar of pre-domesticated or early-domesticated varieties of *V. vinifera*. Bouby (Bouby *et al.*, 2013) highlighted an intermediate form of Roman grape pips, between “highly cultivated” and “primitive cultivars” from wild ancestors, in the South of France.

The *Vitis* pollen retrieved from the Roman amphorae of San Felice Circeo may therefore represent an intermediary stage of domestication, being characterized by thick exine, absence of germinative pores and presence of colpi. This intermediate morphology recalls the morphological variety observed in stamens and pistils of *Vitis* flowers (functional female flowers present either erect but crinkled stamens, or semi- as well as entirely reflexed stamens) [85]. Advanced archaeopalynological analyses are needed for a better understanding of the grapevine evolution from wild, through intermediate to cultivated forms (Cardarelli *et al.*, 2015; Cremaschi *et al.*, 2016; Pecci *et al.*, 2020). This field of research offers new eyes for an innovative archaeological interpretation of the data (Mariotti Lippi and Secci, 2002; Mercuri *et al.*, 2015; Cremaschi *et al.*, 2018).

A second hypothesis to explain the presence of aporate *Vitis* pollen in the Roman amphorae of Circeo concerns the use of archaeological indigenous cultivars to produce fermented grape derivatives. Several modern autochthonous cultivars, such as ‘Loureiro’, ‘Moscato rosa’ or ‘Blatina’, have been documented to compose with a dioecious mating system (Cargnello *et al.*, 1980; Tischelmayer, 2001; Abreu *et al.*, 2006; Maletić *et al.*, 2015; Stupić *et al.*, 2019). Some of them, like ‘Picolit’ and ‘Lambrusco di Sorbara’ are endemic of northern Italy (Lombardo *et al.*, 1978; Mercuri *et al.*, 2021). Since the plants of these refined wines cannot self-fertilize due to infertile pollen from functionally female flowers, grapevine productivity is consequently limited and wine prices may be high (Schamel and Ros, 2021). Our study of modern pollen from different sites near Rome, showing tricolpate aporate grains, supported by Middle Pleistocene records with the same pollen morphology, strongly support the use of indigenous grape cultivars to produce the wine content of the Roman amphorae from San Felice Circeo.

The third hypothesis refers to the nature of the content. As previously described, chemical markers evidenced fermented grape derivatives, which can consist of either wine, vinegar or other beverages,

such as the cooked wine '*defrutum*' or aged sweetened wine '*mulsum*' (Peña, 2007; Arobba *et al.*, 2014; Bernal Casasola, 2015). Some Roman recipes reported in *De re coquinaria V.II.9,* translated and abridged by Feldman (Feldman, 2005), attested of the use of grape wine in traditional cooking made out of roses, spices and fish sauces of *liquamen* or *garum*. Unfortunately, in the absence of *tituli picti*, i.e., a commercial inscription on the surface of the amphorae, the possibilities of wine derivatives remain hypothetical since no chemical biomarker is able to distinguish wine from other traditional grape fermented recipes (Bernal Casasola *et al.*, 2004; García Vargas, 2004). Relying on the botanical evidence, we hereby consider the possibility of *oenanthium*, a flavored wine famous for its medicinal properties. Following Pliny recipe "w*ith the wild grapevine one makes what is called* oenanthia*: one macerates two pounds of wild vine flower in a cadus (30 or 40 liters) of must, one decants after thirty days […]. These grapes, shortly after flowering, are a remedy of singular virtue to temper the heat of the body in diseases*" (Pliny, *N. H.* XIV, 18). Besides attesting the use of wildflowers at Roman times, Pliny gives an interesting justification of *Vitis* pollen in jars through the presence of flowers. More importantly, we have also found stamen filaments (Fig. 33), which support the presence of flowers in the content of the amphorae. Moreover, a few non-arboreal pollen types may account for medicinal aromatization, specifically in SFC5 where *Artemisia* reached 8.3% of the total grains and SFC1 where *Myrtus* has been identified. Such plants are recognized for their medicinal benefits (McGovern, Hall and Mirzoian, 2013; Li *et al.*, 2018). Broadly speaking, there is abundant ethnopharmacological evidence for the common use of herbal concoctions in alcoholic or fermented beverages (Stika, 1996). The use of *Artemisia* for anticancer activity has been reported in Ancient Egyptian herbal wines from Abydos potsherd (McGovern, Mirzoian and Hall, 2009; McGovern *et al.*, 2010). *Myrtus communis* was recognized as a therapeutic drug in ancient Greece (Sisay and Gashaw, 2017) and used as herbal additives in early medieval beers (Nelson, 2005). Nonetheless, the limited pollen representation of *Myrtus* (less than 2%) can also refer to the surrounding vegetation.

In synthesis, our results suggest the use of autochthonous grapevines either cultivated, such as the modern 'Picolit', or wild, as demonstrated by the similarities with *Vitis* pollen from indigenous wild plants, as well as the possible use of grapevines at intermediate stages of domestication. Medicinal wine is another possibility that would explain the presence of *Vitis* stamens, and of *Artemisia* and *Myrtus* pollen, whose plants are used as flavoring. Likewise, *Pinus*, besides ensuring the waterproofing of the amphora, would have flavored the beverage due to its aromatic character. Indeed, herbal wines such as *vinum absinthianum* or *picatum* were common at that time.

5.6. Conclusion

The analyses of the pitch contained in three Roman amphorae from San Felice Circeo illustrates the benefits of applying a multidisciplinary approach. The combined evidence of amphorae typologies, Pliny's testimony regarding the use of *V. sylvestris*, previous archaeobotanical finds indicating the archaeological use of wild grapes, the chromatographic outcomes, and the morphology of *Vitis* pollen and tissues led to new archaeological and anthropological interpretations.

The identification of *Pinus* group *sylvestris* used to produce wood tar for waterproofing, matching the methyl ester diterpenic chemical markers characterized by GC-MS, indicates a non-local origin of the wood tar, as also suggested by ancient historical sources, reporting Calabria and Sicily as important production areas for pitch.

Aside from confirming the usage of Lamboglia 2 as wine containers, chemical analyses highlight the usage of both red and white wines.

The observation of aporate pollen of *Vitis,* compared with different types of fossil and modern wild grapevines, suggests the use of autochthonous grapevines, either wild or cultivated, without excluding a possible intermediary stage of domestication of cultivars still bearing *V. sylvestris* features. It is also possible to conjecture the archaeological presence of a medicinal grape beverage made as an infusion of wild *Vitis* flowers in the must, reported by Pliny as *oenanthium*. However, this hypothesis contrasts with the diverse typology of the amphorae involved in the pitch analyses.

Vitis pollen appears to be a fruitful anthropological indicator of ancient habits by opening a field of archaeological assumptions hitherto inaccessible, such as the inclusion of Roman grapevines into the long process of domestication. Within the long-standing question of distinguishing wild and cultivated grapes from past archives, the archaeopalynological study of *Vitis* may bring new evidence to define the timing and modes of grapevine cultivation.

Since false chemical positive must be tackled by external controls, we provided a straightforward methodology that brought independent evidence of grape derivatives in Roman wine amphorae, based on chromatographical and archaeobotanical tools, allowing to suggest a history beyond the artefacts that could not be identified by single analytical techniques.

Author contribution

Conceptualization, L.C., A.C., C.D., C.V., C.M., D.M.; **methodology**, L.C.; **formal analysis**, L.C., A.C., F.D., C.M., D.M.; **investigation**, L.C., A.C., C.D., F.D., D.M.; **resources**, A.C., C.D., D.M.; **visualization**, L.C.; **supervision**, C.M.; **project administration**, C.M., D.M.; **funding acquisition**, A.C., C.V., C.M., D.M.**; writing—original draft preparation**, L.C., A.C., C.D., D.M.; **writing—review and editing**, all authors

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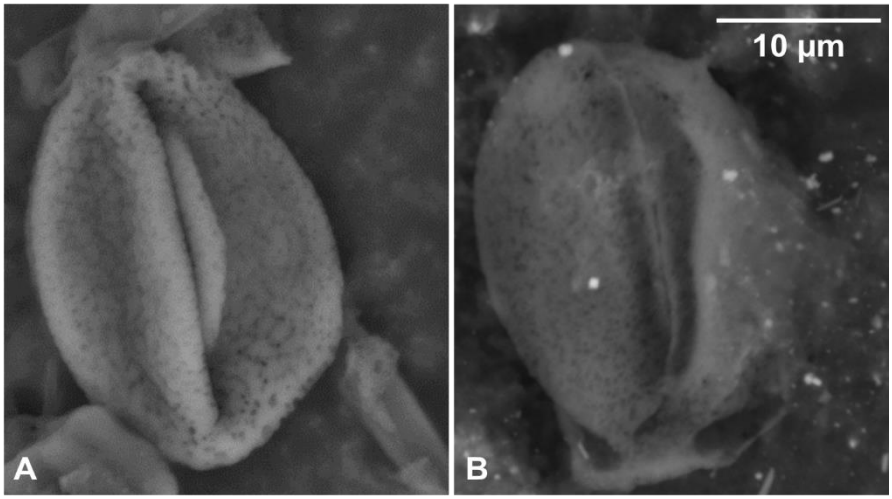
5.8. Supporting information



SI Figure 34. ESEM pictures of Vitis vinifera pollen grains recovered from (A) grapefruits from wild grapes in Tivoli and (B) Fossil sediment from Rignano Flaminio

Chapter 6

Tracing the medicinal content of a Bronze Age Jericho vase: how *Malva parviflora* pollen meet Pinaceae resin markers

Once the multi-analytical approach was developed and conclusive results on grape derivative containers were verified, the transdisciplinary methodology was then extended to non-resinous materials. In this respect, the object under consideration must meet specific requirements, especially regarding post-sedimentary palynological contamination. The archaeological context must indeed ensure that external pollen could not have been deposited following the burial of the object, to allow a reliable palynological analysis. Thanks to the collaboration with Teresa Rinaldi and Lorenzo Nigro from the Sapienza University, we had the opportunity to investigate a small jar used to pour liquids, labelled as 'teapot' due to the important shape similarities with Bronze Age Syrian and Palestinian "caliciform vessels". This vase is all the more interesting as it allows our multidisciplinary methodology to be applied to a distinct spatio-temporal context. The 'teapot' jug comes from the Bronze Age terrestrial site of Jericho (Southern Levant). In this chapter, the inorganic ceramic, rather than the organic waterproofing coating, was the focus of our study, under the guidance of archaeobotanical and chemical analyses. For the first time, the pollen content from the internal surface of the ceramic was investigated with the aim of verifying the methodological feasibility and deciphering the original content of the pot.

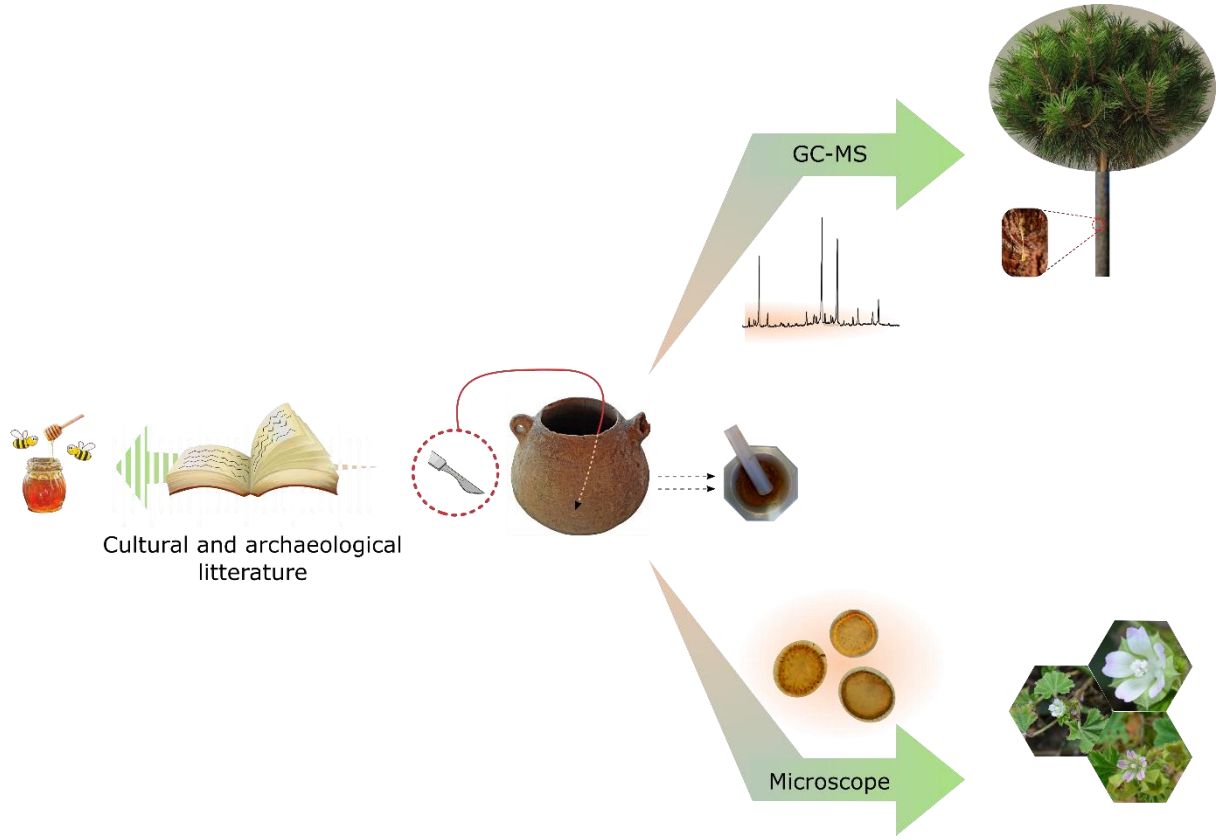


Figure 35. Palynological and chemical analysis applied on the Jericho 'teapot' vase

The following article displays the results obtained from the sampling of the inner ceramics of the Jericho 'teapot'. Given their interesting divergence according to the approach used, different archaeological hypotheses were then considered. The article proposes an exploratory study of the content of the vase through the dissimilar diagnostics established by the multidisciplinary methodology. Contrary to the previous chapter that placed the archaeobotanical outcomes at the center of the interpretation, with chromatography acting as a support, the analyses of the Jericho do not allow the same conclusions to be drawn about the original content. Interestingly, the analyses proved to be complementary, opening on new interpretative horizons that accounted for the equal

participation of molecular characterization and pollen records. The results are discussed considering several archaeological hypotheses. Archaeologist's initial assumptions are therefore tested in light of the analytical outcomes.

Through the extension of pollen identification knowledge to other botanical families than the one previously encountered, I gained learning of palynological formal and morphological analysis. Since the two approaches we employed did not provide corroborating results, I had to deeply investigate historical and scientific literature to blossom the potential archaeological understanding. The diverging palaeoevidence therefore drove further targeting of molecular investigation. The exercise of linking analytical results trained me to ripen the discussion. Besides the application of diagnostic protocols to ceramics, I acquired methodological skills with the aim of interpreting analytical results by including them in a broader archaeological picture. This way, I used the bibliography as a major asset to contextualize the results. Because of the limited amount of material available for the analysis, the chromatographic study could unfortunately not be taken any further, and the lack of material did not allow us to continue with more specific characterization protocols. Although a new sampling is not excluded, the homogeneity of the results remains nonetheless questionable. Further analysis would allow us to clarify certain hypotheses, hence modifying the exploratory structure of the article before submission. The paper might then be submitted in international journals of archaeometry, such as to the *Journal of Archaeological Science*.

Tracing the medicinal content of a Bronze Age Jericho vase: how *Malva parviflora* pollen meet Pinaceae resin markers

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6.1. Abstract

Pollen and chemical analyses were applied on the inner ceramic of a Bronze Age pot (ca. 2300-2000 BC) recovered from the archaeological site of Jericho (Southern Levant). The peculiar shape of the vessel, symbolized by a spouted typology characterized as 'teapot', represents a major asset to the understanding of cultural and daily practices the vessel reverts to. Archaeobotanical analyses revealed a significant amount of *Malva parviflora* pollen and lumps of *Artemisia* and *Malva*. Organic residues, characterized by Gas Chromatography - Mass Spectrometry, highlighted diterpenic markers of Pinaceae resin. As the identified plant are notorious for their health benefits and no vessel coating being macroscopically observed, we interpreted the results as a medicinal concoction. Induced by the archaeologist's hypotheses regarding the functionality of the pouring vessel and supported by the non-overlapping results obtained by palaeobotanical and chromatographic approaches, this study presented exploratory work that considered the various possibilities of liquid content. By obtaining scientific evidence that does not overlap with each other, the multidisciplinary approach then allowed to open the perspectives of anthropological and archaeological interpretation.

6.2. Introduction

Palynology has experienced a great deal of accomplishment in the last decades. The contribution of pollen to the reconstruction of past vegetation (Di Rita, Anzidei and Magri, 2013; Magri *et al.*, 2015; Edwards, Fyfe and Jackson, 2017), the deciphering of palaeoecological environment (Di Rita *et al.*, 2015; Di Rita and Magri, 2019; Mercuri *et al.*, 2020) and to the understanding of ancient cultural practices (Mercuri *et al.*, 2013; Bowes *et al.*, 2015) are not to be boasted anymore. While palynology finds its origins in the early 1940s (Iversen, 1941), the field really developed in the second part of the 20th century, with pioneers studies touting the yielded outcomes through pollen observation (Greig and Turner, 1974; Faegri, Kaland and Krzywinski, 1989).

While palynology is frequently integrated into wider archaeobotanical studies of micro- (non-pollen palynomorphs, spores, diatoms, and phytoliths) and macrofossil (charcoals, leaves, seeds, *etc.*) remains, interdisciplinary approaches that combine distinctive research fields are still too occasionally developed. Besides encouraging a common language between cultural, historical and scientific communities, bridging the gap from independent disciplines greatly strengthens the archaeological interpretation (Bouby *et al.*, 2013; Pecci *et al.*, 2020). As raised by Izdebski et al. (Izdebski *et al.*, 2016), interdisciplinary collaborations innovatively overreach the frontiers between humanities and palaeoscience by providing valuable tools to integrate societal impacts into the deciphering of environmental, cultural and climate changes.

In this respect, the archaeological site of Tell es-Sultan (Jericho West Bank) represents a meaningful pool for multidisciplinary investigations. The recent discovery of a spouted vase, dated back to the Early Bronze Age (EBA)/Intermediate Bronze Age (IBA), offered a rare opportunity to carry out collaborative investigations with the aim of deciphering the original content of the vessel. Indeed, the singular vessel typology, anachronistically designated as a “teapot” (Bunimovitz and Greenberg, 2004; Nigro *et al.*, 2021), outlined the very meaningful archaeological context it may raise. The upper decoration and typological similarity with Bronze Age Syrian and Palestinian “caliciform vessels” suggest a symbolic content that would have been poured (Albright, 1932; Wright, 1971; Dever, 1973). Based on the typological observation, various possible beverages were hypothesized by the archaeologists that performed the excavation campaigns (Nigro *et al.*, 2021), such as water, beer, wine, oil, milk, and even tea. The application of palynological and chromatographic tools aimed at bringing scientific insights to identify the content of the pot advanced by the archaeologists.

The aim of the article is the characterization of the vessel content with scientific lens to confirm and blossom the archaeologist initial hypotheses. Besides the archaeological meaning, the purpose of this study was to verify the possibility of acceding fossil pollen contained in the ceramic of the vase. In this study, Gas chromatography - Mass Spectrometry (GC-MS) and archaeobotanical tools were used to provide the molecular fingerprint of microresidues and pollen impregnated in inner surface of the ceramic.

6.2.1. Archaeological site

The archaeological site of Tell es-Sultan is located in the Southern Levant (31°52'15.99" N; 35°26'39.32" E). Integrated into the Jericho Oasis (Fig. 36), the holy place has early on attracted past civilizations to settle down. The first excavation started during the 19th century, under the supervision of the Captain Charles Warren, before Austro-German archaeologists took precedence on the expeditions during the beginning of the 20th century. They notably discovered the "Cyclopean Wall", the monumental foundation of the rampart, dated to Middle BA III (Wright, 1957). They established the continuous occupation of the site from the 10th to 6th century BC. Few years later, British expeditions uncovered the important necropolis of Jericho, testifying to the prominent place the site occupied in the Fertile Crescent and outlined Jericho’s intense activity during the Mesolithic and Neolithic periods (Garstang, 1932, 1933, 1934). Later on, Kenyon revolutionized the archaeological methodologies employed by developing innovative techniques of excavation (Kenyon, 1953, 1981, 1993). Improving the accuracy of chronological assessment through the analysis of the site stratigraphy, she notably shed light on the site's occupation from the EBA to the Roman period. Since 2017, the archaeological site is excavated in cooperation with Sapienza University of Rome, following an agreement of the Palestinian National Authority (Department of Antiquities and Cultural Heritage

of the Ministry of Tourism and Antiquities (MOTA_DACH)). The focus has been set on the Bronze Age fortifications and residential quarters (Nigro, 2003, 2016; Nigro *et al.*, 2011).

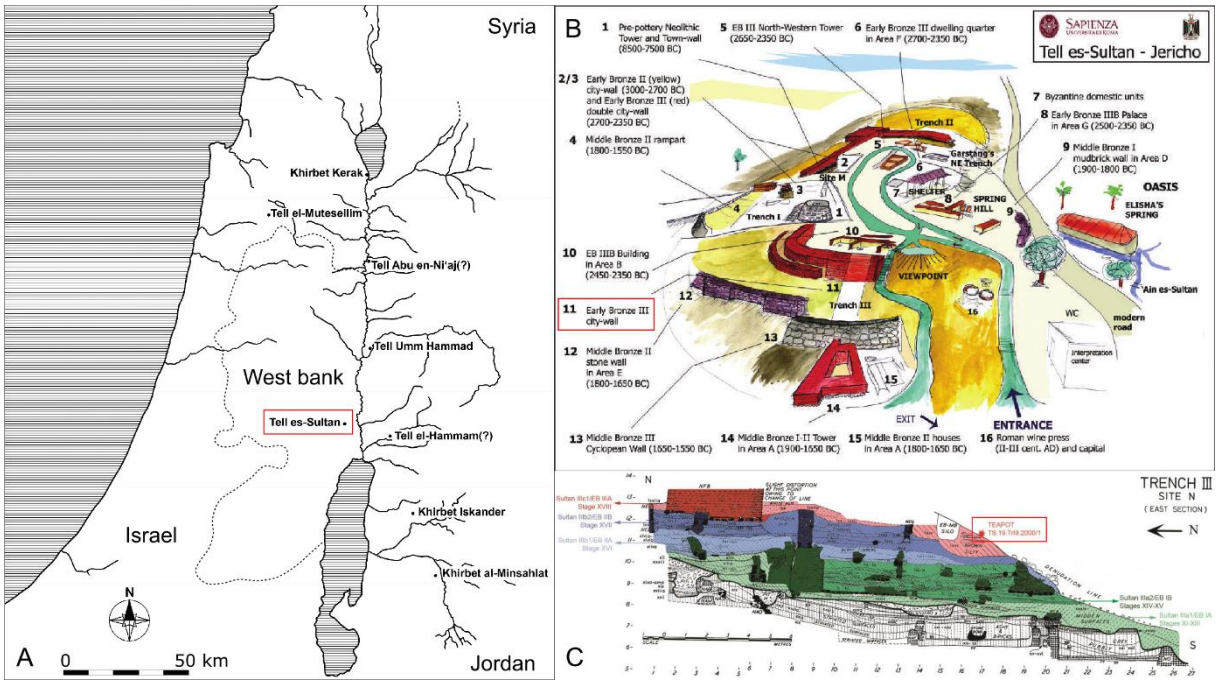

Figure 36. Archaeological site of Tell es-Sultan. A: Location mapping in the West bank (D'Andrea, 2014); B: Schematic reconstruction of Tell es-Sultan (drawing by L. Nigro; © Rome "La Sapienza" Expedition to Palestine and Jordan (www.lasapienzatojericho.it); C: The east section of Kenyon's Trench III outlining the stratigraphic position of the teapot vase (Nigro et al., 2021)

The vessel pot (TS.19.TrIII.2000/1) investigated (Fig. 37) was uncovered during the Italian excavation of 2019. It originates from the stratigraphic analysis of the small village extensions towards the flanks of the tell (Fig. 36). The village and the surrounding sparse dwellings were initially located on the Spring Hill (Montanari, 2019; Nigro *et al.*, 2021). The vase was recovered as embedded in a collapse layer from the advancing rampart stones in the East Section of Kenyon's Trench III. It has been protected by a burnt basket located directly above. The latter was broken, constrained by the crushing of the soil. While the vase typology and the site stratigraphy would indicate the vase belong to the EBA, ca. 2300 BC (Montanari, 2019), radiocarbon dating of a single burnt seed of *Lens culinaris* recovered inside the pot rather dated the vase to 3937 ± 40 BP (Nigro *et al.*, 2021).

6.3. Materials and methods

6.3.1. Vase sampling

The pot clay is reddish-brown (Fig. 37). Small sandy inclusions in the ceramics, already noticed in small jars from Tell es-Sultan, helped in dating the vase to the period Sultan IIId/EBA IVB (Nigro, 2003). The spouted pot is relatively small (12 cm height). It laid on a slightly biconical flat base (8 cm diameter), exhibits an upper hole-mouth with vertical lug-handle (D'Andrea, 2014). The pouring character of the vase reverts to the Northern influences from the late EBA IV/Intermediate BA civilizations (Bunimovitz

and Greenberg, 2004; Joffe, 2018; D'Andrea, 2019). As suggested by the great resemblance with Egyptian and Near East Mesopotamian copper ewers recovered in the tomb of Khasekhemwy, the teapot might be used for ceremonial practices (Schorsch, 1992; Nigro *et al.*, 2021). The lug-handle of the vase that would inconvenience hand holding raises the assumption of a suspended vase that could be used as a dipper or a pithos (Nigro *et al.*, 2021).

Figure 37. Vessel TS.19.TrIII.2000/1 (Nigro et al., 2021). Potter decorations are visible on the upper part

6.3.2. Archaeopalynological analyses

The internal surface of the vase was scratched with a lancet, over 1-2 mm in depth. No black coating was observed. 400 mg of the recovered powder were treated following the standard archaeopalynological procedure (Magri and Di Rita, 2015). HCl 37% (10 mL) was added to remove carbonate compounds and to dissolve the *Lycopodium* tablet that contains a known number of exotic spores to evaluate the pollen concentration. After centrifugation and removal of the supernatant, HF was carefully added to remove silica-based fractions. HCl was then added again to dissolve the potential colloidal silica and silicofluorides generated. NaOH was added after centrifugation and left in boiling water for 10 min to remove humic acids. Washing with water helped regulating the pH to neutral values. The mixture was mounted in glycerol before light microscope observation.

Samples were observed under a Zeiss Axioscope light microscope at 400x and 630x magnifications. Identifications were supported by pollen atlases (Saad, 1960; Culhane and Blackmore, 1988; Bibi *et al.*, 2010), identification keys (El Naggar, 2004; Khalik *et al.*, 2021), and the reference collection of the Laboratory of Palaeobotany and Palynology of Sapienza University of Rome. Pollen morphologies were also analyzed by Environmental Scanning Electron Microscope (ESEM) Hitachi TM-3000 Tabletop operating at 15Kv without previous coating. ESEM pollen images were recorded at magnifications varying from 1.2kx to 4.0kx.

6.3.3. Chromatographic analyses

6.3.3.1. Solvents and reagents

Solvents and reagents were of analytical grade. Dichloromethane (DCM), ethyl acetate, KOH and Na_2SO_4 were supplied by Merck. N,*O*-Bis(trimethylsilyl)trifluoroacetamide / trimethylchlorosilane (BSTFA/TMCS) and hexane were purchased by Sigma-Aldrich.

6.3.3.2. Sample preparation

Two extractive protocols were applied. On the first hand, 100 mg of crushed shard were extracted 3 times with DCM (1 mL) with the ultrasound probe (VCX 130 Vibra-Cell Sonics) for 3 min. After centrifugation, the organic supernatants were combined, filtered with PTFE (0.45 µm) and evaporated to dryness. On the second hand, an alkaline treatment was performed with 100 mg of crushed shard. The sample was extracted three times with 2 mL KOH (1M) with an ultrasound probe for 3 min. After centrifugation, the supernatants were added together and acidified up to pH 2. The organic phase was extracted 3 times with 3 mL ethyl acetate, filtered on a PTFE cartridge (0.45 µm) and evaporated to dryness.

6.3.3.3. Gas Chromatography – Mass Spectrometry

GC–MS analyses were carried out with a Thermo Scientific™ Focus system equipped with a Thermo Scientific Al 3000 autosampler and coupled to a Thermo Fisher Scientific™ ITQ™ 700 Series Ion Trap Mass Spectrometer. The separation was ensured with a 30 m x 0.25 mm internal diameter x 0.25 µm film thickness fused silica capillary column ThermoGOLD™ TG-5MS (5% diphenyl; 95% dimethyl polysiloxane). 1 µL solution was injected in splitless mode at 250°C. The transfer line and the ion trap were respectively maintained at 300 and 200°C. Molecular components were carried by a constant 1 mL/min helium flow. Data treatments were carried out on Xcalibur software. Molecular compounds were identified by retention time, comparison with mass spectrum of commercial molecular standards, with the lab molecular library and with NIST MS Search 2.0 database recorded with an electronic ionization of 70 eV. The oven temperature was held at 50°C for 2 min, increased to 140°C at 8°C/min held for 2 min before reaching 160°C at 2.5°C/min and finally 330°C at 15°C/min and held for 3 min. Spectra were recorded in the 50-650 *m/z* range.

All the samples were trimethylsilylated with BSTFA/TMCS (200 µL; 70°C; 30 min) and evaporated to dryness before being dissolved in a mixture of DCM:hexane (1:1) and injected in splitless mode into the GC-MS apparatus.

6.4. Results and discussion

Palynological analysis revealed a limited pollen concentration content of 242 grains/g sample. The following pollen grains were identified: *Malva* (12 grains + 1 lump); *Artemisia* (1 grain + 1 lump); Brassicaceae (1 grain); Amaranthaceae (1 grain) and *Cedrus* (1 grain).

The palynological content was principally composed of Malvaceae, which yielded 75% of the total count. Following the key steps provided in the literature (El Naggar, 2004; Khalik *et al.*, 2021), Malvaceae pollen were attributed to *Malva parviflora* (Fig. 38). Pollen grains were relatively small, ranging from 50 µm to 62 µm compared to other Malvaceae species. According to Khalik *et al*. (Khalik *et al.*, 2021), the grain dimension provided the first discriminative tool for the identification and several publications address the smallest pollen size to *M. parviflora* (Saad, 1960; Culhane and Blackmore, 1988; Bibi *et al.*, 2010).

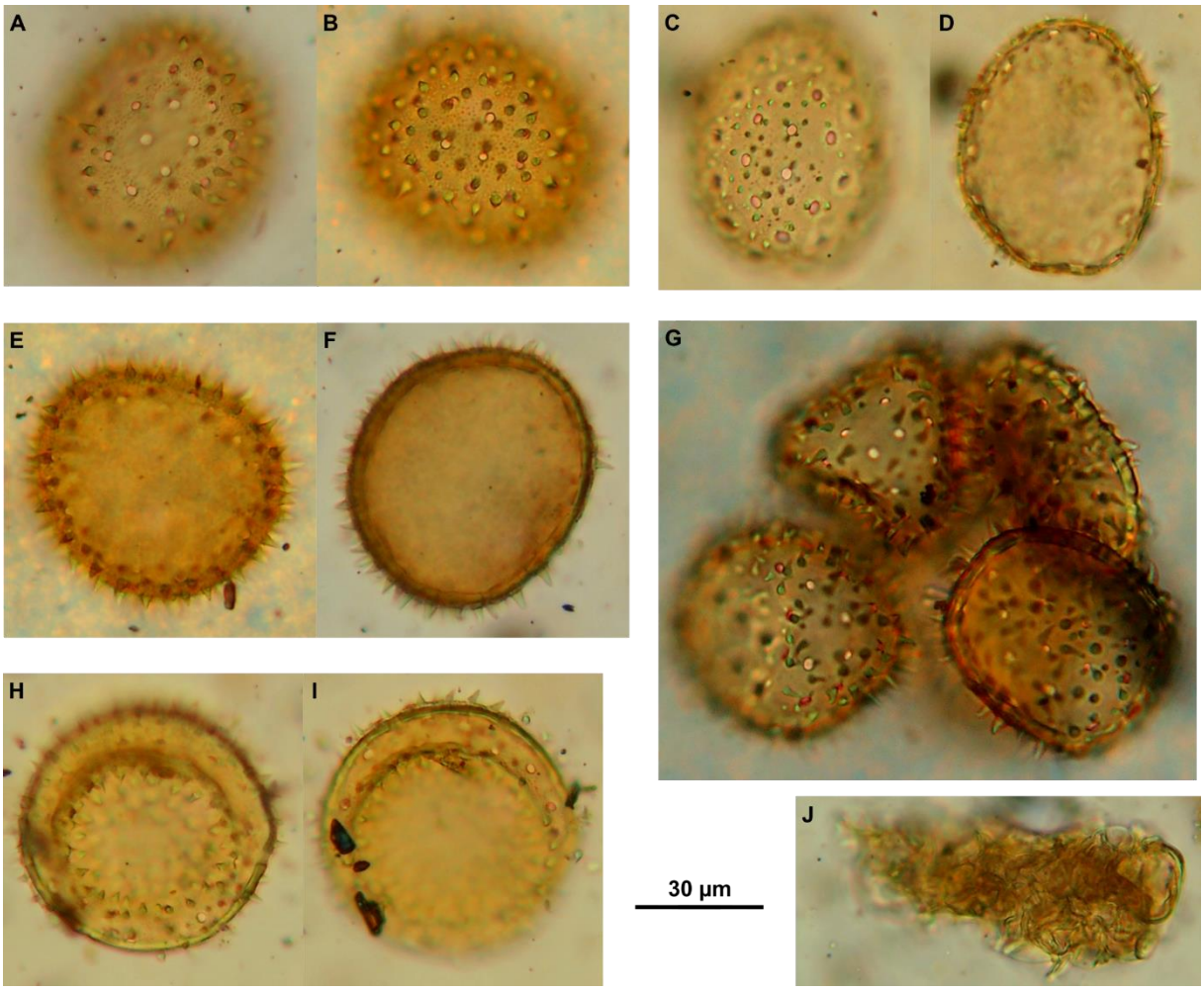

Figure 38. Pollen observed from the vessel. A-B; C-D; E-F; H-I: Malva pollen grains with varying focus; G: Malva lump; J: Artemisia lump

The pollen grains of *Malva* recovered from the vessel are suboblate, monocolpate and pantoporate with numerous aperture features (up to 50) (Fig. 38). Pores are small and circular with pore diameters of 2-(2.44)-4 µm. They are located at the base of the spines, as described by Bibi *et al.* (Bibi *et al.*, 2010). The spines, a characteristic feature of Malvaceae pollen, were relatively small compared to other mallow species. Indeed, El Naggar described *M. parviflora* as the species exhibiting the smallest spikes (El Naggar, 2004). Dimorphism in spike length was observed, with conical and pointed apex spines ranging from 3-(3.72)-6 µm. Spikes are uniformly distributed all over the pollen surface. The exine, clearly separated into sexine and nexine, is relatively thin compared to other Malvaceae species, with values of 2-(3.69)-6 µm (El Naggar and Sawady, 2008). The ornamentation observed under the light microscope is verrucate to microreticulate-punctate. Following Shaheen's identification keys (Shaheen *et al.*, 2009), pollen may also belong to *M. microcarpa* since the discrimination with *M. parviflora* relies

on a pore diameter exceeding 2 µm. However, this species does not live in the Southern Levant, the taxon hitherto occurs in Northern India and West Bengal (Biswa, Lakshminarasimhan and Lokho, 2016). The pollen is additionally rarely described in literature (Azab, 2017; Ali-Shtayeh and Jamous, 2018). *M. sylvestris*, *nicaaensis* and *aegyptia* were excluded on the basis of morphological criteria of size and spine length, despite their great representation in the West Bank (Ali-Shtayeh and Jamous, 2018). Even though Hosni et al. speculated small pollen dimension for *M. neglecta* (Hosni and Araffa, 1999), the hypothesis of an attribution to *M. neglecta* was rejected since other publications stated bigger dimension of 70-100 µm with spine length of 7-9 µm (Culhane and Blackmore, 1988; Shaheen *et al.*, 2009; Bibi *et al.*, 2010).

One pollen grain however exhibited a bigger dimension (ca. 83 µm) even though the diameter could not be accurately measured since the grain was importantly damaged and the grain was opened (pollen not shown). Considerable morphological and structural damages regarding some Malvaceae grains were indeed noticed. The poor state of pollen preservation was notably visible through grain opening or from the loss of spikes all around the exine (Fig. 39A). Since such damages could have interfered with the identification tools at the species level, the concerned grains were not included in the identification. Likewise, chemical treatments and the slide mounting media have been mentioned to alter pollen diameter (Reitsma, 1969; Sluyter, 1997; Meltsov, Poska and Saar, 2008).

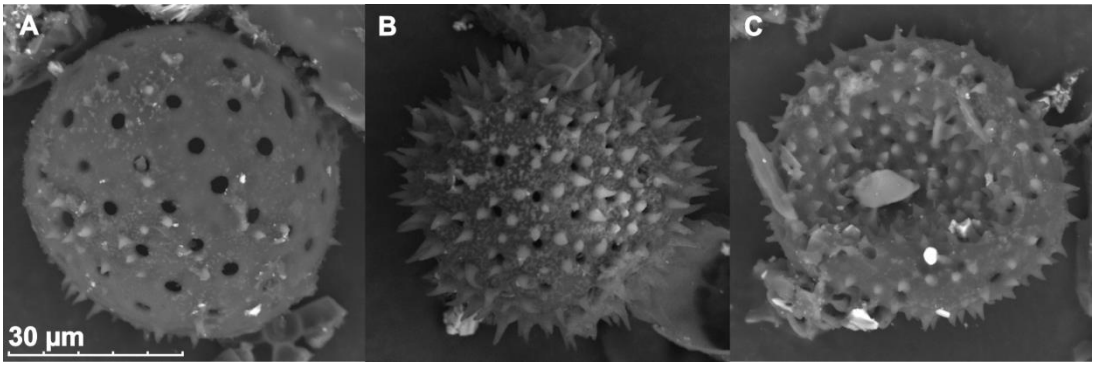

Figure 39. Malva pollen grains observed with ESEM

As reported in Figure 5, chromatographic analyses of the ceramic shard revealed the presence of short chains of dicarboxylic acids, namely adipic, pimelic and azelaic acids. They probably originated from the degradation of unsaturated fatty acids through auto-oxidative reactions triggered by radical mechanism of initiation and hydroperoxide formation (Aillaud, 2001), hence traducing the ageing of the lipid content. Palmitic, stearic and oleic acids were also identified. Additionally, the analyses highlighted the presence of 4-hydroxy benzoic acid, succinic acid and hydrocarbons. Although not specific to *Malva sp.*, such compounds were nonetheless characterized in Malvaceae chemical composition (Hasimi *et al.*, 2017; Al-Snafi, 2019). Hydrocarbons, and more specifically terpene structures, including mono-, sesqui-, di- and more rarely triterpenoids were also evidenced (Cutillo *et al.*, 2006). Since the literature concentrated on the fresh material, unstable compounds such as flavonoids and tannins were reported. Being hardly preserved with time, they are however unlikely to be recovered in archaeological analyses.

The recovery of mericarps attributed to *M. parviflora* in the Upper Palaeolithic archaeological site of Ohalo II in Israel provided evidence of the early on presence of the flower in Southern Levant (Snir, Nadel and Weiss, 2015; Weide *et al.*, 2017). The important extent in which fossil fruitlets of mallows were found (i.e., up to 14% or the current weed species) associated to the meaning recovery location

nearby the grinding stone traduced the flower daily life usage as well as its widespread distribution in the region around 23000 BP (Piperno *et al.*, 2004; Snir *et al.*, 2015). Then, *M. parviflora* was evidenced on the alluvium fields of the late 3rd millennium village of Iktanu, located at the Eastern North of Jericho (de Vries and Bikai, 1993). *Malva* sp. was also identified in the Dead Sea Plain macrofossils recovered from the Middle BA village of Tell el-Hayyat along the Jordan River (Fall, Falconer and Porson, 2019). Such archaeological records attest of the early presence of the flower in the local vegetal environment and its inclusion into past civilizations cultures.

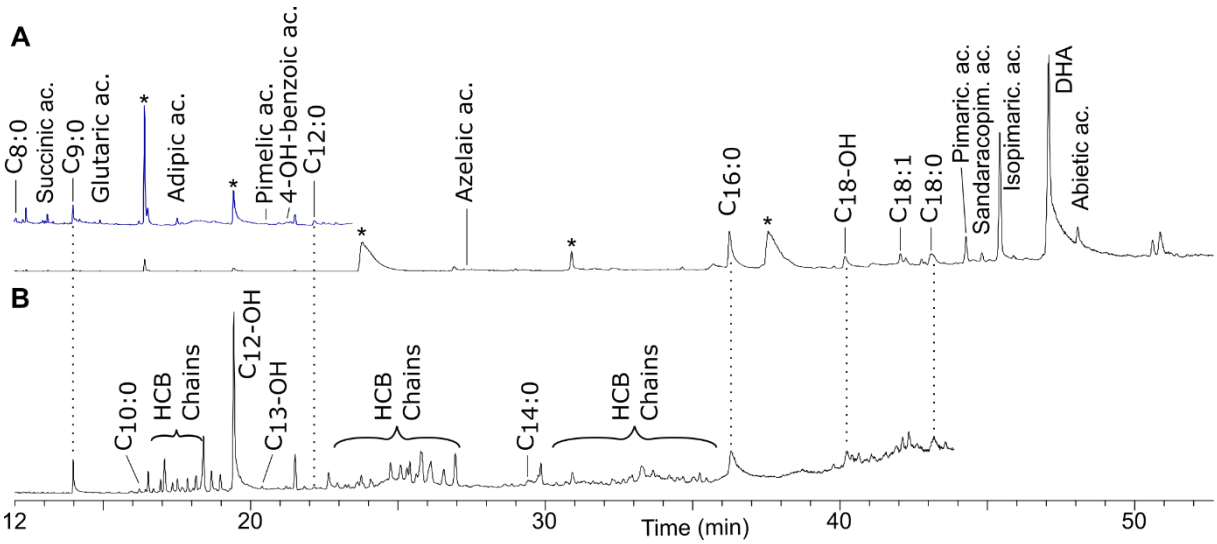

*Figure 40. Chromatograms from (A) Alkaline treatment and (B) DCM extraction.Ac.: acid; HCB Chains: Hydrocarbon Chains; *: Contaminant; $C_{x;y}$: Fatty acid of x carbon atoms and y unsaturations; C_x-OH: Alcohol of x carbon chains*

Moreover, similar pouring vessels were recovered during the excavations of several tombs in the Southern Levant (e.g., Megiddo tombs (Braun and Ilan, 2013), Tell el-Farʿah North (de Vaux and Steve, 1949) or Qiryat Ata (Golani and Bankirer, 2003)). Such discoveries supported the pouring vase relevance in funeral contexts. Bunimovitz (Bunimovitz and Greenberg, 2004) further distinguished the tombs into the one containing teapots and pouring-shaped vessels and the others, rather flourished with jars and four-spouted lamps. The ubiquity of teapot vessels, reported in both mortuary and domestic Canaan contexts, is therefore asserted to the founder role beverages might have played in bringing populations and cultures closer to each other. The typological heterogeneity noticed within the fine ware ceramic (e.g., dimension size, neck presence, horizontal tube handles, bent spout, *etc.*) and the wide distribution all along the Jordan River suggest numerous manufacture production sites and a lively trades of such potteries (Braun and Ilan, 2013). Combined with the evidence of recurrent use of *M. parviflora*, the assumption of a flower functionality in ritual practices and daily use is well grounded. To go further on, the recent bridging with a copper ewer from in the Umm el-Qaab necropolis at Abydos (Egypt) exhibiting similar typology (Nigro *et al.*, 2021) reinforces the ceremonial and symbolic character of `Egyptian mallows`, the common name for *M. parviflora*.

Medicinal benefits of mallows were greatly referenced in literature to chemically address their biological and anti-oxidant properties (Azab, 2017; Delfine *et al.*, 2017; Akbar, 2020; Sharifi-Rad *et al.*, 2020). *Malva* sp. is still currently consumed in decoction, as attested by current ethnopharmacological surveys of the millennial character of the plant (Ali-Shtayeh *et al.*, 2008; Gasparetto *et al.*, 2012). This way, indigenous civilizations already used mallows to cure respiratory and digestive diseases with herbal decoctions (Said *et al.*, 2002). Bible recipes referred to the ethnobotanical use of mallow leaves

and flowers: Egyptians used poultice pounded leaves of *M. parviflora* to diminish arachnid's stings (Duke, 1983). Roman consumed them as edible vegetable or infused as tea substituent, as boasted in Palestinian folkloric songs which designated *M. rotundifolia* as the commonest salad herb in the Middle Age (Crowfoot and Baldensperger, 1932). In the Northern Jordan, local people conferred to *M. parviflora* an important economic, floristic and cultural feature (Nawash *et al.*, 2014). This current high use value can find its roots in the remarkable flower representation in the vegetal landscape.

The recovery of pollen lumps of *Malva* and *Artemisia* (Fig. 38G; 3J) enhanced the assumption of medicinal herbal tea flourished with flowers, since lump agglomerates can only originate from the flower itself. Moreover, *Artemisia* has long been employed for its anti-inflammatory, anti-microbial and anti-oxidant properties (Said *et al.*, 2002; Khan and Khatoon, 2008). Brassicaceae and Amaranthaceae were assigned to the environmental vegetation and did not help in characterizing the content functionality.

Independently from the *Malva* species, ethnobotanical investigations in the Siran Valley (Pakistan) focused on the pharmacological benefit regarding sexual disorders (Shah and Khan, 2006; Ahmad *et al.*, 2009). Historically, Pliny the Elder (*Naturalis Historia*, XX, 84) reported the curative and aphrodisiac benefits of mallow consumption, the plant being already well-known from Greeks. He widely detailed healing recipes against animal venom, ulcer and even toothache by leaving the plant macerating with urine or even lichen and honey mixing. In fact, the archaeological presence of honey would alternatively provide a valuable explanation to the presence of pollen grains and lumps in the fossil record. Even though a significant amount of sugar in *M. sylvestris* flowers (i.e., 10-12 mg over 10 flowers) was measured (Jablonski and Koltowski, 2001), *Malva* are not the privileged flowers for bee foraging compared to *Cirsium*, *Trifolium* or even *Centaurea* (Fussell and Corbet, 1992; Rösch, 2005). However, being an integral part of the Jericho floral environment and widely distributed as indicated by the palynological analyses of Neolithic beehives from Tel Rehov, Israel (Weinstein-Evron and Chaim, 2016), it is very likely that mallows afforded bee-forages. In that sense, other archaeological contexts such as Italian charred honeycombs dated to the Iron Age provided evidence of *Malva* occurrence (Castellano *et al.*, 2017). Rösch interpreted the great pollen diversity recovered from a Bavarian BA bowl as an indicator of archaeological mead or honey, assigning *Artemisia* as the most frequent anemophilous pollen foraged (Rösch, 2005). In the hypothesis of a medicinal herbal content, honey would have a real meaning on health benefits in parallel of sweetening the taste. In this case and considering the small size of the pot, only a limited amount of honey would have been added to the vase, which explains the mitigated number of pollen grains identified and the low pollen concentration. *De facto*, pollen grains undergo differential degradation due to the different chemical compositions of the sporopollenin (Campbell, 1999; Twiddle and Bunting, 2010). To the best of our knowledge, Malvaceae have never been included in partial degradation studies. Nonetheless, a better preservation of mallow pollen, combined with the widespread presence of *Malva* in the natural environment may address the major distribution of *Malva* in the overall counting and explain the damage state observed for *Malva* grains. However, chromatography did not molecularly support honeybee assumption since no linear long-chain alkanes, fatty acids or alcohols, nor fatty acyl monoesters and hydroxyl fatty acyl monoesters were characterized (Roffet-Salque *et al.*, 2015). More targeted extractive protocols should be further applied to definitively address such hypothesis.

Alternatively, the presence of wine was tested since the grape beverage was reported as a vector of medicinal recipes (McGovern, Mirzoian and Hall, 2009). Based on the butylation protocol that was previously reported to surely trace tartaric acid (Chassouant *et al.*, unpublished paper), the wine hypothesis was refuted in the Jericho vase since no grape-fermentation markers were identified in the inorganic shard (data not shown).

The West Bank being close to the Bible birthplace and Israel being referred as '*A Land Flowing with Milk and Honey*' (Exodus 3; 8), the vessel could contain milk: this hypothesis, providing a ymbolic understanding of the content, needs to be verified by proteomics analysis.

Reverting to Figure 5, diterpenic markers of pimarane and abietane skeletons were characterized by chromatography. Dehydroabietic acid (DHA), together with pimaric, isopimaric, sandaracopimaric and abietic acids gave insight into the archaeological presence of Pinaceae resin in the vase. Given to the fact that no macroscopic coating was noticed during the sampling and corroborated to the absence of *Pinus* pollen in the vase, one reasonable hypothesis considered the resin addition to the mixture, made on purpose either as coating or external addition. Different aims can be suggested. First, the Pinaceae resin may have been used to modify the taste of the concoction, as for resinated wine (McGovern *et al.*, 1996). Then, the oleoresin fraction may have conferred anti-microbial and anti-inflammatory virtues. The essential oil fraction being compounded of mono and sesquiterpenoids, it may not have survived over archaeological times, leaving only the diterpenic resin fraction visible. Moreover, common is the ethnopharmacological evidence of resin-derivatives used as traditional drugs, as much for turpentine oil as for pine tar (Lev and Amar, 2002). For instance, the Turkish *Çam* preparation is a traditional curing mixture made of pine resin and honey (Satil, Selvi and Polat, 2011).

Since *Pinus* pollen has a large dispersal radius due to its saccate morphology (Schwendemann *et al.*, 2007), its absence in the palynological analyses of the vase highlighted the will of uniquely using the natural exudate. Indeed, if the resin had undergone any calorific treatment such as wood pyrolysis, then pollen would have been recovered, as demonstrated by the important proportion of *Pinus* pollen in the waterproofing coating of archaeological amphorae recovered from the ancient anchorage of San Felice Circeo (Chassouant et al., unpublished paper). In this respect, no methyl ester DHA and retene markers were characterized by chromatography although the protocols applied have shown their extractive efficiency in the past (Pecci, Cau Ontiveros and Garnier, 2013). Such molecules allow wood distillation and high temperature treatment to be traced (Colombini *et al.*, 2005). The absence of oxidized DHA derivatives additionally indicates the resin did not suffer from ageing (Mezzatesta *et al.*, 2021b).

Interestingly, *Cedrus* pollen was also identified in the vase. The teapot having been uncovered in a close room space in the excavation field, collected with care and maintained in close and clean conditions (Nigro *et al.*, 2021), the possibility of post-deposition contamination was excluded although *Cedrus* is widely spread in Lebanon. The pollen should therefore originate from the vessel itself. *Cedrus* essential oil, namely "*Cedrium*", was mentioned in Egyptian cultural contexts, notably for mummification rituals (Koller *et al.*, 2003; Charrié-Duhaut *et al.*, 2007; Łucejko *et al.*, 2012). The resin exhibits anti-microbial properties strongly appreciated for embalming, structural buildings and medicinal treatments (Kizil *et al.*, 2002; Termentzi, Fokialakis and Leandros Skaltsounis, 2011). The chemical composition mainly accounts for sesquiterpenic skeletons, with himalachene considered as biomarker of true cedar resin (Loizzo *et al.*, 2008; Sarret *et al.*, 2017). Back to the chromatographic analysis, no himalachene derivatives were identified, hence highlighting the more pronounced instability of sesquiterpenoids towards diterpenoids. Although the absence of such *Cedrus* markers did not rule out hypothetical cedar exudate in the teapot, notably plausible through the added value of health benefits of the resin, no certainties can either be formulated regarding its presence. Further investigation involving aged cedar exudate may provide a deeper understanding of the molecular alteration triggered by the resin degradation over time.

6.5. Conclusion

This study provided valuable evidence of the consumption of mallow at Bronze Age time in the Levant. Pollen analysis revealed the extensive presence of pantoporate spiked grains, identified as *Malva parviflora*. Pollen grains were relatively small compared to other Malvaceae. Fossil records additionally attest of *M. Parviflora* wide contribution to the local vegetal environment at Paleolithic times.

Chromatographic analyses did not explicitly confirm the presence of Malvaceae but did not invalidate it either: hydrocarbon chains, alcohols and acids belong to the common composition of plants. Short chains dicarboxylic acids witnessed the degradation of fatty acids that certainly occurred during archaeological times. Organic residues additionally indicated the presence of diterpenic markers. The absence of aged and heated molecular derivatives gave credits to the exudate nature of Pinaceae resin. Besides its exploratory character, this study provided valuable scientific evidence to draw the attention on a medicinal beverage, notably raised by the archaeobotanical records. Supported by *Malva* and *Artemisia* pollen lumps that can only originate from flower anthers, results were interpreted as traces of an ancient medicinal concoction. Enhanced by archaeological assumptions regarding the spouted character of the vessel and the biological properties of the botanic species identified, this multidisciplinary approach provided great signs of ancient cultural practices employing *Malva parviflora* and *Artemisia* flowers with Pinaceae resin.

Since palynological and chromatographic results did not cross-check each other, further investigations would be requested to unravel archaeological recipes and cultural consumption. The lack of material did not allow organic residue analysis to be pushed with more focalized technics to straightly investigate the presence of beeswax, fatty compounds such as long aliphatic chain carboxylic acids or terpenoid typical of herbal plant or cedar. Molecular markers, although tremendously necessary to evidence Pinaceae unnoticeable with palynological tools, probably suffered from degradation and leaching out. More in-depth analyses with solid phase extractions are encouraged to molecularly address the presence of cedar or honey, until then hardly identifiable with standard chromatographic methods (McGovern, Mirzoian and Hall, 2009; Mezzatesta *et al.*, 2021a).Typological resemblances with teapots recovered from funeral contexts alternatively suggested the symbolic role of the archaeological preparation, that could be supported by the presence of *Cedrus* pollen since cedars are highly emblematic in the nearby Lebanon.

The present study accounted for the development of collaborative approaches in order to provide valuable insights from independent fields of research. In this regard, archaeometric tools provided meaningful bridges to overcome gaps raised by single-centered investigations.

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Author contributions

Conceptualization, L.C., A.C., T.R., C.M., D.M.; **methodology**, L.C.; **formal analysis**, L.C., F.D.; **investigation**, L.C., F.D., C.M., D.M.; **resources**, A.C., T.R., L.N.; **visualization**, L.C.; **supervision**, C.M., D.M.; **project administration**, A.C., C.M., D.M.; **funding acquisition**, C.V., C.M., D.M.**; writing** - **original draft preparation**, L.C.

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Chapter 7

The analysis of oil amphorae

The analytical background provided by the IRPNC unit's expertise in optimizing protocols extractive capacities was clearly established through the characterization of fatty acid composition of amphorae or human mummies in previous PhD thesis. As with the organic residue analysis detailed earlier for grape derivatives content, lipid residues are of significant value in the interpretation of amphorae. Even though the protocols regularly mentioned remain traditional, notably saponification and lipid extraction, the need for a robust protocol is necessary in order to not bias archaeological interpretation raised by the absence of molecular markers. Applying a similar approach than the one developed for grape-derivative containers to characterize the fatty acid content of oil amphorae, the following chapter deals with a protocol comparison of the extractive capacities. This study focuses on the ceramic shard of amphorae supposed to have contained oil that were recovered in the cargo of Planier 3.

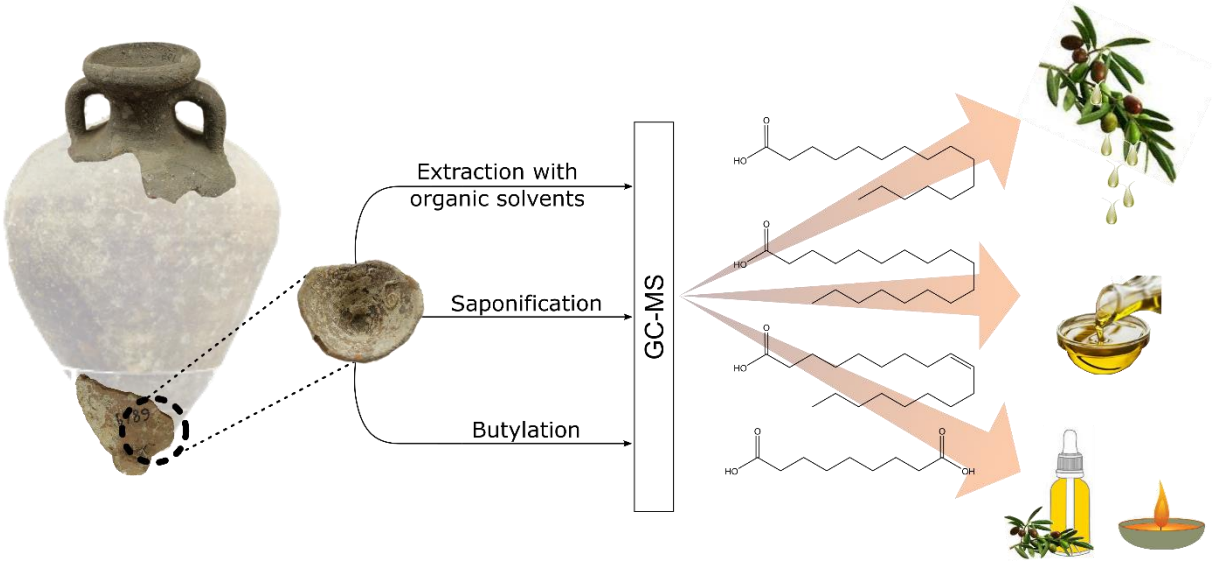

Figure 41. Protocol comparison applied on oil shards

Aside from adapting the methodology we already developed to different archaeological material, I trained myself on the analysis of oil content. Since the results we obtained by chromatography were not straightforwardly addressing olive oil, as initially expected, I had to put them in perspective. Since ambiguous results are always more difficult to explain at first sight, the analysis of the oil shards allowed me to detail different archaeological hypotheses with the help of the bibliography and the archaeologist advice. In the same way as the previous chapter, this study presents an exploratory work that puts forward different archaeological hypotheses in order to contextualize the molecular analyses. This study is not currently intended to be published in an international journal although the possibility is not ruled out.

7.1. Introduction

Lipid residue analysis have greatly participated to gain knowledge on ancient civilizations. The research field strongly participated in decoding the diet from animal and vegetal sources (Copley *et al.*, 2003, 2005; Craig *et al.*, 2004; Evershed, 2008; Whelton *et al.*, 2021). For instance, Dunne et al. (2019) shed light on ruminant milk used to feed children using FA isotopic $\delta^{13}C$ values from Bavarian Bronze and Iron Age vessels, specifically meant for children alimentation. Dealing with animal milk nourishment, they managed to address ruminant domestication at prehistorian time (Dunne *et al.*, 2019). Furthermore, it remarkably helped in assessing anthropological activities, products manufacture and ancient savoir-faire (Drieu, 2017; Rageot *et al.*, 2019). For instance, lipid residues played an interesting role in vessel use identification (Urem-Kotsou *et al.*, 2002; Connan and Nissenbaum, 2003; Regert *et al.*, 2003; Stern *et al.*, 2008). Supported by archaeological considerations regarding the shape, the design, the ceramic clay and the manufacture mode of production, archaeometry yielded to clarify the specific use vessel was meant for. Fatty acid analyses conducted with GC - Combustion Isotope ratio - MS allowed the distinction between ruminant (ovine or bovine), non-ruminant carcass fats (swine and equine) and fish or dairy fats (Steele et al., 2010; Manzano et al., 2016) to be realized. Stable carbon isotope ratios of palmitic and stearic acids provided a means to trace back fat's origin (Mottram and Evershed, 2003; Colombini and Modugno, 2009). $\delta^{13}C$ values were nevertheless collected from modern samples, leaving some doubt on the possible transposition to FA ratios in archaeological context since degradation alters the chemical profile of lipids, especially for plant oils (Steele *et al.*, 2010). The distinction between ruminant and porcine adipose is usually evaluated thanks to $\Delta^{13}C$, the difference in isotopic carbon measurements from stearic to palmitic (i.e., $\delta^{13}C_{18:0} - \delta^{13}C_{16:0}$) (Evershed, 2009).

Moreover, lipid analysis highly supplied unravelling civilization management strategies and population moves (Correa-Ascencio and Evershed, 2014; Carrer *et al.*, 2016; Matlova *et al.*, 2017; Suryanarayan *et al.*, 2021). This way, the isotopic compositions of hundreds of potsherds were integrated into radiocarbon dating, environmental, farming and climate understanding of the past civilizations. The agricultural reconstruction can then be scaled-up from determined spatiotemporal sites to broader environments (Roffet-Salque *et al.*, 2017), notably integrating lipid residues into complementary studies of zooarchaeology, archaeobotany, petrography, *etc*. Assessing the origin of fatty acids from ruminant, non-ruminant, plant oils or waxes, dairy or aquatic derivatives helped in understanding the advantages the civilizations took on their environments. However, such interpretations need solid and equivocal results. Indeed, caution must be taken while using lipids as biomarkers since they are widespread in molecular composition of edible products.

Altough the lipid source can be ambiguous, it is worth noting the ability of some very specific fatty acid to trace characteristic vegetal oil. Such lipidic patterns, as well as their degraded relatives, have actually been identified in archaeological context (Romanus *et al.*, 2008; Garnier *et al.*, 2009; Pecci, Cau Ontiveros and Garnier, 2013). For instance, erucic and gondoic acids were identified as traces of brassica, linseed and sesame seeds oil in Egyptian lamps (Colombini, Modugno and Ribechini, 2005; Copley *et al.*, 2005). Ricinoleic acid revealed castor oil in Late Antique amphorae of *Spatheia Bonifay* type (Pecci, Salvini and Cantini, 2010). More recently, even FA from $C_{16:0}$ to $C_{30:0}$ indicated ben oil from *Moringa* sp. in Ostia LIX African amphorae (Djaoui, Garnier and Dodinet, 2015).

Furthermore, unsaturated lipids are likely to degrade, turning their structures to not be preserved overtime (Philp and Oung, 1988). Degraded patterns can nevertheless be used as markers since they can allow the original fatty acid to be identified. For example, oxidized derivatives such as dihydroxycarboxylic, ω-hydroxycarboxylic or α-ω-dicarboxylic acids were identified with accelerated

ageing performed on fresh oil (Colombini, Modugno and Ribechini, 2005), hence highlighting the main degradation pathways.

Since degraded molecules proved to be meaningfull, robust protocols are favored for the extraction of any marker, degraded or not, when it comes to achaeological artefacts analysis. For this reason, a special interest was taken in investigating the extraction capacities of the protocols employed. The aim of the study was twofold: first, the comparison of the extractive capacities of traditional lipid extraction with organic solvents, saponification and the microwave-assisted butylation; then, the characterization of the lipid content of amphorae shards that supposedly contained oil. Gas chromatography – Mass Spectrometry was employed to supply the analysis of lipid residues extracted. The comparison was performed on oil ceramics recovered in the Planier 3 shipwreck. The archaeological corpus exhibited different typologies to favor the representability of the protocol comparison.

7.2. Material and methods

7.2.1. Materials

In this section were considered 11 amphorae from the Planier 3 cargo (see Table 9). 4 of them were classified as Early African (6781a; 6823; 1005 and 6849), 1 was a Brundisium amphora (579) and all the 5 others belong to the ovoid typology (747; 6849b; 598a; 728; 6561 and 561). Objects are referenced in the Chapter 1.5 “Cases of study” and archaeological profile drawings are shown in the appendix 1).

Typology	Sample	Amphorae pictures
Early African	6781a 6849 1005 6823	 6781a
Brundisium amphora	579	 579
Ovoid	747 561 728 598a 6561 6849b	 561

Table 9. Oil amphorae shards analyzed

7.2.2. Protocols

Among the protocols most mentioned to extract the lipid fractions, the following three were evaluated. First, a lipid extraction (LE) proposed by Mottram et al. (Mottram *et al.*, 1999); then the same lipid extraction followed by butylation (Garnier and Valamoti, 2016) and finally saponification (Mezzatesta *et al.*, 2021). The amphorae were always sampled bearing in mind the possible human

contamination, especially for Planier 3 artefacts that were excavated longtime ago, passed into the hands of various protagonists and stored in several places. The thin external layer of the shard clay was removed with a cleaned lancet before scraping the ceramic over 1-2 mm in depth. The clay was then crushed into powder before starting any extractive protocols.

Adapted from Mottram *et al.*, 100 mg of crushed shard were, on the first hand, extracted 2 times with 1.5 mL of $CHCl_3$:MeOH (2/1 v/v) with the ultrasound probe for 5 min, centrifuged during 5 min at 6000 rpm and 2 other times with 1 mL DCM following the same extractive pathway (Mottram *et al.*, 1999). Organic extracts were all mixed up together and evaporated to dryness.

On the second hand, the lipid extraction performed by microwave-assisted butylation was well-detailed in the chapter 4 (see "Protocol comparison for organic residue analyses from waterproofing materials and shards of Roman archaeological amphorae"). 100 mg of powder shard were extracted first with 1 mL of DCM:MeOH (1:1 v/v) using the ultrasound probe for 3 min. After centrifugation, the supernatant was taken apart and the extractive step was performed two times more. This first step gave rise to the first lipid extract (1LE) that is not detailed below since the extraction looks really similar to the one above-mentioned with $CHCl_3$:MeOH. After the remaining powder was dried enough, the second step of the lipid extraction (2LE-MW) could be performed. A mixture of BF_3 diethyl etherate complex, butan-1-ol and cyclohexane (1:2:4 v/v/v) was added to the dried powder, placed in the CEM Discover LabMate MW synthesizer (single dynamic cycle of 5 min; 80°C). After 3 repetitions, the mixture was neutralized with a saturated Na_2CO_3 solution. The organic phase was extracted twice with DEE and washed twice with Milli-Q water before being dried over sodium sulphate and evaporated to dryness.

Finally, 100 mg of shard amphorae were extracted 3 times with THF (1 mL) under 5 min US and centrifuged (5 min, 5000 rpm). Supernatant was recovered and maintained at 65°C for 60 min with 2 mL of a KOH 10% solution in MeOH:H_20 (9:1 v/v). After one third evaporation of the solution, 3 mL Milli-Q H_20 and 1 mL HCl (5M) were added. The organic phase was extracted 3 times with 5 mL DEE and dried with anhydrous Na_2SO_4 before filtration, evaporation under N_2 stream.

For all the protocols, after the extraction was achieved and extracts were evaporated, samples were trimethylsilylated (BSTFA/TMCS; 70°C; 30 min) before evaporation to dryness, dissolved in 1 mL hexane:DCM (1:1 v/v), filtered on PTFE cartridge (0,45 µm) and then injected splitless in GC-MS.

7.3. Results and discussion

As the first step, GC-MS highlighted the presence of diterpenes for 5 of the 11 samples investigated. As presented in Table 10 and Fig. 42, molecular markers such as DHA and/or DHAM were identified in the amphorae 6781a; 598; 6561 and for 1005 (at the trace level), hence unravelling the presence of archaeological Pinaceae wood tar within the amphorae. Retene, a marker of intense heating treatment (Mills and White, 1989), was simultaneously identified in the same samples. Although only 7-oxodehydroabietic acid methyl ester (7-oxo-DHAM) was characterized in the sample 6849, this amphora was surely coated with diterpenic wood tar since the molecule arose from the oxidation of DHAM (see Fig. 13) (van Den Berg *et al.*, 2000). Such highly oxidized compound indicates the important ageing character of the coating.

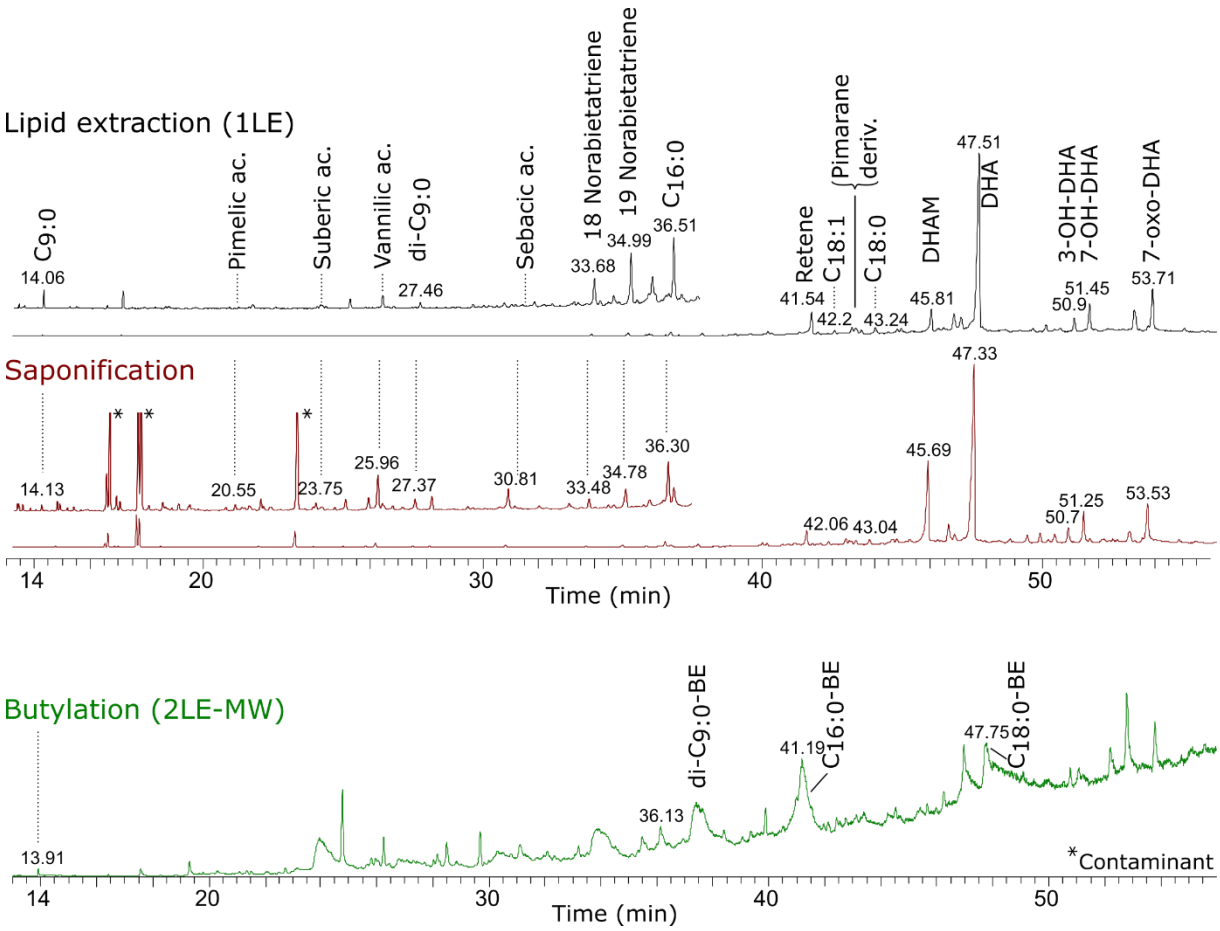

*Figure 42. 6781a TIC Chromatograms obtained from the analysis of the sample 6781a after lipid extraction (labelled 1LE), saponification and butylation (2LE-MW). Butylated patterns cannot be time-compared to lipid extraction and saponified compounds since molecular structures have been esterified. di-$C_{x;y}$-BE: dicarboxylic fatty acid of x carbon atoms and y unsaturation, Butyl ester; OH-DHA: hydroxy-DHA; DHAM: DHA methyl ester; Ac.: acid; deriv.: derivatives ; *: Contaminant*

Although the use of Pinaceae pitch is well established for waterproofing wine amphorae, its occurrence in oil containers is interestingly less reported in literature. Invisible to the naked eyes, the detection requires analytical tools which can explain the lately on emergence of evident cases (Romanus *et al.*, 2009; Pecci, Cau Ontiveros and Garnier, 2013; Djaoui, Garnier and Dodinet, 2015; Pecci *et al.*, 2017, 2021; Manhita *et al.*, 2020). Waterproofing linings were characterized, though, in Late Roman amphorae (Garnier, Silvino and Bernal Casasola, 2011), Dressel 20, Early and Late African (Woodworth *et al.*, 2015; Allevato *et al.*, 2017). Some research groups questioned the capacity of oil to dissolve the coating. This would justify the absence of waterproofing linings at a macroscopic scale, and its presence at the molecular level since the chemical markers were trapped in the shard (Peña, 2007). However, the coating would have damaged the taste of oil (Garnier, Silvino and Bernal Casasola, 2011). Based on lab ceramics to model fats diffusion through the ceramic layer, Romanus argued its infeasibility considering the tendency of oil to leak from the inner to the outer surface after 45 days (Romanus *et al.*, 2009). According to him, the coating would have impermeabilize and prevented the loss of oil good while trading.

In a second instance, chromatographic analysis after Lipid Extraction (1LE) also revealed the presence of fatty acids, mainly palmitic and stearic acids, as reported in Table 10. Oleic acid was only extracted for the samples 6781a and 598a, not as a major compound though. Since the lipid profile did not

correspond to the one reported for olive oil (Romanus *et al.*, 2009), further extractive protocols with chemical treatment were pursued. Saponification hydrolyzes the TAGs, hence liberating FA that were primarily bound to it. Since GC-MS does not permit high molecular weight compound volatilization, saponifying archaeological samples rends visible the bound FA and lipid distribution might be different from the global lipid extraction. The butylation catalyzed by BF_3 Lewis acid partially breaks down the inorganic fraction of the ceramic to rend accessible lipids that are trapped in. Initially developed for archeological wine potsherds (Garnier and Valamoti, 2016), esterification was also applied on lipid ceramics to access the polymerized or insoluble markers, hence invisible using traditional protocols (Djaoui, Garnier and Dodinet, 2015). Indeed, the same covalent bounding mechanism that ensured lipid surviving after centuries also turned them into solvent insoluble markers (Regert *et al.*, 1998).

Sample	Main FAs in order of decreasing concentration	Azelaic Acid	DHA	DHAM	Retene
6781a	$C_{16:0}$ $C_{18:0}$ $C_{18:1}$	X	X	X	
1005	$C_{16:0}$			traces	traces
6849	$C_{16:0}$ $C_{18:0}$			deriv.	traces
6823	$C_{16:0}$				
579	$C_{16:0}$				
747	None				
728	None				
6561	$C_{16:0}$ $C_{18:0}$		X	X	traces
561	None				
598a	$C_{16:0}$ $C_{18:0}$ $C_{18:1}$	X	X	X	
6849b	None				

Table 10. Molecular evidence of the content of Planier 3 oil shards

As detailed in Table 10, the benefits of both the saponification and the esterification remained mitigated. Regarding FA, none of them allowed $C_{18:1}$ extraction, except for 6781a where the molecule was already characterized with lipid extraction. Identical lipid profiles from 1LE and saponification suggested the absence of TAG within archaeological samples and highlighted oxidative pathways to generate oil degradation.

Moreover, the preponderance of palmitic over stearic acids firstly introduced by lipid extraction is maintained with saponification and butylation, here too indicating a degraded pattern. Uniformly, palmitic acid is recovered in larger extent compared to stearic acid. Recently, palmitic to stearic acid ratios showed their relevance, especially for identifying the nature of drying oils in paintings (Bonaduce *et al.*, 2017; van Dam *et al.*, 2017) and the degradation advancement (Helwig *et al.*, 2014; La Nasa *et al.*, 2018; van den Berg, Burnstock and Schilling, 2019). Generating structural distortion within the polymerized network formed during the drying of oil, the palmitate over stereate ratio can be an indicator of the damage undergone. Comparing the original ratio present in the oil with the metal palmitate over stearate ratio is a way to express the overall degradation state. The ageing effect on the chemical composition is integrated in the FA degradation ratios of azelaic to palmitic acid and through the calculation of the total dicarboxylic acid content (Manzano *et al.*, 2011). Indeed, azelaic acid arises from the oxidation of oleic acid on the unsaturated carbon, hence producing diacids molecules of 9-carbon chains. However, since no oleic acid was characterized from the lipid analysis of amphorae, palmitate over stearate ratios would not be indicative. Quantitative analysis was consequently not conducted for shards.

Although no characteristic lipid profile can be extracted, some words can nonetheless be said on their comparative benefits. The butylation of the samples notably promoted the extraction of $C_{16:0}$, $C_{18:0}$ for 5 samples (728; 561; 6561; 6823 and 1005) and azelaic acid only for 1005 compared to lipid extraction (see the appendix 2 for the molecular relative quantity extracted). Saponification supported the extraction of stearic and azelaic acids, primarily absent. The extraction of palmitic acid is more quantitative with butylation than with traditional lipid extraction for the amphorae 6823 and 1005 while it did not provide supplementary information for the samples 6849; 598a and 6781a. Finally, butylating allowed $C_{16:0}$, $C_{18:0}$ and azelaic acid to be either newly or more quantitatively extracted for 6781a; 598a and 6849 whereas it did not provide any difference for 1005 and 6823. Important is also to notice that none of the protocols highlighted the presence of sterols such as β-sitosterol or stigmasterol, despite its great distribution in plants and its frequent recovery in ceramics (Kimpe *et al.*, 2004; Pecci *et al.*, 2018; Reber, 2021; Whelton *et al.*, 2021).

Presented in Fig. 20, various molecules can be generated from FA degradation. Specific care was hence taken for FA and degraded derivatives identification, sinking specific mass fragments. Independently from the protocol applied, no straight trend could be drawn. No extractive protocol was consequently favored to provide valuable insight on the content. The absence of unequivocal FA distribution pointed again the specific character of archaeological amphorae, underlining the individual care the characterization required. Degraded markers were carefully targeted since they helped in diagnosing either the initial good or the underwent conditions. Markers of auto-oxidation, namely pimelic, azelaic, sebacic and/or undecanedioic acids provided insights on heat and light exposure as marker of natural degradation (Den Dooren De Jong, Dauvillier and Roman, 1961; Frankel *et al.*, 1977; Frankel, 2010; Pecci *et al.*, 2018). Many evidence of short chains degraded products were also linked to the use of archaeological ceramics (Regert *et al.*, 1998; Drieu, 2017; Blanco-Zubiaguirre *et al.*, 2018). Although the entire reactional mechanisms are still unclear, the respective α, ω-dicarboxylic acids distribution mirrors the oxidative pathway undergone. From unsaturated FA precursors, direct cleavage of the double bond, hydration or oxidation followed by oxidative cleavage as well as a successive oxidation, hydration and bond cleavage were reported by Regert *et al.* (Regert *et al.*, 1998). Then, dihydroxy-FA recovered from archaeological artefacts traduced degradation occurring via dihydroxylation. Relative products are dominated by 8-carbons chain patterns (Regert *et al.*, 1998). Despite the degraded aspect revealed by the analysis of Planier 3 shards, no dihydroxy-FA was identified by any protocol.

Operating in aerobic conditions, ß-oxidation could address the over-representation of $C_{16:0}$ over the other FA, indicating a stepwise degradation in living organisms (Aillaud, 2001). Similar FA profile with palmitic dominance were reported in glass bottle dated back to the 79 A.D. Vesuvius eruption. Stearic and oleic acids were identified in equivalent amount, quantitatively estimated to a quarter of the palmitic acid. Such results highlighted the degraded effects on olive oil lipids over the last 20 centuries (Sacchi *et al.*, 2020). Although degradation markers could provide valuable insights on the initial content following the biomolecular concept, it unfortunately remained complicated to trace back the content based on FA distribution. The chemical composition is evolving with molecules alteration. Many oxidative degradation pathways exist. Forecasting alteration would be insufficient to understand FA lifecycle (Heron and Evershed, 1993; Drieu, 2017). Moreover, the loss of material over time is inevitable, even without apparent leaching by groundwater percolation or microorganisms presence (Reber and Evershed, 2004; Evershed, 2008; Spiteri, Heron and Craig, 2011).

7.3.1. The amphorae ceramics: concealed indirect indications of the content

Since archaeological vessels need to be investigated as a whole, specific care was given to the petrographic nature of the ceramics themselves to gain indirect knowledge on the production sites, the typologies expected with the aim of suggesting a content. Based on typological considerations from the shape of the amphorae, shards were classed in different groups, distinguishing Early Africans from ovoid and Brundisium amphora (see Fig. 43). Global color and mineral incrustations are different from each other. Broadly speaking, the ovoid typology refers to amphorae exhibiting similar body shapes whose identification and origin are not straightforward. They have an oval body, overhung by beaded edges and round sections handles. Brundisium amphorae belong to the ovoid although the provenance is well established. According to Peacock and Williams, Brundisium amphorae usually contain quartz and limestone incrustations, and to a lesser extent fossiliferous particles, pyroxenes and mica (Peacock and Williams, 1986) and they have a beige to grey color. Intensively produced in the Brindisi region from the 1st century BC, they were meant for oil and were transported all over the Mediterranean Sea (Manacorda, 1981). Recent archaeological excavations of Giancola kilns gave valuable insights on the impressive organization regarding the production through the occupation of the workspace. Among the few production sites in Brindisi, Giancola was the more influent compared to la Rosa, Apani or even Masseria Marmorelle (Manacorda and Cambi, 1994). In Giancola, the work was collectively divided into pottery shaping and kilning. The wide expansion of Brundisium amphorae in the Roman Empire traduced the effectiveness of the oil production and stated the economic power of the production site. Under the supervision of *Visellius*, relative to *Ciceron*'s family, the intensive production at this time ensured by slaves, allowed farming and exportation monopoly in the region (Roselaar, 2012). Although Brindisi is famous for the important number of epigraphists reported on amphorae, no information could be drawn out since the only sample coming from Brindisi (i.e., 579) did not exhibit such written indication.

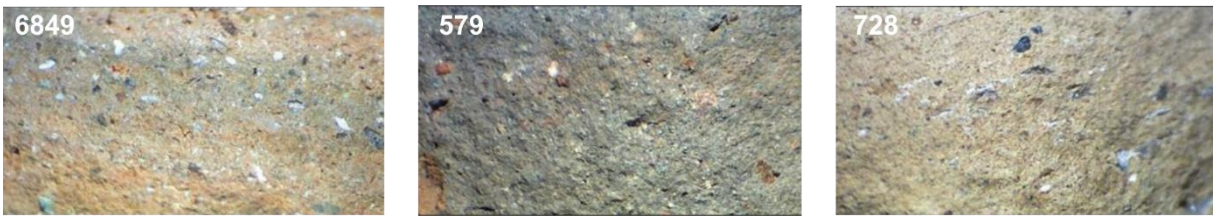

Figure 43. Shard sections from Early African (sample 6849), ovoid (sample 728) and Brundisium amphora (sample 579). (© M. Branger (Branger, 2012))

Early African amphorae exhibit a compact light red to beige ceramic, with fine particles of quartz. Petrographic comparison of the ceramics of Early Tripolitan and Tunisian amphorae supported the great similarities already observed through their oval shape typologies, chronological and geographical production dated back to 150 BC (Ben Jerbania, 2013; Contino and Capelli, 2013; Vargas *et al.*, 2019). Historically talking, they both correspond to the timescale of the economic boom of goods trading amphorae exportation had initially advanced. After the Macedonian and Celtiberian wars and the destruction of Corinth dated to the middle of the 2nd century BC, Roman economy, hereupon led by the elites, took the opportunity of growing through merchandising in the Adriatic Sea (Manacorda, 1986). Trades were enhanced by the emergence of the ovoid shape inspired from Corinthian models, hence displacing the economic hubs to the Adriatic. The commercial growth that went hand in hand with agrarian and amphorae production was greatly supported by the important colonial presence, notably Apulia and North Africa. Ovoid amphorae implemented the change from multi-located trading

all around the Mediterranean to a single center located in Rome (Vargas *et al.*, 2019). After clustering amphorae typologies together as Early African, Contino *et al.* concluded that part of the production was surely located in the Northern Africa, in Tunisia. They considered the site of El Mnihla, Carthage as the production site before the production expansion to the African coast (Contino and Capelli, 2013). Indeed, recent excavations of a ceramic dump at El Mnihla near Tunis shed light on the probable production site within the Carthage-Tunis-Utica triangle (Ben Jerbania, 2013). Little is nonetheless known from archaeometric analysis, epigraphy information and kiln production site (Bonifay, 2007) and too much interest was devoted to olive oil trade (Bonifay, 2004). Highlighted by the discovery of olive fruits from the Plemmirio B wreck in Sicily, Bonifay *et al.* argued the possibility for African amphorae to have contained fruits instead of oil (Bonifay *et al.*, 2015). Indeed, Pliny's testimony[9] targeted the higher quality of Italian olives to produce oil compared to overseas fruits, that are preferred for the table as staple food. Facing the absence of olive oil profile in Ostia LIX, Djaoui *et al.* hypothesized the olive preservation in brine. Indeed, olive as a fruit would not leak on the internal surface and not leave chemical traces since FA markers are contained in the flesh (Djaoui, Garnier and Dodinet, 2015). They assumed the amphorae provenance from Thabraca, Tunisia, before the redistribution in Rome and the recycling in Egypt with Moringa oil to explain the presence of Moringa markers besides the absence of FA markers and the natural distribution of *Moringa* sp. out of Africa. From the best of our knowledge, little was reported on olive trades except the recent publications aforementioned. Regarding the Early African amphorae investigated (6781a; 6823; 1005 and 6849), the hypothesis of a non-edible use remained nonetheless relevant. Indeed, since the oil produced in Tunisia seemed to be of poor quality as pointed out by Pliny, it is not unlikely that it was used in other ways, such as consumable for oil lamps. Several historic writers like Pliny[10], Theophrastus[11] or Strabo[12] reported the contribution of olive oil for supplying lamp lighting combustion (Copley *et al.*, 2005). Considering the archaeological site of Pompeii, Griffiths provided a first estimation of the oil fuel quantities necessary to supply artificial lights in domestic and commercial activities (Griffiths, 2019). No less than 20 586 L of olive oil were necessary to ensure the lighting of the 1884 lamps. The discovery of two ceramic lamp workshops within the city (Cerulli Irelli, 1977; Peña and McCallum, 2009) additionally attested of the predominance of nocturnal activities, hitherto underestimated. Scaled-up to the entireness of the Roman Empire, the impressive amount of fuel raises interrogations on the supplying possibilities. Olive oil was consequently produced, with production area expanded all over the Mediterranean basin. Olive farming became a major contribution of the Roman economy (Bowman and Wilson, 2013; Marzano, 2013). Olive was hence integrated in daily life from the production to the consumption (Mattingly, 1988a, 1988b).

Another underestimated aspect of oil consumption in the Roman Empire regards the body-caring and cosmetic. Olive oil was indeed a 'star' ingredient in the preparation of hygiene, medicaments and beauty products (Griffiths, 2019). Romans valuated olive oil for massages and skin care, referred as 'Beauty baths' (Gorini *et al.*, 2018). Cosmetics were prepared on the base of olive oil, sometimes perfumed for make-up usage (Pérez-Arantegui *et al.*, 2009) or using with white lead pigments to cure hair loss (Armocida, 1991; Rudock, 2019). Olive oil was considered as a 'high moisturizing medicine' for skin treatment (Sarkar *et al.*, 2017).

Porosity investigations gave reliable insights on the ceramic absorption towards lipids. Experiments conducted on ceramic replica demonstrated the pore-size effect on the preservation of lipids and the

[9] Pliny the Elder, Naturalis Historia, XV, 16 "Quam ob causam Italicis transmarinae praeferuntur in cibis, cum oleo uincantur"

[10] Pliny the Elder, *Naturalis Historia*, XV, IV, 5

[11] Enquiry into Plants IV: 2, 9

[12] Geography VII, 1, 35

impact of the operational-chain on the lipid distribution (Rosiak, Kałużna-Czaplińska and Gątarek, 2020). Ceramic pores act as trapping holes for molecules. Lipid absorption ensures their survival over archaeological time, as well as it diminishes the probability of leaching and degrading because of microorganisms and/or oxidation. Heating, even at low temperatures increased the porosity by altering the stone microstructure. Lipids are consequently better absorbed. So is the chance of profiling lipids to trace back the original content with thermally induced porosity (Charters *et al.*, 1993). Moreover, lipid accumulation differs within the vessel itself, conducting higher absorption in the upper body and the rims rather than in the base (Charters *et al.*, 1993; Evershed, 2008). Finally, the lipid concentration also differs from a ceramic to another, basically because of the porosity absorption, but also because of the dependence of lipid preservation towards the porosity (Namdar, Stacey and Simpson, 2009; Drieu *et al.*, 2019). Micropores strengthen lipid concentration because lipids are physically preserved from biological and chemical attacks such as hydrolysis, oxidative reaction or radical oxidation (Dudd, Regert and Evershed, 1998; Evershed, 2008). Although lipid residue analysis is widely reported in ceramics (Heron, Evershed and Goad, 1991; Dudd, Regert and Evershed, 1998; Namdar, Stacey and Simpson, 2009; Allegretta *et al.*, 2017; Drieu *et al.*, 2019), and even though mineralogy and petrography allowed the ceramic to be geographically located (Medeghini *et al.*, 2016; Medeghini and Nigro, 2017; Botticelli *et al.*, 2020), little has been studied on the dependency of lipid absorption towards the different kinds of ceramics, starting from petrography analyses.

7.3.2. Archaeological interpretations from enigmatic chemical markers

Another hypothesis arised from the re-use of amphorae. As raised by Pecci, this aspect is effectively rarely considered in the ceramic life-cycle (Pecci *et al.*, 2017). Little are the evidence of reusing containers. Peña assumed the recycling as storage containers, to pack food or non-food substances or even for water storing (Peña, 2007). In the Northern necropolis of Potentia (Italy), different purposes were mentioned for amphorae second lives, such as burial goods, funerary libations or even grave markers (Monsieur, 2007). Cases of Dressel 2-4 reuses were also evidenced at Villa B Oplontis in Southern Italy since residue analysis of the empty amphorae unraveled wine markers. Containers were recovered in upside down positions, ready to be filled again before the Vesuvius eruption occurred (Pecci *et al.*, 2017). Secondly, olive oil lipid profile was identified together with grape beverage markers, hence revealing the second use of wine amphorae for plant oil (Pecci, Salvini and Cantini, 2010). It would be unlikely to fill oil container with wine due to the bad taste oil would confer to the ceramic. In the same way the ceramic absorbs wine, the wine would absorb oil residues trapped in the ceramic (Pecci, 2009; Romanus *et al.*, 2009). Organic residue analysis, although being a powerful reliable tool, seems to show its limits since markers are identified all together and cannot be chronologically distinguished from each other. Tracing the original content can present some issues if the container was reused or the content mixed with some other goods. Re-use occurrence is consequently difficult to address. One striking example, mentioned earlier, needs however to be noticed since they interpreted the absence of chemical markers in Ostia LIX as a matter of fact (Djaoui, Garnier and Dodinet, 2015). Surprisingly, wine markers were characterized, together with FA, diacids, cholesterol, vegetal sterols and long-chain alcohols. Since the transport of olive fruits in brine would not leave chemical markers on the ceramic, the extractive outcomes were conjectured as a sign of the second life of the amphorae. Containers were reused after olive fruits were traded from North Africa with red wine, dairy products, plant oil and/or *Moringa* sp. oil. Although spatio-temporality may face some uncertainties because residue analysis did not provide straightforward outcomes easing the

interpretation, they attempted to take advantage of the ambivalence, filling the knowledge gap and providing innovative suggestions to understand trade at a global level.

7.4. Conclusion

In conclusion, little was described in literature about the oxidized profile lipid residue analysis may suffer while analyzing oil amphorae. The situation we are facing in Planier 3 is all the more interesting since it opens a field of interpretation. First of all, no protocols used in this study seemed to give valuable outcomes. Even though solvent extraction, butylation and saponification have different reaction mechanisms and chemical approaches for lipid extraction, none of them provided relevant insight to straightforwardly understand the object history. A universal protocol does not exist. Results showed an important heterogeneity regarding the molecules extracted. Palmitic acid usually dominates the lipids distribution, followed by stearic acid. The frequent absence of oleic acid, together with the presence of degradation markers of azelaic acid, indicated oxidative reactions. From the degraded pattern, different hypotheses can be raised. The fatty content could be meant for body caring or lamp illuminant, instead of edible purpose. The early African amphorae might have contained olive fruits, since North Africa's olives were preferred to be consumed at table compared to Italian fruits that gave tastier oil. Finally, the hypothesis of reusing amphorae cannot be discarded although organic analysis of the residues did not provide any further indication on the initial content.

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Author contributions

Conceptualization, L.C., F.O., C.M.; **methodology**, L.C., C.M.; **formal analysis**, L.C.; **investigation**, L.C., C.M.; **resources**, F.O.; **visualization**, L.C.; **supervision**, C.M.; **project administration**, C.M.; **funding acquisition**, C.V., C.M.**; writing—original draft preparation**, L.C.

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Chapter 8

General discussion, conclusion and perspectives

8.1. Calling for thesis objectives

As a short reminder, the objective of this PhD thesis regarded the development of innovative analytical tools to address the archaeological content of amphorae. The collaboration with archaeologists, scientists and cultural offices aimed at providing a common language to better understand the object history with the specific purpose of including the assessments into broader anthropological, ethnocultural and societal considerations. To that end, we needed to overcome the analytical barrier inherent in the objects: the artefacts having been immersed underwater or underground for thousands of years, the search for traces, both macroscopic and microscopic, proved difficult.

The main part of the thesis focused on grape-derivative containers. Grape-beverages were indeed ubiquitous during the Roman Empire, in terms of production and consumption. Wine trading was so important that Panella and Tchernia (Panella and Tchernia, 1994) estimated that half of the traded amphorae would have contained wine in 50 BC, considering Italian and Roman provinces in the selected context of Rome and Ostia. This proportion remarkably increased to 65% before the generalized economic decline Roman trading suffered around 200 AC (Bowman and Wilson, 2009). Produced in such extent, wine naturally became one of the pillars of the imperial economy and of the Roman culture. Roman writers such as Columella, Virgil, Cicero, Cato the Elder or Horace abundantly referred to grape in all its forms. Grape agriculture was notably detailed by Pliny the Elder who, among others, reported the common grapevine cultivars[13] as well as the different types of presses used for winemaking[14] (Rossiter, 2008). Wall paintings and decorative features attested of the cultural daily custom extensiveness and its related socioeconomic impacts on the society (Fleming, 1997; Feige, 2021). Its use is also greatly detailed, from grape fruits consumption to numerous wine Mediterranean recipes, including resinated, cooked and medicinal wine beverages (Hostetter, Beck and Stewart, 1994; Garnier *et al.*, 2003; Feier *et al.*, 2019). Abundant is the archaeological evidence of ceramics having contained grape derivatives considering uses and transport (Pecci, Cau Ontiveros and Garnier, 2013; Pecci *et al.*, 2018). For instance, fermented grape-beverages have been reported in several Roman Dressel amphorae ranging from the 1st century BC to the 4th century AD (Condamin and Formenti, 1978; Pecci, Salvini and Cantini, 2010; Arobba *et al.*, 2014; Pecci *et al.*, 2017). Archaeological wine over-representation nicely turned the research field of grape derivatives into a promising anthropological approach to better understand past civilizations cultures and ancient Mediterranean trade routes.

Another field of investigation regarded oil amphorae. Oil was indeed one of the 'star' ingredients in the Roman Empire (Finley, 1973; Hitchner, Amouretti and Brun, 2002). Among the many usages, oil was widely mentioned for cooking, as lamp fuel for lighting, for lubrication, for perfuming or skin-caring, for ritual or religious practices (Mattingly, 1988; Edmondson, 2015; Ayllon-Martin, Perez Gonzalez and Remesal Rodriguez, 2019). Historical writers abundantly reported its contribution in daily life practices, stating on its physical and culinary benefits, as for example in the renowned Apicius' recipes book of *De Re Coquinaria*. Forbes and Foxhall (Foxhall and Forbes, 1982) estimated the Roman yearly olive oil consumption around 50 L per person. Rough estimation of the consumption as light fuel to supply domestic and nocturnal commercial activities of Pompeii exceeded 20000 L per year, which corresponds to 275 Dressel 20 containers (Griffiths, 2019). To supply such important demand, the production of olive oil was largely scaled in Southern Spain, Tunisia and Lybia (Mattingly, 1988).

[13] *Naturalis Historia* III ; XIV
[14] *Naturalis Historia* XVIII

8.2. Methodology development and protocol optimization

The diagnosis approach we established was twofold. We first concentrated on the development of chemical protocols able to extract meaningful molecules from shard and waterproofing matrices. We then focused on the archaeobotanical content of amphorae to support and flourish the initial assumptions raised by molecular biomarkers.

8.2.1. Organic residue analysis: the improvements

Regarding the preponderance of wine amphorae in the sample corpus (i.e., 16 amphorae over the 27 artefacts), the extraction optimization was axed on fermented grape-beverages and wine markers. Several protocols were tested on archaeological shards and organic linings with the aim of comparing their relative extractive capacities. Protocols were chosen regarding their ubiquity in literature. They exhibited different chemical mechanisms to access the markers bound to the original matrix. The organic extraction with traditional solvents does not aim at reacting with the sample but rather extracts molecules present therein. Ultrasound generates highly energetic waves that travel within the extractive medium. The alternation of high- and low-pressure cycles creates acoustic cavitation, which enhance the extraction of compounds when the cavitation bubbles implode at the solid surface. The turbulences generated by ultrasound improve mass transfer from the matrix to the medium. Secondly, alkaline treatment promotes the release of tartaric acid from the corresponding salts. The mechanism involves alkaline ions to shift the solubility equilibrium of tartrate salts, which favors the chemical cleavage with the residual matrix (Pecci *et al.*, 2013). Thirdly, butylation eases the extraction of molecular compounds in the organic phase by esterifying the acid function. The protocol accounts for the presence of a harsh Lewis acid that partially breaks down the matrix to rend more accessible the compounds bonded to it (Garnier and Valamoti, 2016).In a first step, the analysis of grape markers focused on the microresidues impregnated in shards, as suggested by the esterification publication we got inspired by. As neither Garnier and Valamoti, nor the next publications detailed the heating method they used (Garnier and Valamoti, 2016; Frère and Garnier, 2017; Drieu *et al.*, 2020, 2021), we had to develop the esterification protocol by ourselves. The parent publication indeed mentions 2 hours of heating at 80°C with butanol, BF_3 and cyclohexane. The latter solvent having a boiling point at this same temperature, we proposed microwave (MW) to ensure the butylation rather than a reflux set-up or an oil bath. The choice was highly motivated by the cost-effectiveness of the technique, the technology being common in organic chemistry to extensively reduce reaction times and energy costs (Kappe, Dallinger and Murphree, 2008). To not compromise precious archaeological material, we exploited commercial molecular standards of grape acids to set-up the MW parameters necessary to complete the esterification. We used tartaric acid as the major asset to decide the operating parameters and then extended the protocol to the other standards. The esterification advancement was followed by thin layer chromatography and dibutyl tartrate was further controlled with FT-IR, NMR (^{1}H, ^{13}C, ^{19}F) and GC-MS injection. Based on the dibutyl tartrate we characterized, we were able to conclude on the MW parameters in addition to obtaining chromatographic and molecular information of the compounds to be searched for in further archaeological analyses. Grape compounds were identified as butylated and derivatized with the GC-MS (Fig. 44). Retention times and mass fragments of derivatized and butylated-derivatized acids are shown in Table 11. Mass spectra are further detailed in the Appendix 3.

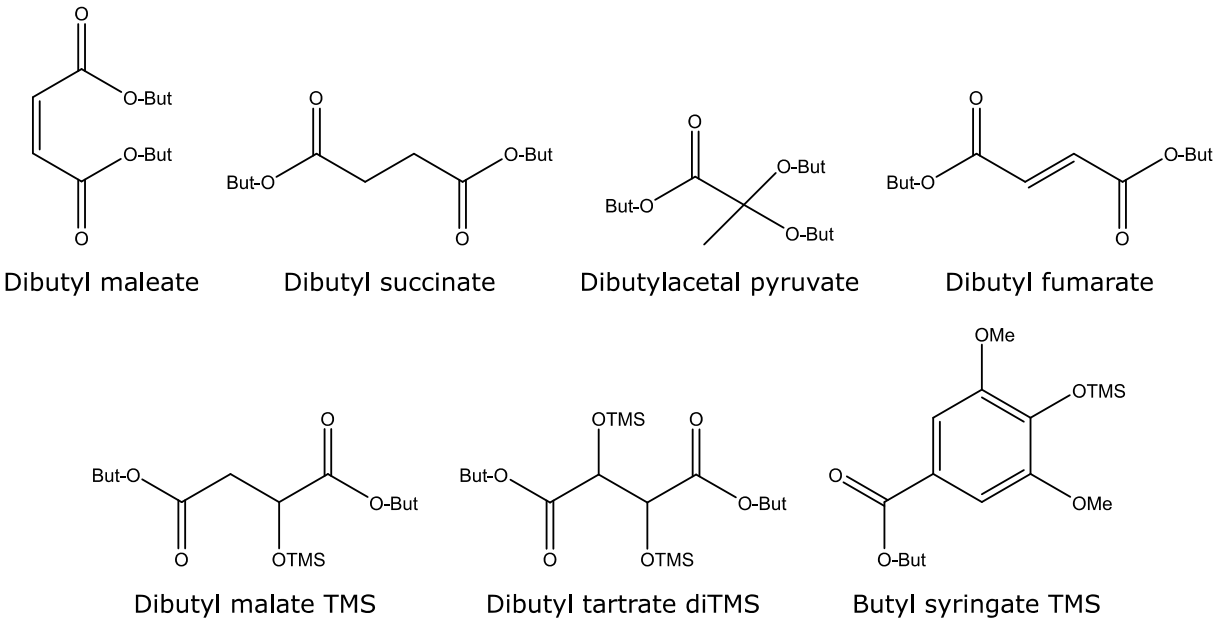

Figure 44. Molecular structures of grape-fermented acids after butylation and derivatization

Reaction time was therefore decreased from hours of heating to 3 dynamic cycles of 5 min each. We alternatively verified the molecules did not degrade depending on the heating source by performing the same reaction, with the different heating set-up. The identical chromatograms we obtained from the standards of tartaric acid and colophony resin after butylation validated the use of MW.

TMS			**Butyl ester TMS**		
	Retention time t_r (min)	Mass fragments		Retention time t_r (min)	Mass fragments
Maleic acid diTMS	12.96	**147**; 215; 245	**Dibutyl maleate**	18.47	41; **99**; 117; 229
Succinic acid diTMS	13.16	44; 73; **147**; 262	**Dibutyl succinate**	19.51	55; **101**; 157; 231
Pyruvic acid TMS	13.57	45; 66; **147**; 217	**Dibutylacetal pyruvate**	20.02	43; **61**; 117; 145; 173; 201; 274
Fumaric acid diTMS	13.82	41; **147**; 261	**Dibutyl fumarate**	20.09	41; 53; 55; 99; **117**; 155; 229
Malic acid triTMS	17.10	**73;** 147; 149 ; 335; 351	**Dibutyl malate TMS**	24.89	**73**; 101; 117; 145; 161; 217; 219; 319
Tartaric acid tetraTMS	21.78	**73**; 147; 189; 292; 305; 423	**Dibutyl tartrate diTMS**	29.96	**73**; 133; 147; 189; 217; 233; 276; 407
Syringic acid diTMS	30.92	253; **297**; 312; 327; 342	**Butyl syringate TMS**	36.50	**240**; 296; 311; 326

Table 11. Grape-fermented acids retention time and mass fragments after derivatization and butylation followed by derivatization. Only the main mass fragments of 15% intensity compared to the base peak (bold) are listed (except for malic and tartaric acids and their butylated derivatives for which fragments of 30% intensity compared to the base peak are listed). The last mass corresponds to the peak molecular weight.

Once the parameters were optimized for the commercial standards, the butylation was smoothly run on the archaeological shards of Planier 3 with the aim of characterizing grape markers impregnated in the ceramic. Since the esterification proved to be efficient, the research question then concentrated on the ability of butylating the grape acids when it came to deal with organic coatings. The same MW protocol was therefore tested on an archaeological pitch sample that had already been considered for a protocol development in a previous PhD thesis (Fujii, 2018). The Dressel 2-4 amphora labelled C98, originating from Pompeii, already gave excellent results in terms of grape markers. The choice to use this object as a vector for the coating adaptation was additionally justified by the important material quantity, sufficient to supply the protocol adaptation and any further adjustment if needed. Grape derivative markers of tartaric, syringic, malic and glutaric acids, initially identified by alkaline treatment in the previous study, were also recovered after butylation. The esterification led to the identification of pyruvic acid, hitherto unnoticed. The fruitful characterization of fermented grape derivatives highlighted the robustness of the butylation to extract bound acids from organic coatings, especially the very meaning tartaric acid.

After the protocol parameters were developed on the archaeological coating of the sample C98, they were tested on the lining of a Dressel 1 originating from Planier 3 (i.e., sample no. 1014), and then applied on the other artefacts of the cargo. The amphora 1014 indeed exhibited enough material to initialize the coating-butylation attempts among the corpus samples investigated in the theses. Results proved to be successful as well, as identified by the non-butylated and butylated compounds in Fig. 45.

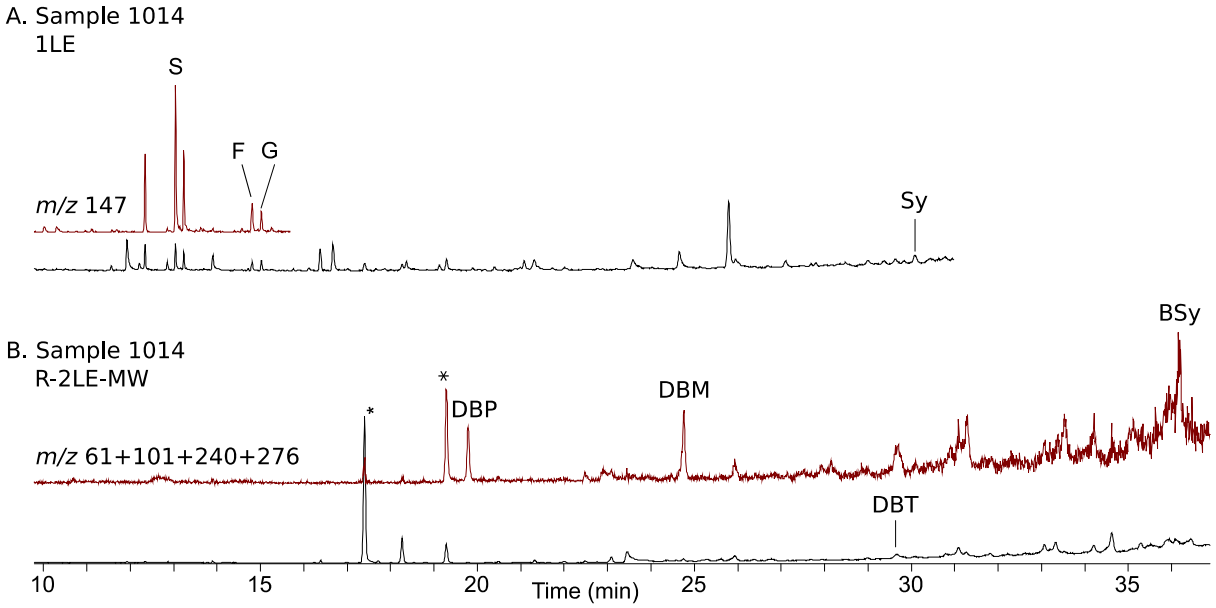

Figure 45. TIC (black) and m/z (red) chromatograms of the sample 1014 after (A) the extraction with traditional solvent (labelled 1LE) and (B) the butylation directly applied on the coating.S: Succinic acid ; F: Fumaric acid ; G: Glutaric acid ; Sy: Syringic acid ; DBP: Dibutylcetal pyruvate ; DBM: Dibutyl malate ; DBT: Dibutyl tartrate ; BSy: Butyl synringate.

At this point of the protocol development, one indication needs to be detailed. Besides the great characterization of fermentation and grape markers, the ability of the butylation protocol to completely characterize the object, from the content to the coating nature, was questioned. The colophony standard resin we initially butylated gave us the insurance that esterification was unable to surely support the identification of diterpenic compounds, independently from the heating source. The

reaction conditions, as much with MW than reflux heating or oil bath, indicated diterpenoid side-reactions, thus avoiding the molecular characterization of diterpenic markers. Figure 46 presents the chromatograms obtained from reflux and MW, demonstrating the production of identical compounds, hardly identifiable though. Further on, the same protocol was distinctively applied on molecular standards of pimaric, sandaracopimaric, isopimaric and 7-oxo-DHA acids. Results unequivocally showed a bench of molecular compounds exhibiting diterpenic skeletons, confirming the unreliability to provide insights on the chemical composition of the waterproofing material with esterification. Indeed, the diterpenic markers are the molecular trackers allowing to distinguish *resin*, *pitch* and *wood tar* by the presence of heating and oxidized markers and wood pyrolysis with the presence of methyl ester derivatives, respectively.

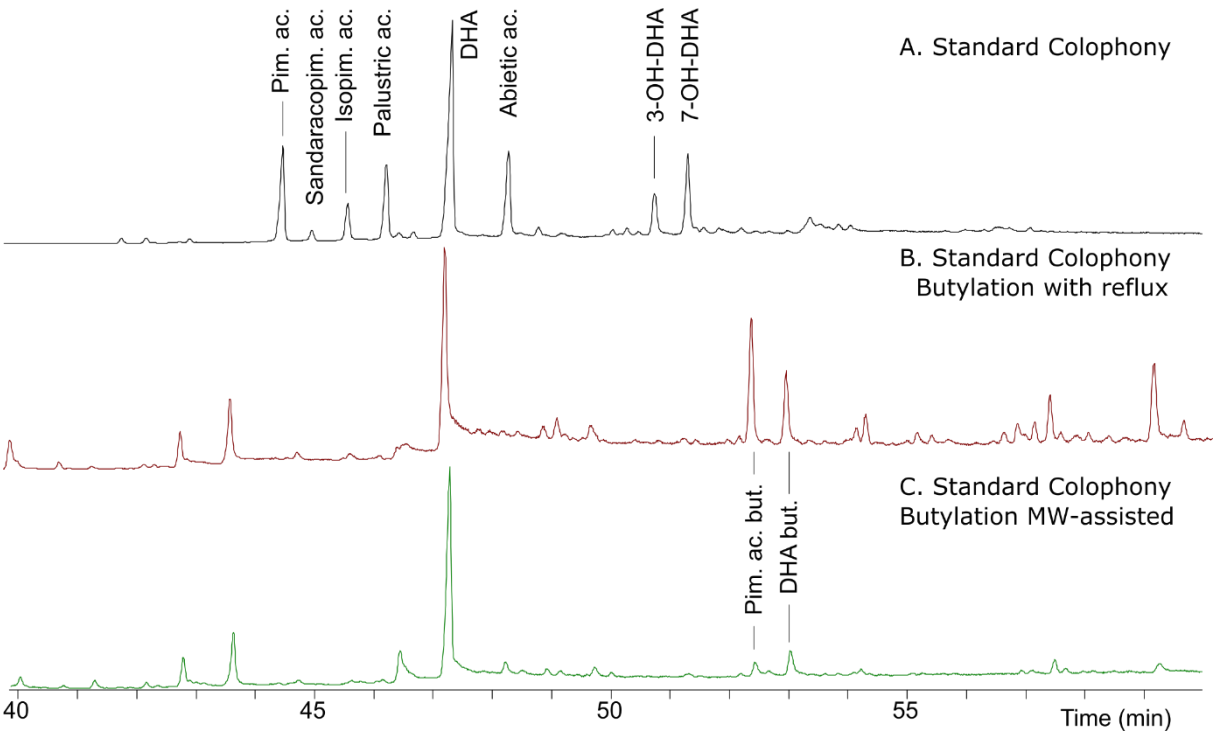

*Figure 46. Chromatograms of standard colophony with different chemical treatments: A. No butylation; B. Butylation completed with reflux (2h; 80°C); C. Butylation MW-assisted (3*5 min; 80°C). Pim.: Pimaric; Sandarcopim.: Sandaracopimaric; Isopim.: Isopimaric; ac.: acid; But.: Butylated*

To reach a complete characterization of the archaeological material (content and lining nature), the optimized MW-butylation was strengthen up with other extractive pathways. Even though we raised the number of characterized compounds to a total of 97 molecules, the decision was taken to concentrate on highly valued molecules while elaborating the extractive protocols to apply since not all of them help in diagnosing the original content and coating. The main molecules are listed with relative retention times and MS fragments in the appendix 4). For example, fatty acids and diacids, small organic acids recurrent in living organisms (e.g., benzoic acid and its derivatives, vanillic acid, isovanillic acid, *etc*.) or tricyclic derivatives from diterpene structures (e.g., norabietatriene derivatives, 5β–podocarpatrienoic acid derivatives, *etc*.) were not considered. 22 molecules were therefore targeted based on their ability to trace archaeological grape derivatives content, fermentation and to give insight on the nature of the waterproofing coating and the treatment it went through for formulation. The molecules considered were detailed in the fourth chapter, together with the development of the extractive protocols considered. Several attempts performed on archaeological materials of Planier 3 and San Felice Circeo (i.e., 10 shards and 8 coatings) shed light on the most

fruitful couplings to apply. For the analysis of shards, a two-step protocol consisting of a lipid extraction with traditional solvents followed by MW-butylation reached better outcomes. For the analysis of organic resins, simultaneous alkaline treatment and acid-catalyzed butylation directly applied on the coating material are favored (Fig. 47).

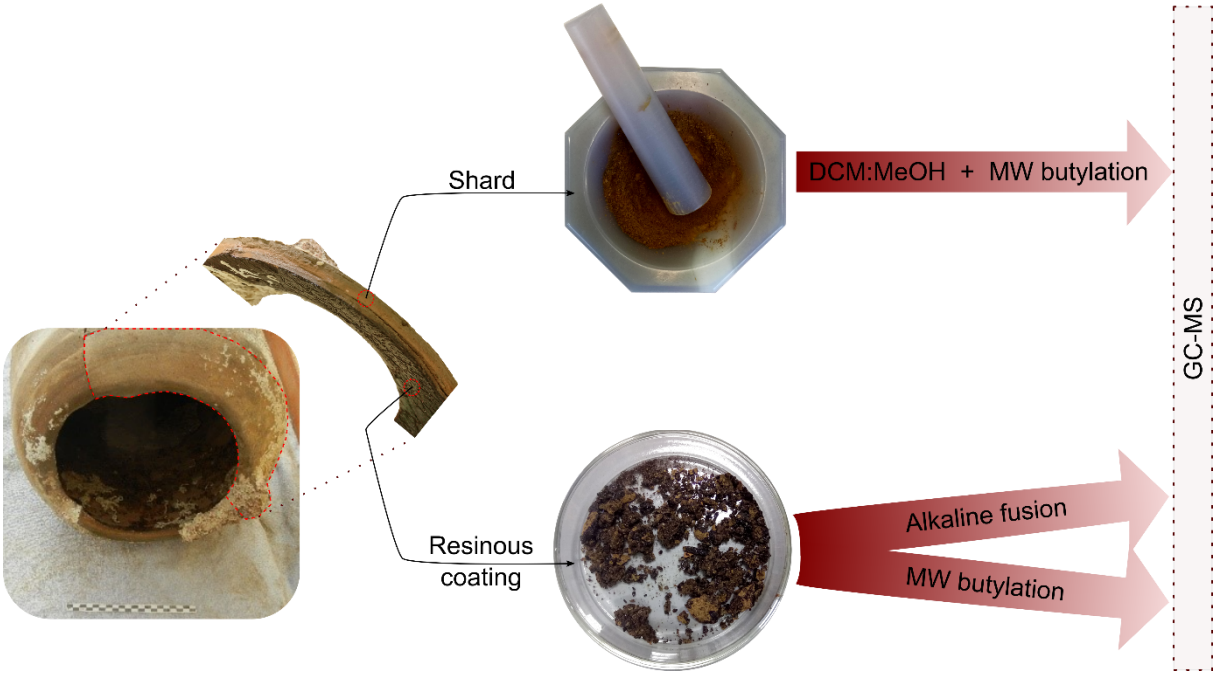

Figure 47. Protocols to apply for the extraction of grape and fermentation markers

8.2.2. Archaeobotanical analysis

The archaeobotanical analyses focused on plant remains contained in the amphorae. In this regard, an analytical protocol aiming at dissolving the pitch and collecting the botanical residues it contained was initially developed. The treatment combined the dissolution of the pitch with organic solvents and traditional palynological methods to physically and chemically breakdown the matrix, remove undesired compounds and concentrate archaeobotanical features. The methodology was first applied to 7 archaeological coatings and then to a teapot shard which had a particular excavation context assuring of a low risk of contamination from post deposition.

In conclusion, the methodological novelty arises from the reinforcement of analytical protocols able to extract highly significant molecules, considered as markers of the original content and the application of such protocols on organic coatings and inorganic shards of amphorae, until now never considered with a multidisciplinary perspective. The interference of archaeological considerations, chemical and archaeobotanical evidence was developed to encourage further investigation.

8.3. Archaeological perspective: From the object to the interpretation

As reported at length through the analyses of several archaeological items from different contexts, organic residues and archaeobotany demonstrated their collaborative richness in terms of scientific support. To the best of our knowledge, such fruitful collaboration has been too occasionally tested regarding the number of organic residue analysis applied on vessels and pottery. Sparse is the evidence of multi-analytical approaches although botanical investigation empowered the archaeological interpretation, both to avoid misinterpretation due to contamination and to attain a global understanding of the material studied. In this section, we will revert to the artefacts investigated, paying particular attention to the benefits provided by the application of a multidisciplinary methodology.

For the amphorae labelled SFC1, SFC2 and SFC5 coming from the ancient anchorage of San Felice Circeo (discussed in the fifth chapter), organic residues revealed fermented grape derivatives and diterpenic markers associated to the presence of Pinaceae wood tar. *Pinus* fossil pollen present in considerable extent, together with wood fragments, substantiated the assumption of resinous tree pyrolysis in addition of providing more accurate insights on the botanical nature of the coating allowing the identification up to the subsection including *P. mugo*, *P. nigra* and *P. sylvestris*. The geographical distribution of such species, virtually absent in Central Italy, provided great evidence of the non-local origin of the *Pinus* used to prepare the pitch, hence shedding light on exportations and Roman trading. Palynological observation of the coatings showed aporate tricolpate pollen grains, identified as *Vitis vinifera*. Grapevine stamens were also identified, hence confirming the archaeological grape derivatives content. The morphological singularity of the *Vitis* pollen was interpreted as a possible trace of autochthonous vineyards used to prepare grape beverages. Aporate *Vitis* pollen additionally interrogate the state of domestication of the grapevines, giving insights on the possibility to use pollen grains as an indicative tool to understand grape domestication. Alternatively, the speculation of a medicinal infusion prepared with wild *Vitis* flowers and referred in historical testimonies as *oenanthium* could not be discarded. Although this hypothesis is unlikely since the three amphorae had different geographical origin, it would corroborate the herbal pollen of *Artemisia* and *Myrtus* recovered in the amphorae. Such interpretation could not have been raised without the support of transdisciplinary investigations, with archaeological considerations bridging the gap between molecular markers that certified the presence of fermented grape derivatives and archaeobotanical observations that attested of peculiar *Vitis* morphology.

For the spouted vase of Jericho discussed in the sixth chapter, diterpenic markers unraveled the presence of Pinaceae in the teapot, unnoticeable with palynological analysis. Since *Pinus* pollen are easily dispersed, the absence of such pollen in the teapot was hence interpreted in the light of natural resin, confirmed by the absence of oxidized diterpenoids and heating-preparation markers. Regarding the pollen content, *Malva parviflora* grains were abundantly recovered in the vessel, suggesting a medicinal beverage. Such interpretation was supported by the presence of *Artemisia* grains and lumps of pollen that could originate from flower anthers or honey. Both species additionally exhibit healing properties. Even though molecular markers of cedar were not identified by gas chromatography, *Cedrus* pollen identified in the vessel enhances the symbolic character, already conferred to the vase from typological considerations. The morphological resemblance with teapots recovered from funeral contexts indeed outlines the cultural functionality the vessel could have afforded. Giving new insights on ancient medicinal recipes consumed at Bronze Age times, pollen and molecular compounds

interrogate ethnopharmacological practices and broaden the anthropological understanding of the Jericho civilizations.

Despite the analysis of the resinous coatings of the samples nos. 1014, 749, SFC3 and SFC4 during the thesis, results were not presented earlier in the manuscript since the interpretation in the light of transdisciplinarity remains mitigated. Pollen analysis clearly identified *Pinus* although the extent diverged among the samples (Table. 11). Although an important heterogeneity in terms of *Pinus* dimensions avoided the classification into Mediterranean and highland pines, *Pinus* pollen accounted for a third of the overall 24 grains identified in the sample SFC3 (pollen content: 104 pollen/g). SFC4 only exhibited a single *Pinus* pollen (pollen concentration: 46 grains/g), statistically insufficient to trace any geographical origin. On the contrary, high percentages were recovered in the amphorae from Planier 3 even though the samples 1014 and 749 showed a limited pollen content. 1014 only contained *Pinus*, with 8 grains identified in total (grain concentration: 293 pollen/g). 32 grains were observed in 749 (257 pollen/g) with *Pinus* representing 85% of the count. Supported by the presence of methyl ester diterpenoids identified by GC-MS, the assumption of Pinaceae wood tar was hereby verified in all the 4 samples. *Pinus* grains identified in 1014 and 749 were importantly damaged, which did not allow a more precise identification (Fig. 48A-B-E). No further indication of the botanical origin of the pith could be obtained.

	Aceraceae	*Asteroideae*	*Betulaceae*	*Brassicaceae*	*Euphorbiaceae*	*Fagaceae*	*Pinaceae*	*Vitaceae*	Total pollen grains	Pollen concentration (pollen/g)
SFC3	*Acer* 4%			Brassicaceae 4%		*Quercus* 50%	*Pinus* 29% *Cedrus* 13%		24	104
SFC4		*Artemisia* 25%			*Mercurialis* 13%	*Quercus* 38%	*Pinus* 13%	*Vitis* 13%	8	46
1014							*Pinus* 100%		8	293
749			*Alnus* 3%			*Quercus* 3%	*Pinus* 85% *Cedrus* 9%		27	257

Table 12. Pollen grains recovered from the analyses of amphorae SFC3, SFC4, 1014 and 749

In addressing the content, pollen analysis did not help in understanding the original food product. Pollen identified was attributed to the environmental landscape, notably the great representation of *Quercus*, identified in samples SFC3 (50%) and SFC4 (38%) while grape fermented markers were characterized by chromatography. Although not sufficient to solely address the archaeological content by itself, one tricolporate grain of *Vitis vinifera* was nevertheless identified in the amphora SFC4 (Fig.

48C-D). Supported by the presence of pyruvic, malic and tartaric acids, and considering the moderate dispersion of *Vitis* pollen (Turner and Brown, 2004), the grain can be interpreted as a supplementary indication of an archaeological grape derivative content.

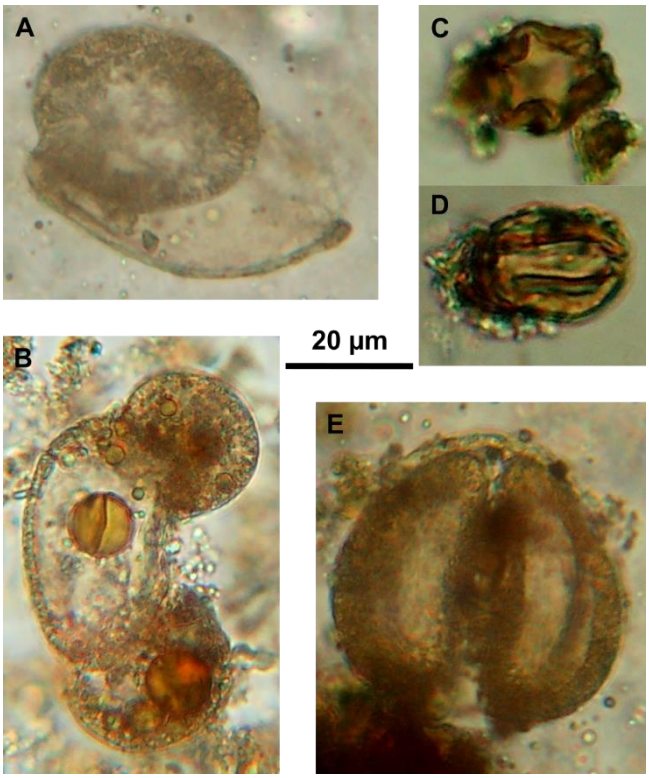

Figure 48. Pollen recovered from the archaeological wood tar. A. Pinus sacchus from the sample 749; B. Pinus from the sample 1014; C-D. Tricolporate Vitis vinifera from the sample SFC4; E. Pinus from the sample 749

The sample SFC3 needs to be further detailed. The Mañà C2 typology addressed by the archaeologist belong to the North African Punic style (van der Werff, 1986; Martin-Kilcher, 1999). Such amphora certainly came from the region of Carthage / Tunis, as suggested by a recent excavation that uncovered a dump of ceramics and coarseware shards with a relevant representation of Mañà C type amphorae (Ben Jerbania, 2013). The important extent in which amphorae were discovered (i.e., up to 80% over the 150 shards identified) was interpreted in the light of a manufacture center or a local production site. During the 2nd century BC to the first half of the 1st century AC, they were fairly distributed in the Western Mediterranean basin, from Portugal to the coastal towns of Tunisian Sahel (de Almeida and Arruda, 2005; Dias, 2010; de Sousa and Arruda, 2013). Mañà C2 were reported for the trading of fish preserves from the Bay of Cádiz to the Empire (Barrios, 1996; Arruda, Viegas and Bargão, 2005). Supported by fish and fish by-products installations discovered in the Mauretania Tingitana archaeological sites of Cotta and Lixus, it seems reliable to integrate the preparation of North African *salsamenta* and *garum* into the Baetic economic activity (Ponsich and Tarradell, 1965; Ponsich, 1988). As firstly pointed by Ponsich (Ponsich, 1975), the production of North-African amphorae was probably disconnected from the production sites of salted fish sauces: fishing activities and fish-derivatives preparation would have come from Tingitana while the amphorae were manufactured on the European side of the sea, in Gades (Iberian Peninsula), which corresponds to the transition from the Baetican amphora Dressel 7-11 to autochthonous forms such as Mañà C24 (Barrios, 1996). The empty

amphorae leaving Gades were sent to Tingitana to be filled with fish sauces, then returned to Gades before being exported throughout the Mediterranean as a product of Baetican origin (Ponsich, 1975; Étienne and Mayet, 1998). This theory of usurpation finds its roots in the absence of kilns in Mauretania Tingitana (Teichner and Pujol, 2008) despite the presence of *tituli picti* (e.g., CO(r)D(ula) LIX(itana) VE(tus) or CO(r)D(ula) TING(itana) VET(us)) attesting of the African origin of the containers (Liou, 1987; Martín-kilcher and Thierrin-Michael, 1994; Martinez Maganto, 2000).

Oil has alternatively been mentioned in Mañà C containers (Peña, 2018), but no chemical profile looking like olive oil or other plant oil was identified from chromatographic analysis. Palmitic, oleic, stearic and azelaic acids were recovered, with important quantities of unsaturated fatty acids that do not fit fresh olive oil profile. Also, in the hypothesis of oil, the coating would have been dissolved by the fatty content, altering the original taste (Bonifay, 2007). Even though one waterproofed Dressel 20 oil amphora was reported with macroremains of the black lining (Manhita *et al.*, 2020), oil supposition was discarded based on chemical markers. The absence of *Olea europea* pollen further discredits the assumption of oil.

From the organic residue analysis, no fish fats could be identified although the $C_{18:1}$ characterized could account for elaidic acid (i.e., the oleic acid trans-isomer $C_{18:1}$ trans-9) or vaccenic acid (i.e., $C_{18:1}$ trans-11), both being naturally present in fish and animal milk fed with fish oil (AbuGhazaleh *et al.*, 2003; Islam *et al.*, 2018; Dutta and Dutta, 2019). Further investigation is be needed to surely address the isomerism, notably with MS^2 approach since a second fragmentation would distinguish patterns from isomers or with Matrix Assisted Laser Desorption Ionization-Time of Flight/MS (MALDI-TOF/MS) equipment (Gómez-Cortés *et al.*, 2009; Peršurić *et al.*, 2018). Chemical reactions can be an alternative to identify the double bond position in lipid isomers, as stated in recent publications calling for meta-chloroperoxybenzoic acid epoxidation coupled with collision-induced dissociation MS-MS devices (Feng *et al.*, 2019) or ozonolysis with electrospray ionization MS (Thomas *et al.*, 2007).

However, chromatography revealed the presence of grape and fermentation markers (Fig. 49), giving valuable insights on the archaeological fermented grape beverage that was originally contained in the amphorae. It is worth noting that palynology has not provided any indication on aromatic plants despite their common use in fish preparations. Indeed, *garum*, *liquamen* and other fish preserves called for coriander, leek, cumin, celery, *etc*. to bitter the flavor (Feldman, 2005). Supported by diverging evidence from archaeology and organic residues, the hypothesis of reuse, although barely considered in archaeology, becomes even more probable. For instance, the *Officina del Garum degli Umbrici* in Pompeii provides another great example of reuse assessment based on archaeological consideration regarding the number of amphorae discovered, their upside-down positions and the original content originating from typology assumptions. In a specific room of the rear courtyard of a house considered as the manufacture center for fish derivatives preparation and direct sale, more than 200 amphorae were stored (Peña, 2007; Bernal *et al.*, 2014). Among them, olive oil containers of Mañà C type, Ostia and Tripolitanian were recovered empty in reversed position which suggests a further filling with local fish preserves (Peña, 2018). Worth considering historical literature, little is described about amphorae reuse although rare evidence was testified by Pliny[15]. For example, he reported the use of oil containers freshly dried for transporting *halmyrídia*, a maritime specie of cabbages to date known as sea kale (*Crambe maritima* L.). Interestingly, such packaging was reported to better conserve the vegetables and ensured them a green color despite the lengthy maritime journey. Following Peña's understanding of the scarce evidence of container reuse (Peña, 2018), the presence of an epigraphic indication on the tomb of *Gaius Commissius Succesus* attested of his retail activities in the 2nd century

[15] *Naturalis Historia* XIX 41

AD. The merchant, whose epitaph clearly designated him as a jug and small amphorae dealer at the *Portus Vinarius*, was unequivocally involved in buying and selling used wine amphorae in Rome.

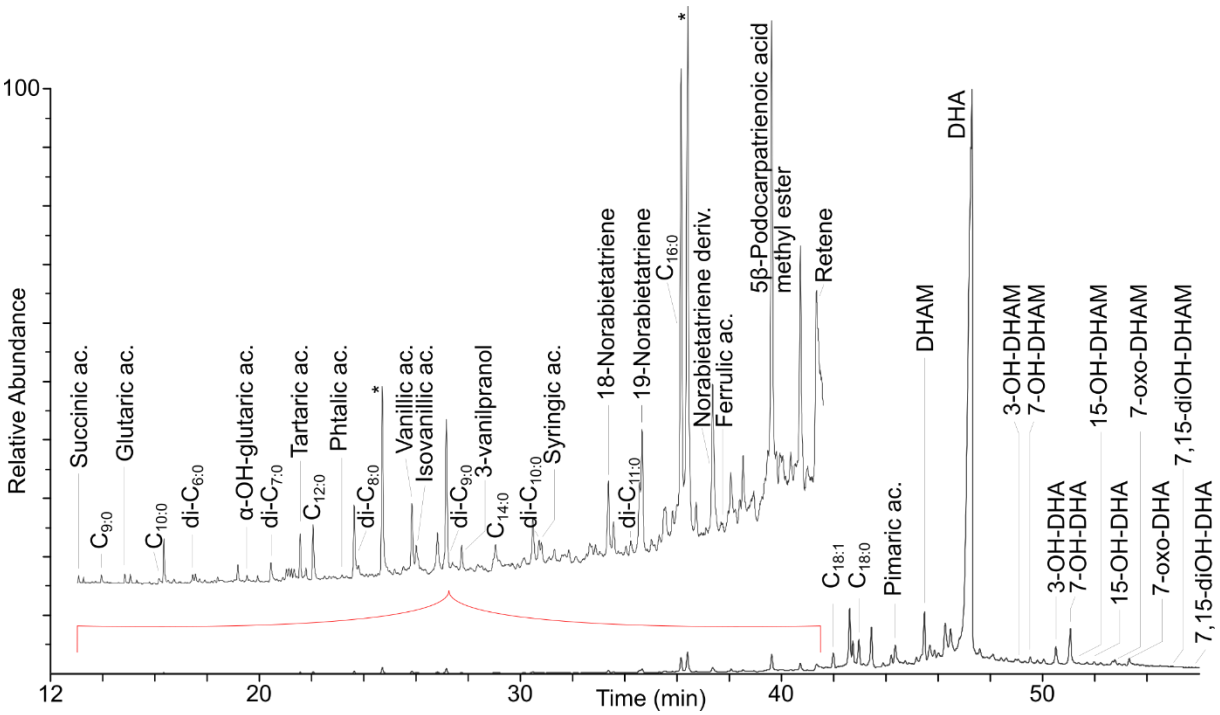

Figure 49. TIC chromatogram of the Mañà C2 amphora (sample no. SFC3)

As highlighted by the residue analysis of the above-mentioned Dressel 20 from Pax Julia Civitas (Lusitania) (Manhita *et al.*, 2020) or from the Keay 62A amphora from the La Palud 1 shipwreck (France) (Garnier, 2007) that unexpectedly revealed pitch coating together with olive oil fatty acids interpreted as a sign of amphora reuse, with wine or fish preserves as prime-content, the recycling of amphorae is complex to consider since it raises several aspects. No standard practices can be assumed, and the reuse can have different faces. Recycling may be considered for the same substances, but it may also be part of a wider supply chain, considering amphorae as a vector of packaging. In the case of reuse, a lower quality regarding the second content can be hypothesized, as amphorae already used may have been cheaper compared to fresh ones and presumably altered the taste (Peña, 2007). Local wine meant for nearby regional supply was hence suggested for Dressel 2-4 re-filling in the wine bottling warehouse of Oplontis Villa B in Pompeii (Pecci *et al.*, 2017).

The extent to which amphorae were re-employed is also questionable. Rarely considered in literature, the analysis undertaken on containers reflect the dearth of attention paid to these possibilities. Apart from the economic advantage of using low-priced containers, there may also be geographical reasons to justify the reuse. It is worth considering the need of amphorae as a packaging support for the export of local production. Assuming that local manufacture of amphorae was non-existent or insufficient, then reuse might have played an important role (Peña, 2018). If the re-assemblage of amphorae was indeed common, then it becomes essential to adapt the applied archaeometric analyses. In this regard, general and global analysis has already been encouraged, in order to understand the object as a whole and not to bias the interpretation by specific and too targeted analytical research (Drieu *et al.*, 2020). In this way, not only will the understanding of the object be more relevant, but also its integration into an economic, fluvial, geographical, chronological and anthropo-sociological scheme will be more pertinent.

In other respect, the 11 amphorae from Planier 3 that were supposed to have contained oil were only analyzed with chromatographic traditional methods and results were discussed in the seventh chapter. Lipid residues showed a remarkable distribution of saturated fatty acids, with a clear predominance of palmitic over stearic acid. Despite its major contribution in fresh olive oil, oleic acid was hardly ever recovered while azelaic acid was identified in several shards. The different molecular compounds extracted underlined the degraded state of the original oil. Understanding why such oxidative processes occurred relies on the assessments of archaeological interpretations regarding the content and the objects' history. This way, several hypotheses were suggested even though none could be verified. Characteristic fatty markers that would have highlighted a vegetal source differing from olive (e.g., castor oil, rapeseed oil, *etc*.) were not identified. Amphorae could have transported olive fruits since the flesh does not release fatty acids. Such consideration was supported by the Northern provenance of the early African amphorae, famous to produce table olives rather than oil. Otherwise, amphorae could have been filled with degraded olive oil on purpose: the function of the good not being associated with consumption and cooking, the oil did not require high quality. For example, body lotions or lamp fuel accounted for poor quality of oil. Olive oil being a basic food source during Roman times, its occurrence was alternatively reported in several derivatives products such as medicaments, soaps or perfumes (Rowan, 2019).

In the case of the Planier 3 oil amphorae, results illustrate the limits of a single analytical methodology. Chromatography highlights the degraded profile of the contents without bringing additional evidence of an exotic or atypical product. The application of a multi-analytical approach would then be interesting because it could open the field of interpretation, as previously demonstrated with the wine amphorae from San Felice Circeo or the Jericho vase. Although little considered in archaeometry, the example of perfumed oil could provide valuable explanation. Such derivatives were indeed used as a base for archaeological cosmetics and olive oil was initially scented for preparation (Pérez-Arantegui *et al.*, 2009). In this case, palaeobotany could be an excellent option to address this hypothesis since pollen would be the witness of flower or herbal flourishment

However, as the search for pollen requires a reliable and well-detailed archaeological environment, the main risk being obviously the integration of post-sedimented grains in the interpretation of the results, the selection of objects and sampling are essential for a valuable palaeobotanical analysis. Most of the amphorae from Planier 3, all contents included, corresponded to shards with no apparent resinous coating (i.e., 19 over the 22 artefacts), which were recovered underwater. Unfortunately, these objects could not be included in palynological analysis for various reasons. Firstly, because the superficial seabed does not represent a terrain free of post-sedimentary pollen contamination and the objects were excavated more than 50 years ago. Secondly, because they were fragments of amphorae, some with several centimeters of concretion on the surface of the ceramic. Indeed, objects recovered in their integrity are favored when dealing with shard pollen analysis, always with the idea of limiting contamination. When archaeological excavations ensure a physically bordered environment that drastically reduces the risks of contamination, such as domestic or funerary contexts for example, palynological analysis from the shard becomes possible.

Since pollen analysis of pottery has rarely been carried out so far, the first challenge we faced was to verify the possibility of such an approach, using the residual pitch contained in amphorae. Once confirmed, we then validated the feasibility of pollen analysis from the ceramic shard itself. In this regard, we carefully chose an object whose archaeological context ensured a low risk of post-sediment contamination.

8.4. Conclusion

The following table gives an overview of the main results obtained from chromatography and archaeobotany for all the artefacts studied all along the different chapters.

The twofold analytical approach undoubtedly allowed more quantitative information on the investigated samples to be acquired. The methodology greatly participated to a better definition of the *Pinus* origin of diterpenic markers (for the samples no. 1014 and 749), to the identification of the Mediterranean subfamily of the *Pinus* waterproofing coating (samples no. SFC1, SFC2 and SFC5). The multi-analytical approach additionally promoted the contextualization of the presence of grapes in certain amphorae, thus joining anthropological perspectives to the archaeometric analysis of the objects.

Finally, the scientific lens we adopted showed a new aspect through the analysis of the Jericho vase, by providing information that do not overlap with each other. Rather than corroborating each other, chromatographic and archaeobotanical analyses proved to be complementary and opened up a new interpretative field regarding the recipes of a medicinal beverage.

Sample	Picture	GC-MS	Archaeobotany	Hypothesis	Cf. Chapter
6545 Dressel 5 Kosamphora Greece		Grape markers Fermentation markers Diterpenes Oxidized diterpenes Methyl ester diterpene Retene	-	Fermented grape beverage (wine) Pinaceae wood tar	4
6828a Lamboglia 2 Adriatic coast Italy		Grape markers Fermentation markers Diterpenes Oxidized diterpenes Methyl ester diterpene Retene	-	Fermented grape beverage (wine) Pinaceae wood tar	4
6828b Lamboglia 2 Adriatic coast Italy		Grape markers Fermentation markers Diterpenes Oxidized diterpenes Methyl ester diterpene Retene	-	Fermented grape beverage (wine) Pinaceae wood tar	4
6828c Lamboglia 2 Adriatic coast Italy		Grape markers Fermentation markers Diterpenes Oxidized diterpenes Methyl ester diterpene Retene	-	Fermented grape beverage (wine) Pinaceae wood tar	4
6565 Lamboglia 2 Adriatic coast Italy		Grape markers Fermentation markers Diterpenes Oxidized diterpenes Methyl ester diterpene Retene	-	Fermented grape beverage (wine) Pinaceae wood tar	4
6566 Lamboglia 2 Adriatic coast Italy		Grape markers Fermentation markers Diterpenes Oxidized diterpenes Methyl ester diterpene Retene	-	Fermented grape beverage (wine) Pinaceae wood tar	4
1014 Dressel 1B Tyrrhenian coast Italy		Grape markers Fermentation markers Diterpenes Oxidized diterpenes Methyl ester diterpene Retene	Pollen of *Pinus*	Fermented grape beverage (wine) *Pinus* wood tar	4 Discussion
749 Lamboglia 2 Adriatic coast Italy		Grape markers Fermentation markers Diterpenes Oxidized diterpenes Methyl ester diterpene Retene	Pollen of *Pinus, Cedrus*	Fermented grape beverage (wine) *Pinus* wood tar	4 Discussion
6570a Dressel 1B Tyrrhenian coast Italy		Grape markers Fermentation markers Diterpenes Oxidized diterpenes Methyl ester diterpene Retene	-	Fermented grape beverage (wine) Pinaceae wood tar	4
6793 Lamboglia 2 Adriatic coast Italy		Grape markers Fermentation markers Diterpenes Oxidized diterpenes Methyl ester diterpene Retene	-	Fermented grape beverage (wine) Pinaceae wood tar	4
6904 Chios amphora		Grape markers Fermentation markers Diterpenes Retene	-	Fermented grape beverage (wine) Pinaceae pitch	4

Sample	Picture	GC-MS	Archaeobotany	Hypothesis	Cf. Chapter
6561 Ovoid Adriatic coast Italy		Fatty acids Diterpenes Oxidized diterpenes Methyl ester diterpene Retene	-	Degraded olive oil, Olive fruits Pinaceae wood tar	7
747 Ovoid Adriatic coast Italy		-	-	-	7
561 Ovoid Adriatic coast Italy		-	-	-	7
6849b Ovoid Adriatic coast Italy		-	-	-	7
6781a Early African Tunisia		Fatty acids Diterpenes Oxidized diterpenes Methyl ester diterpene	-	Degraded olive oil, Olive fruits Pinaceae wood tar	7
6823 Early African Tunisia		Fatty acids	-	Olive fruits	7
6849 Early African		Fatty acids Methyl ester diterpene Retene	-	Degraded olive oil, Olive fruits Pinaceae wood tar	7
579 Brindisium amphora		Fatty acids	-	Olive fruits	7
1005 Early African Tunisia		Fatty acids Methyl ester diterpene Retene	-	Degraded olive oil, Olive fruits Pinaceae wood tar	7
598a Ovoid Adriatic coast Italy		Fatty acids Diterpenes Oxidized diterpenes Methyl ester diterpene	-	Degraded olive oil, Olive fruits Pinaceae wood tar	7
728 Ovoid Adriatic coast Italy		-	-	-	7

Sample	Picture	GC-MS	Archaeobotany	Hypothesis	Cf. Chapter
SFC1 Late Greco-Italic / Dressel 1A Tyrrhenian coast Italy		Grape markers Fermentation markers Diterpenes Oxidized diterpenes Methyl ester diterpene Retene	Pollen of Mediterranean *Pinus*, aporate tricolpate *Vitis, Myrtus* *Vitis vinifera* tissue of the filament for the flower stamen	Fermented grape beverage (wine) *Pinus* wood tar	4, 5
SFC2 Dressel 1A Tyrrhenian coast Italy		Grape markers Fermentation markers Diterpenes Oxidized diterpenes Methyl ester diterpene Retene	Pollen of Mediterranean Pinus, aporate tricolpate Vitis, Cedrus Charred wood of *Pinus*	Fermented grape beverage (wine) *Pinus* wood tar	4, 5
SFC3 Mañá C2		Grape markers Fermentation markers Diterpenes Oxidized diterpenes Methyl ester diterpene Retene	Pollen of *Pinus, Cedrus*	Fermented grape beverage (wine) Amphorae reuse *Pinus* wood tar	4 Discussion
SFC4 Greek-Italian		Grape markers Fermentation markers Diterpenes Oxidized diterpenes Methyl ester diterpene Retene	Pollen of *Pinus,* Tricolporate *Vitis*	Fermented grape beverage (wine) *Pinus* wood tar	4 Discussion
SFC5 Lamboglia 2 Adriatic coast Italy		Grape markers Fermentation markers Diterpenes Oxidized diterpenes Methyl ester diterpene Retene	Pollen of Mediterranean *Pinus*, *Pinus* lump, aporate tricolpate *Vitis* Charred wood of *Pinus*	Fermented grape beverage (wine) *Pinus* wood tar	4, 5
Teapot Jerico Bronze Age		Pimarane diterpenes Short chains diacids (C_6-C9) Fatty acids (C_8-C_{18}) Linear alcohols (C_{12}-C_{18})	Pollen of *Malva parviflora*, *Artemisia*, *Cedrus*	Medicinal beverage	6

Table 13. Chromatographic and archaeobotanical results and interpretations of the artefacts studied

8.5. Perspectives

The current demand for a better understanding of archaeological objects has stimulated the search for powerful and cutting-edge protocols for the characterization of ceramic vessels and pottery. Beyond the innovative analytical aspect, this thesis aims at filling knowledge gaps between cultural, archaeological and scientific fields. The objectives concerning the development of reliable analytical protocols, both in terms of extraction of organic residues and in terms of archaeobotanical observations, have been achieved. The main challenge therefore relies in the object itself and directly infers on the decision of the analytical treatment to follow.

The multi-analytical approach allows to better define the archaeological and anthropological contours of the studied contexts. The collaboration of independent techniques remarkably promotes the consideration of new archaeological hypotheses, from the nature of the product itself to its integration in the understanding of goods trading in the Mediterranean basin. The application of such a methodology on a larger scale would open new horizons. Indeed, as objects do not equally survive archaeological time, the application of a strict multidisciplinary method cannot be sufficient on its own. It is therefore necessary to use and corroborate the observations provided by different analyses.

In this regard, DNA, and more precisely ancient DNA (aDNA) have recently joined the field thanks to polymerase chain reactions that amplify the remaining genetic material. The archaeological artefacts considered for analysis must be carefully selected and sampled to avoid contamination or misinterpretation from false positives. Climatic, environmental and storage conditions can play an important role in DNA preservation. Cold, dry and anoxic atmosphere are favored (Schlumbaum, Tensen and Jaenicke-Després, 2008). Following Di Donato et al. (Di Donato *et al.*, 2018), aDNA proved to be a valuable tool to address plant evolution and domestication of modern crops. Genetic approaches revolutionized the understanding of plant adaptation and migration (Gepts, 2004; Doebley, Gaut and Smith, 2006; Meyer, DuVal and Jensen, 2012; Guasch-Jané, 2019; Zhou, Muyle and Gaut, 2019). aDNA analyses of grapevines revealed sequence insertions into the nuclear genome of *Vitis vinifera*, hence outlining genetic introgression from local wild species (Wales *et al.*, 2016). Furthermore, grape, oil and herbs genetic markers were successfully identified in Classical Greek containers of a 2400-years old shipwreck (Hansson and Foley, 2008; Foley *et al.*, 2012). The inorganic building material seems to act as a support to which DNA binds (Briggs, 2020). As for tartrate salts in organic residue analysis, the binding with the ceramic medium would protect genetic markers from degradation (Grunenwald *et al.*, 2014). Additionally, the analysis of aDNA from fermentation bacteria provide a new avenue for studying prehistoric beverages. aDNA fragments of *Saccharomyces cerevisiae*, the main yeast used in fermentation processes have been identified in 5000-years-old Egyptian jars, hence providing new evidence on the archaeological wine contained herein (Cavalieri *et al.*, 2003). Such investigations, although they are on the cutting edge of science and technology are still barely conducted on archaeological pottery. Most promising would be the integration of genetic markers and DNA fingerprinting in multi-analytical approaches to decipher the intricate history beyond the archaeological ceramic.

8.6. References

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Appendix 1. Drawings of several amphorae from Planier 3

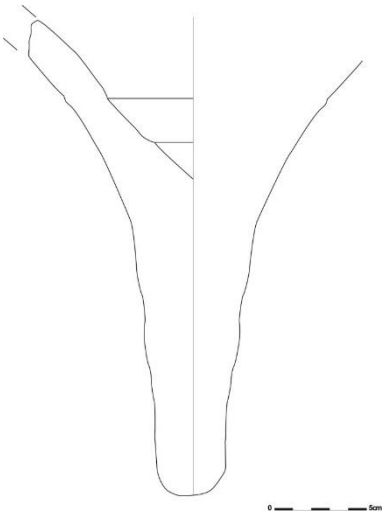

Fig. A1. Lamboglia 2 foot (amphora 6565) (drawn by M. Branger)

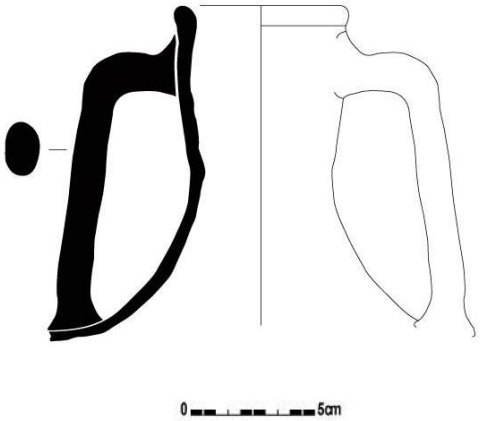

Fig. A2. Chios amphora (amphora 6904) (drawn by M. Branger)

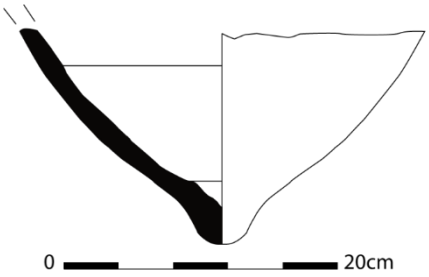

Fig. A3. Ovoid foot (6849b left) (drawn by M. Branger)

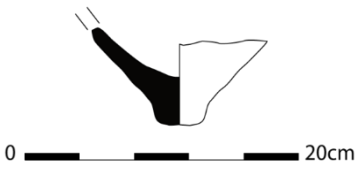

Fig. A4. Early African foot (6849 right) (drawn by M. Branger)

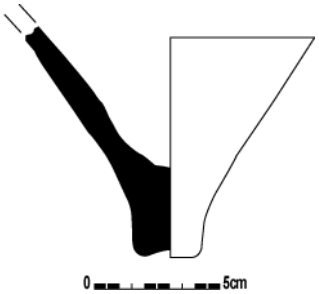

Fig. A8. Early African (amphora 6781a) (drawn by M. Branger)

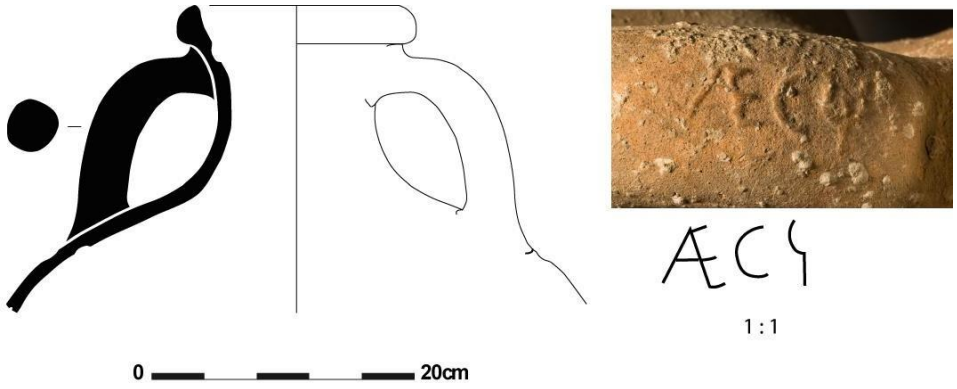

Fig. A6. "CAEC" stamp from an ovoid (amphora 6561)
(drawn by M. Branger and stamp picture from C. Durand, CCJ, CNRS)

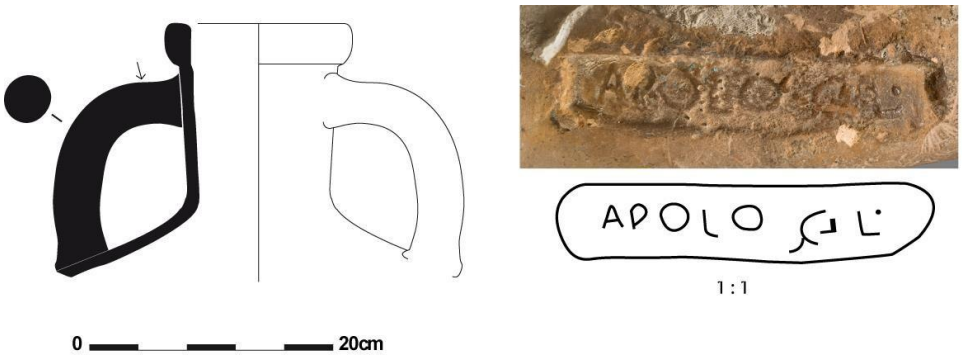

Fig. A7. "APOLO.CAL" or "GAL" stamp from an ovoid (728)
(drawn by M. Branger and stamp picture from C. Durand, CCJ, CNRS)

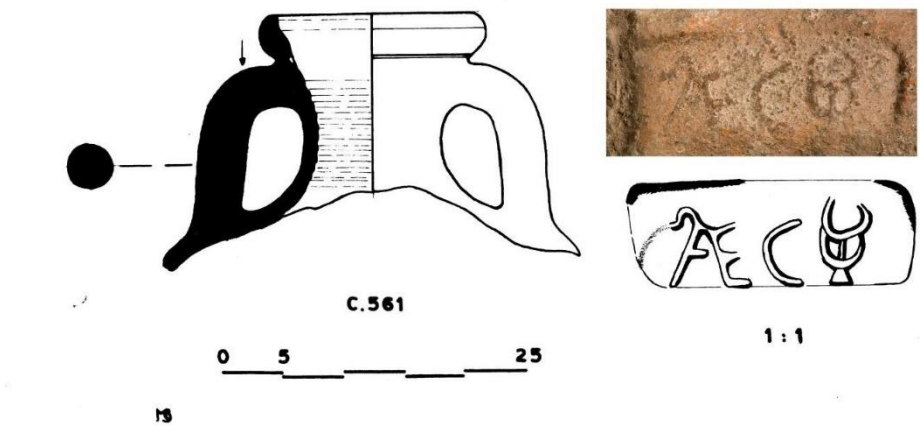

Fig. A8. "CAEC" stamp from an ovoid (561)
(drawn by M. Branger and stamp picture from C. Durand, CCJ, CNRS)

Reference

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Appendix 2. Fatty acids and derivatives identified in the oil amphorae

			6781a			1005			6849			6823		
Sample	Retention Time	Main Mass Fragments	1LE	Butylation	Saponif.	1LE	Butylation	Saponif.	1LE	Butylation	Saponif.	1LE	Butylation	Saponif.
$C_{9:0}$	14.1	75; 217	Second	Second	Second	Minor	Minor	Minor	Second	None	Major	Minor	Minor	Minor
di-$C_{9:0}$	27.5	149; 317	Second	None	Second	None	None	Second	None	None	None	None	None	Minor
$C_{16:0}$	36.5	117; 313	Major	Second	Major	Minor	Minor	Major	Major	None	None	Minor	Minor	Major
di-$C_{9:0}$ BE	37.4	170; 227	-	Second	-	-	Second	-	-	None	-	-	Minor	-
$C_{16:0}$ BE	40.9	185; 237	-	Major	-	-	Major	-	-	Major	-	-	Major	-
$C_{18:1}$	42.2	117; 339	Second	None	Second	None	None	None	None	None	None	None	None	None
$C_{18:0}$	43.2	117; 341	Second	None	Second	None	None	Minor	Second	None	None	None	None	Minor
$C_{18:0}$ BE	47.9	185; 285	-	Major	-	-	Major	-	-	Major	-	-	Minor	-
9,10 diOH $C_{18:0}$	-	317; 517	None	None	None	None	None	None	None	None	None	None	None	None

	579		747	728		6561		561		598A			6849b
Sample	1LE	Saponif.	Saponif.	1LE	Butylation	1LE	Butylation	1LE	Butylation	1LE	Butylation	Saponif.	1LE
$C_{9:0}$	None	Major	None	Minor	Minor	Minor	Minor	Minor	Minor	Second	Second	Second	Major
di-$C_{9:0}$	None	None	None	None	None	None	None	None	None	Second	None	None	None
$C_{16:0}$	Major	None	None	None	Major	Major	Minor	None	Minor	Major	Second	Major	None
di-$C_{9:0}$ BE	-	-	-	-	None	-	Second	-	Minor	-	Second	-	-
$C_{16:0}$ BE	-	-	-	-	Major	-	Major	-	Major	-	Major	-	-
$C_{18:1}$	None	None	None	None	None	None	None	None	None	Minor	None	None	None
$C_{18:0}$	None	None	None	None	None	Minor	None	None	None	Second	None	None	None
$C_{18:0}$ BE	-	-	-	-	Major	-	Major	-	Major	-	Major	-	-
9,10 diOH $C_{18:0}$	None	None	None	None	None	None	None	None	None	None	None	None	None

Table A1. Fatty acids identified in oil amphorae from Planier 3. 1LE: First Lipid Extraction; Saponif.: Saponification; $C_{x:y}$: FA of x carbon atoms and y unsaturations; di-$C_{x:y}$: dicarboxylic acid of x carbon atoms and y unsaturations; BE: Butyl Ester; diOH: dihydroxy FA. "Major" refers to the more intense peaks of the TIC chromatogram; "Second" refers to secondary peaks; "None" means the compound was not identified (neither with mass fragments searching); "-" refers to butylated molecules that could not be identified with non-butylation protocols.

Appendix 3. Mass spectrum of standard molecule

This appendix provides the mass spectra of the main molecules characterized and discussed in the thesis. The molecules are classified according to their chemical family and appear in increasing order of retention time. The following molecules are further detailed:

Fatty acids

Palmitic acid TMS

Oleic acid TMS

Stearic acid TMS

Azelaic acid TMS

Terpenes

Retene

Pimaric acid TMS

Sandaracopimaric acid TMS

Dehydroabietic acid methyl ester

Isopimaric acid TMS

Palustric acid TMS

Dehydroabietic acid TMS

Abietic acid TMS

7-oxo-dehydroabietic acid TMS

Organic acids

Succinic acid TMS

Fumaric acid diTMS

Malic acid triTMS

Tartaric acid tetraTMS

Syringic acid diTMS

Butylated acids

Dibutyl malate

Dibutyl succinate

Dibutylacetal pyruvate

Dibutyl fumarate

Dibutyl malate TMS

Dibutyl tartrate diTMS

Butyl syringate TMS

Dibutyl azelaic acid

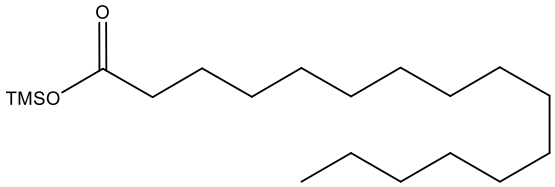
Fatty acids

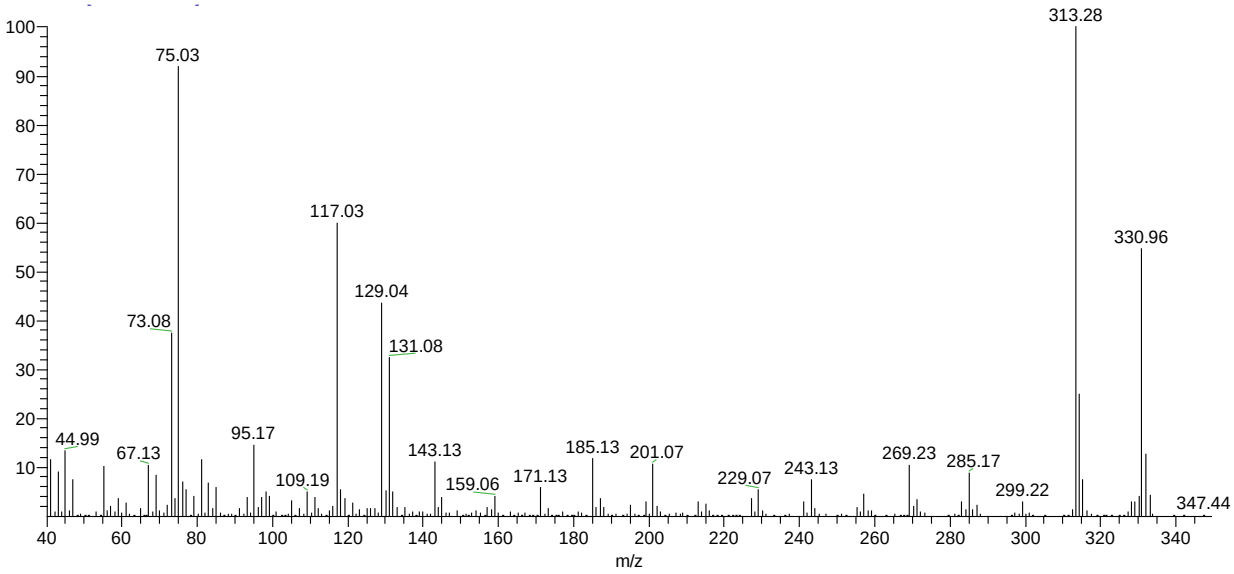
Palmitic acid TMS

$C_{19}H_{41}O_2Si$

Molecular weight: 328.61 g.mol^{-1}

Retention time: 36.54 min



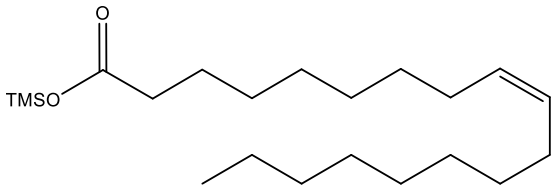


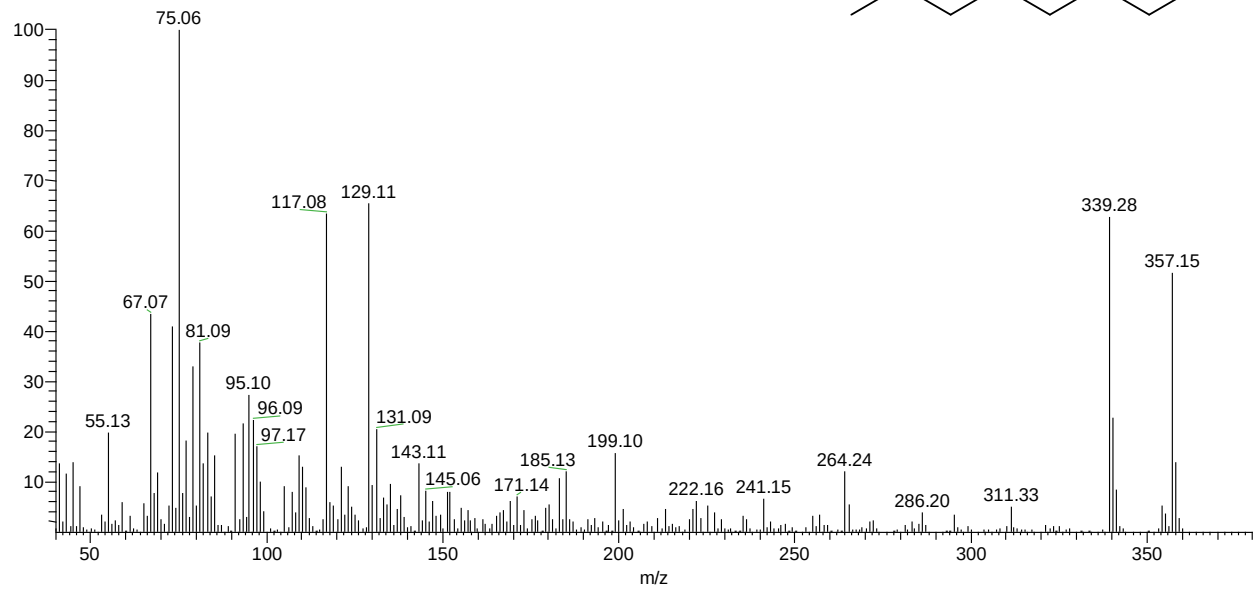
Oleic acid TMS

$C_{21}H_{42}O_2Si$

Molecular weight: 354.65 g.mol^{-1}

Retention time: 42.22 min



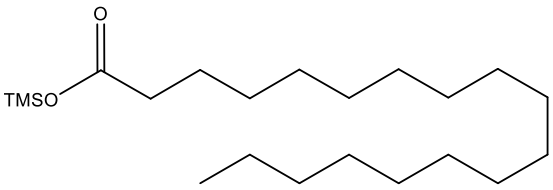


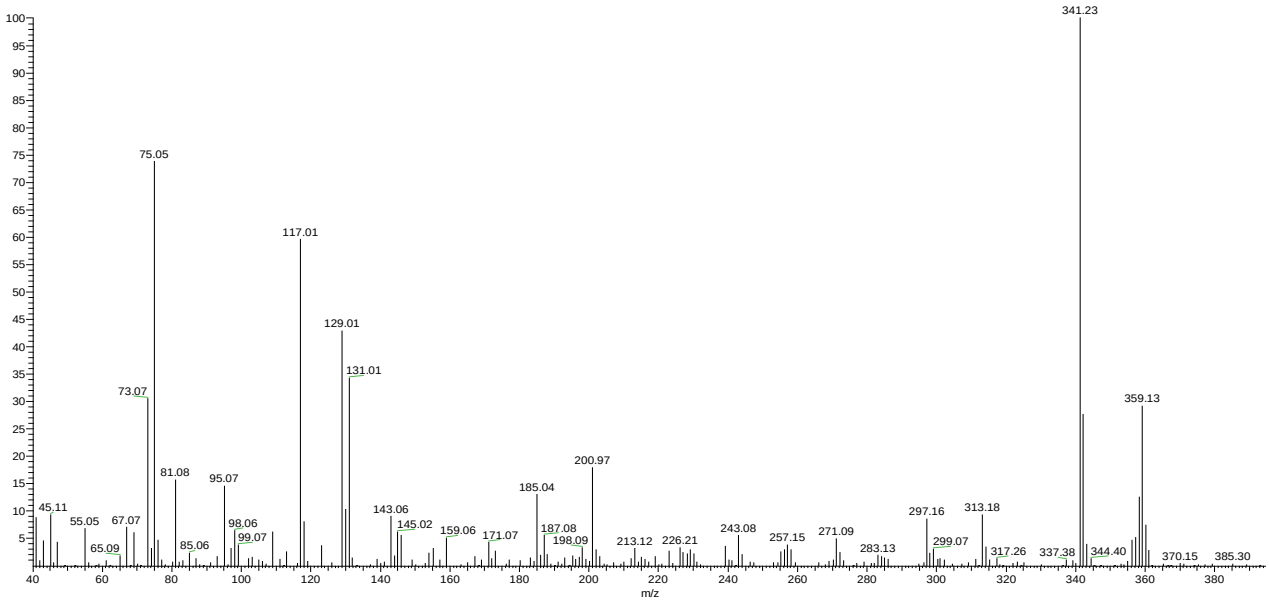
Stearic acid TMS

$C_{21}H_{44}O_2Si$

Molecular weight: 356.67 g.mol^{-1}

Retention time: 43.22 min



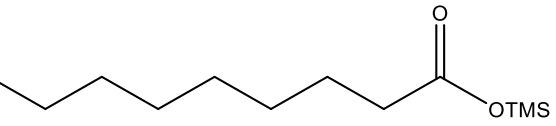


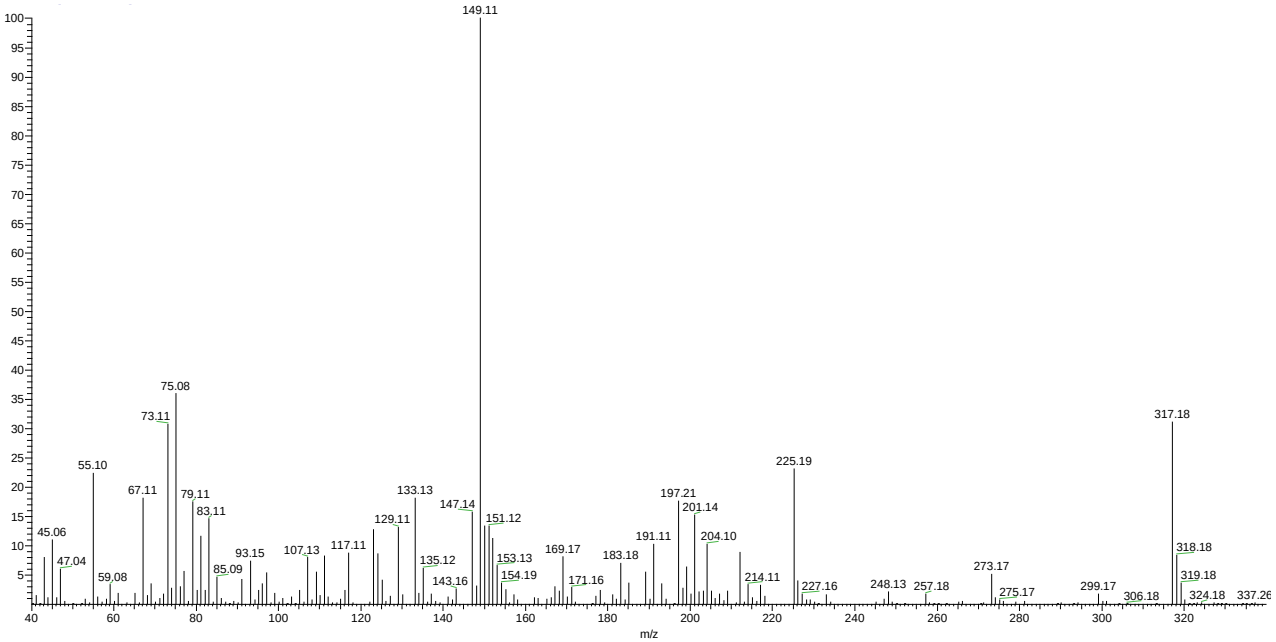
Azelaic acid diTMS

$C_{15}H_{32}O_4Si_2$

Molecular weight: 332.59 g.mol^{-1}

Retention time: 27.5 min





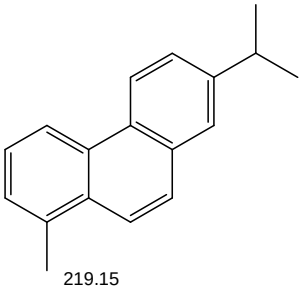
Terpenes

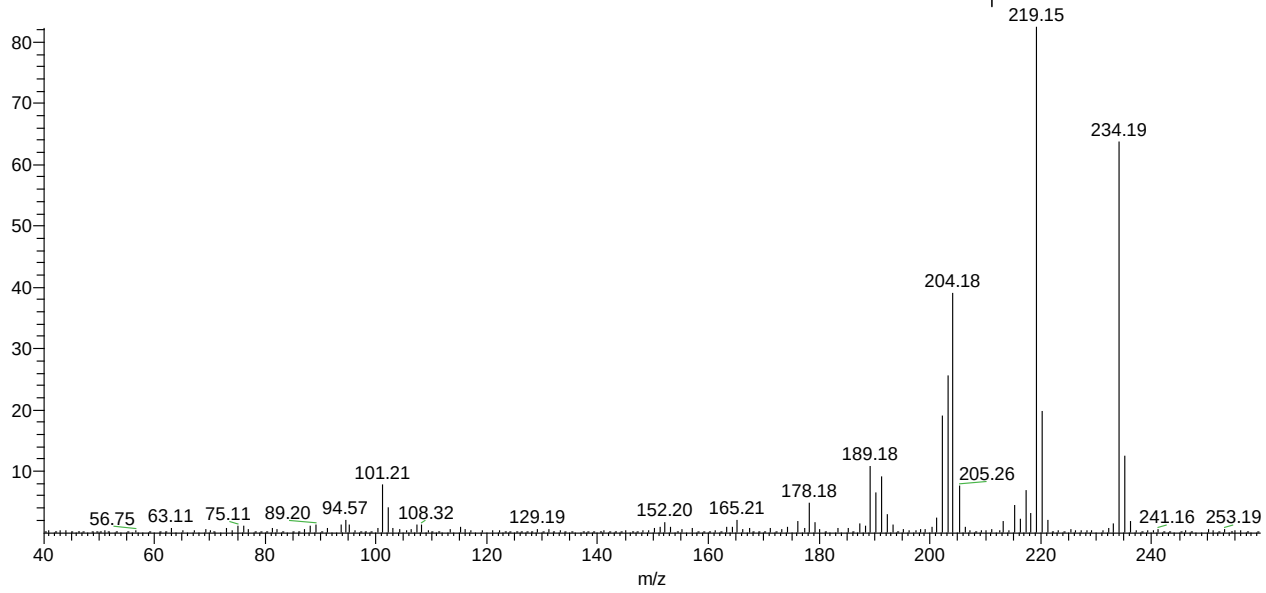
Retene

$C_{18}H_{18}$

Molecular weight: 234.34 g.mol^{-1}

Retention time: 41.50 min



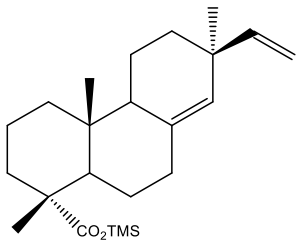


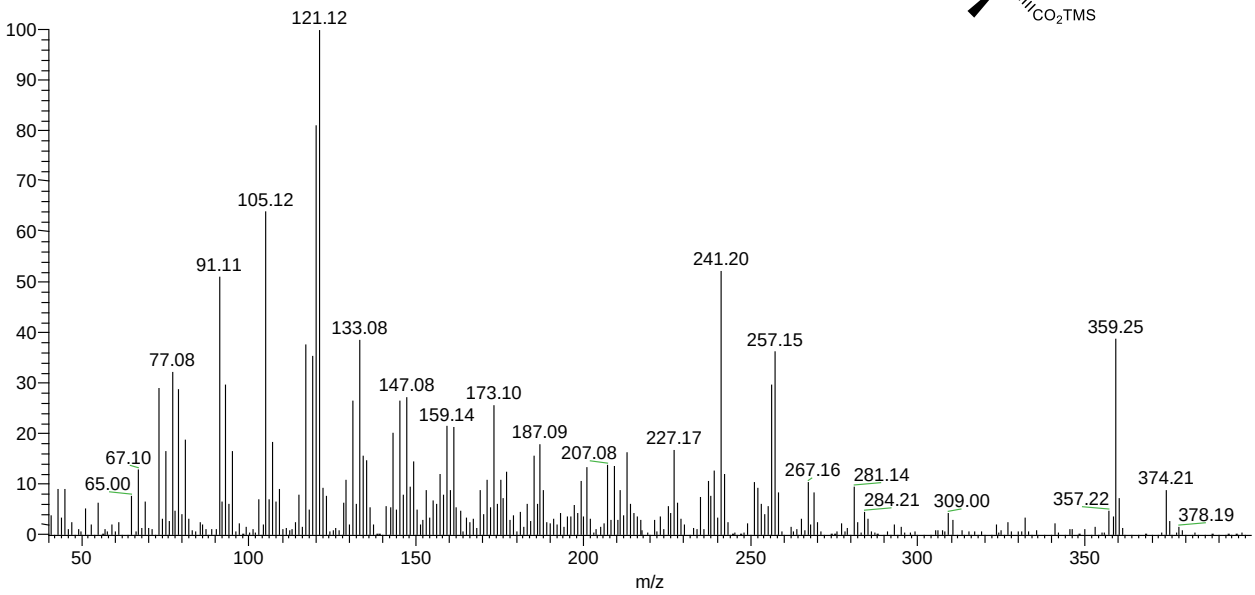
Pimaric acid TMS

$C_{23}H_{38}O_2$ Si

Molecular weight: 374.64 g.mol^{-1}

Retention time: 44.46 min



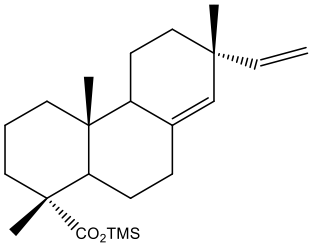


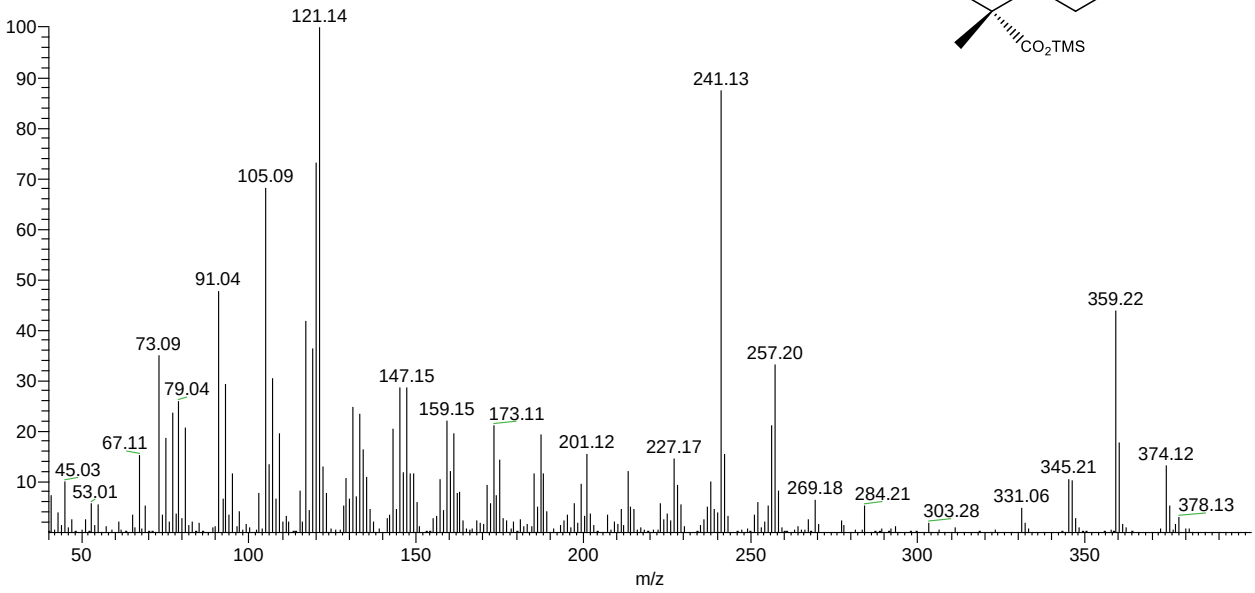
Sandaracopimaric acid TMS

$C_{23}H_{38}O_2Si$

Molecular weight: 374.64 g.mol^{-1}

Retention time: 45.04 min



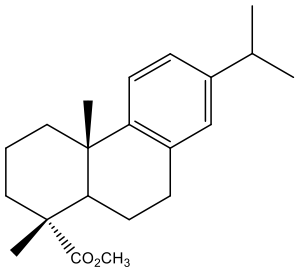


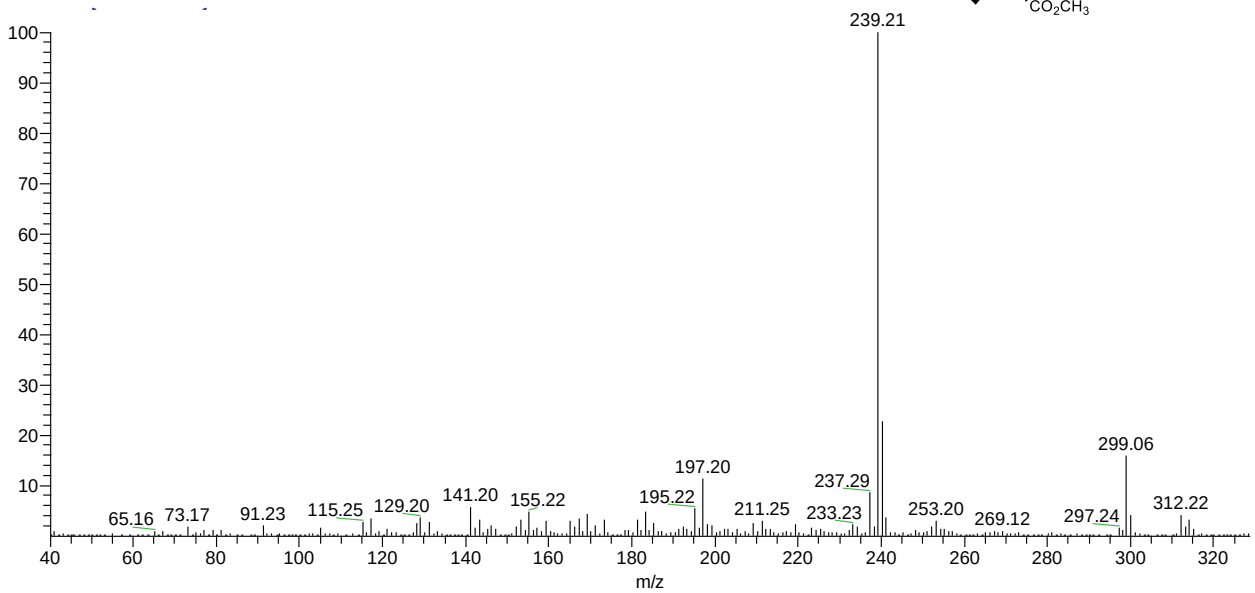
Dehydroabietic acid methyl ester

$C_{21}H_{30}O_2$

Molecular weight: 314.47 g.mol^{-1}

Retention time: 45.48 min



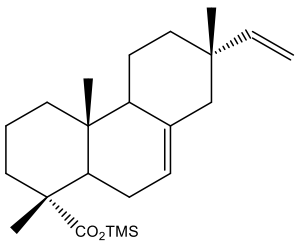


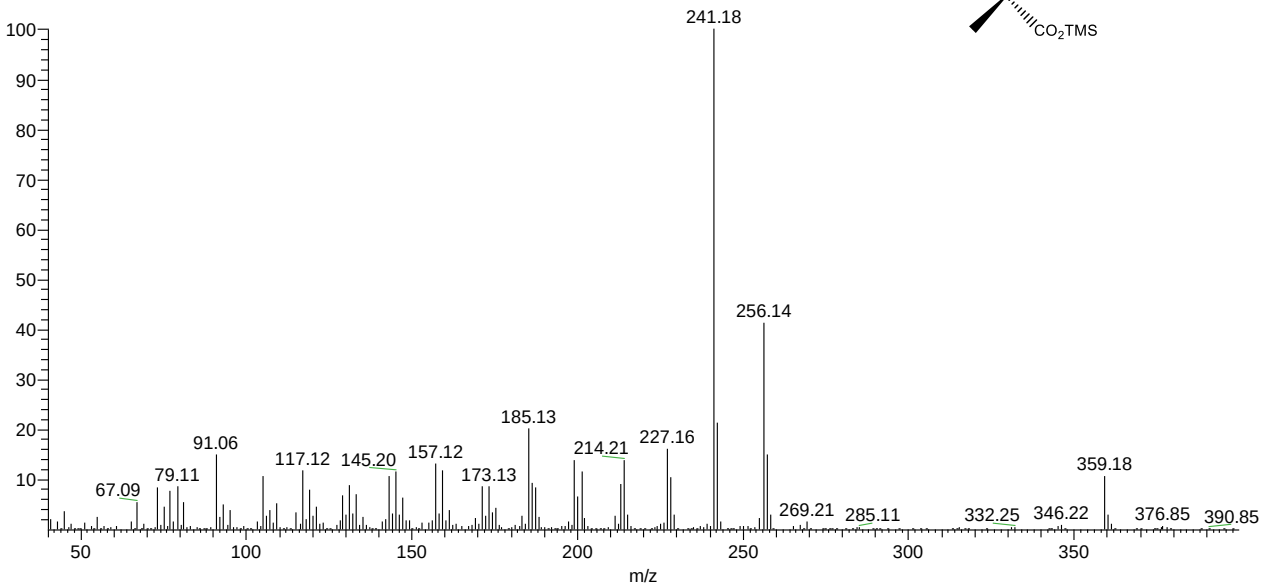
Isopimaric acid TMS

$C_{23}H_{38}O_2Si$

Molecular weight: 374.64 g.mol^{-1}

Retention time: 45.62 min



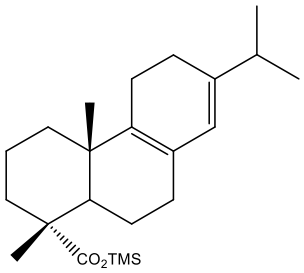


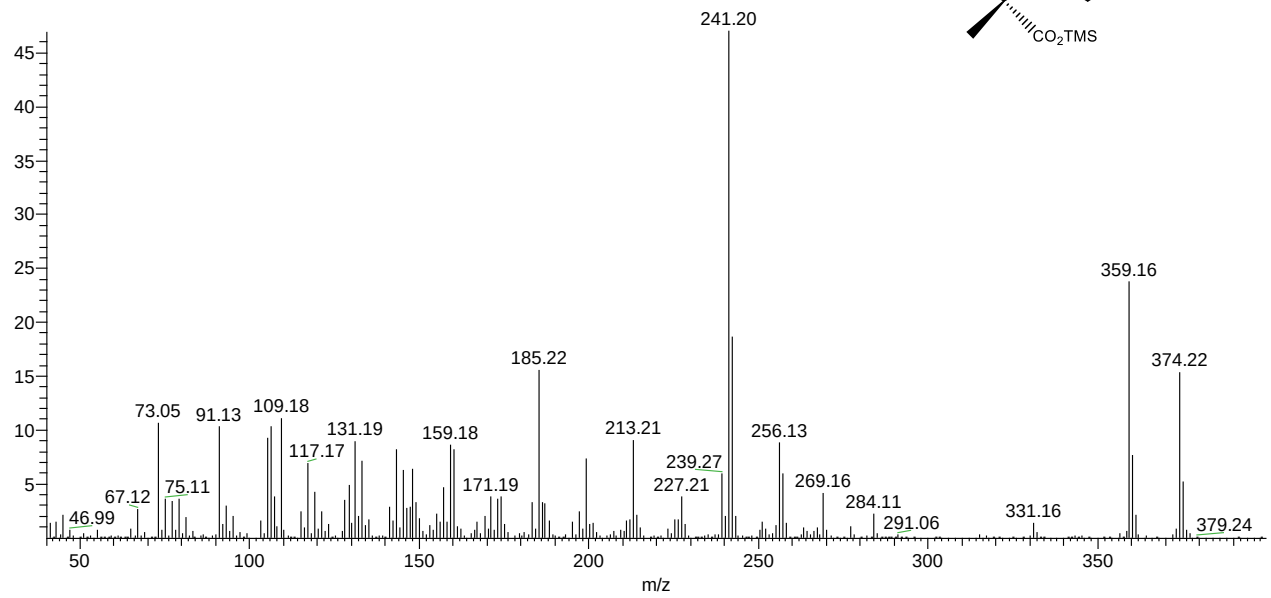
Palustric acid TMS

$C_{23}H_{38}O_2Si$

Molecular weight: 374.64 g.mol^{-1}

Retention time: 46.28 min



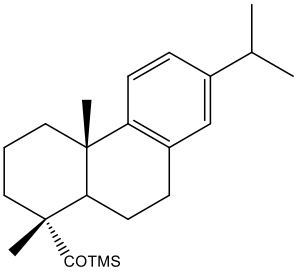


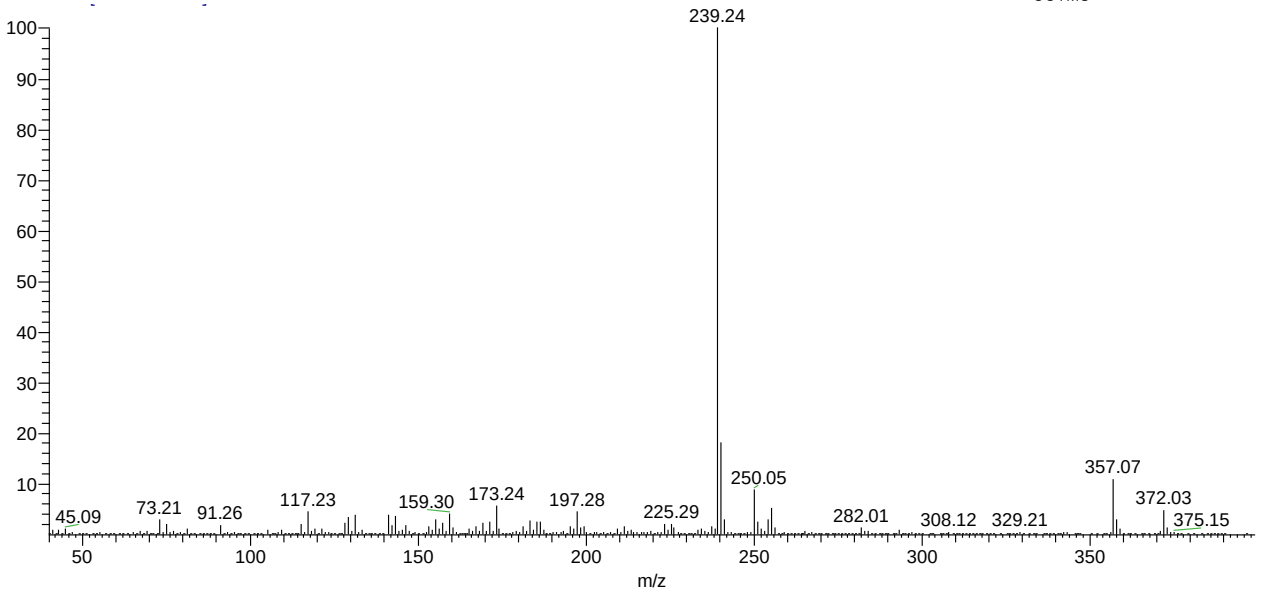
Dehydroadietic acid TMS

$C_{23}H_{36}O_2Si$

Molecular weight: 372.62 g.mol^{-1}

Retention time: 47.11 min



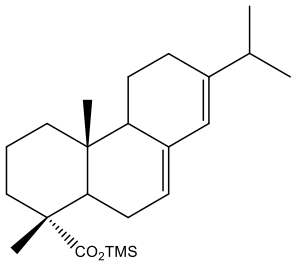


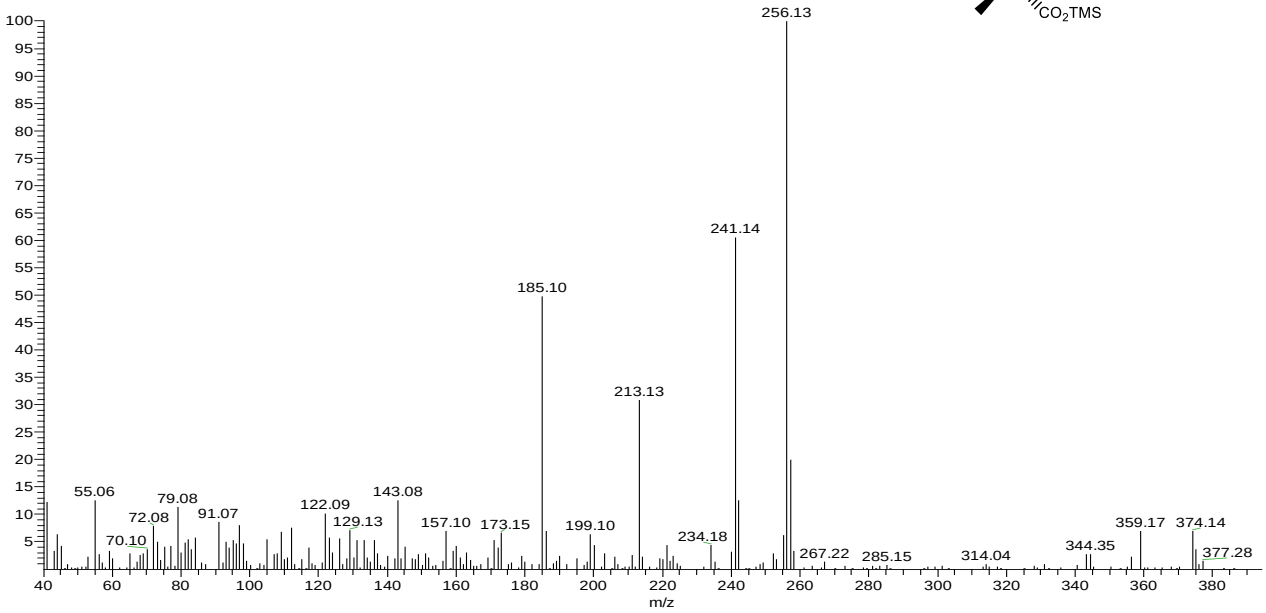
Adietic acid TMS

$C_{21}H_{44}O_2Si$

Molecular weight: 356.67 g.mol^{-1}

Retention time: 48.18 min



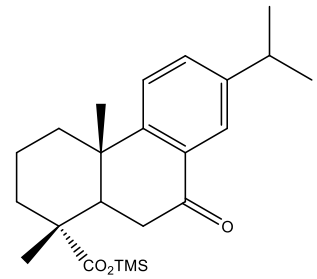


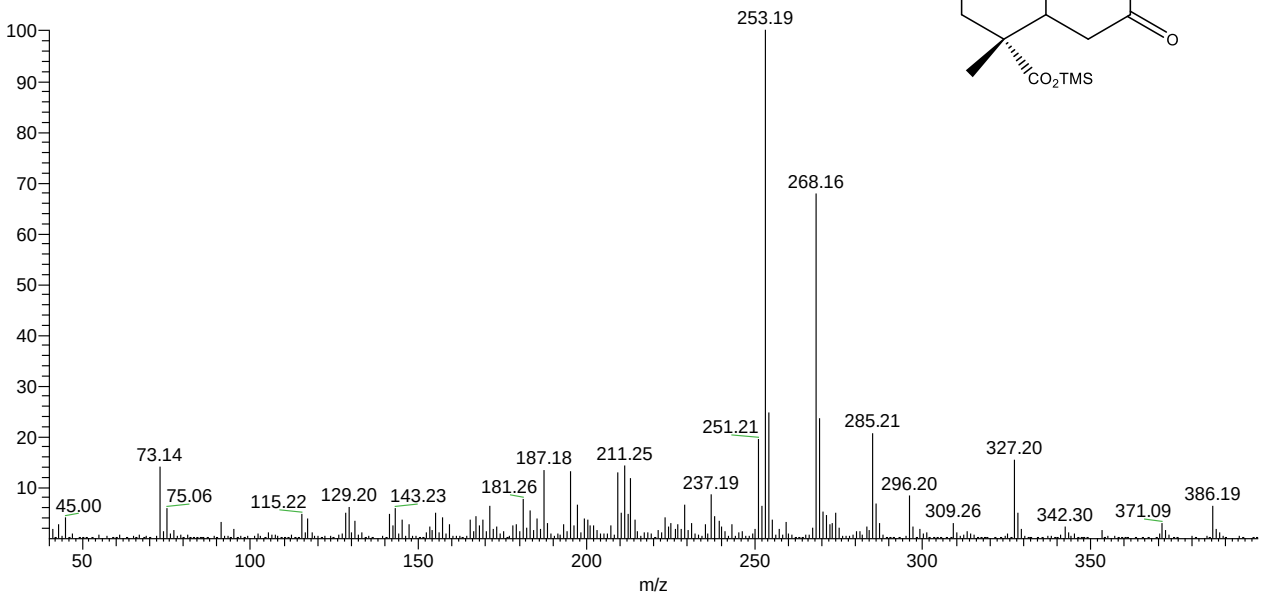
7-oxo-dehydroadietic acid TMS

$C_{23}H_{34}O_3Si$

Molecular weight: 386.61 g.mol^{-1}

Retention time: 53.72 min





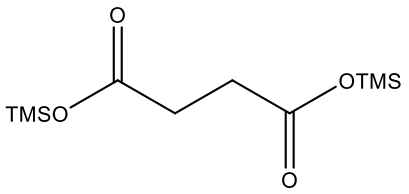
Organic acids

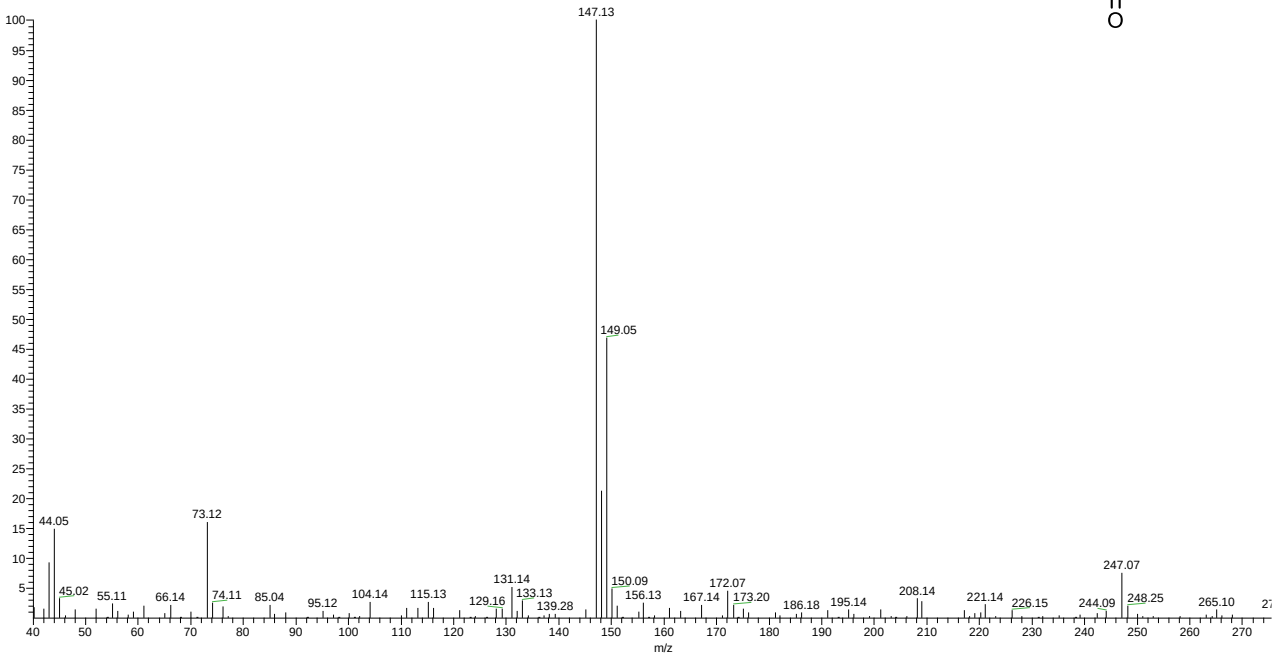
Succinic acid TMS

$C_{10}H_{22}O_4Si_2$

Molecular weight: 262.45 g.mol^{-1}

Retention time: 13.16 min



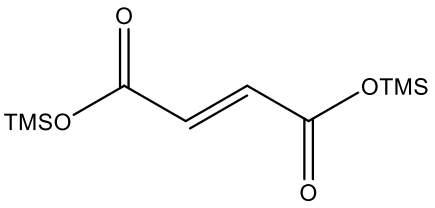


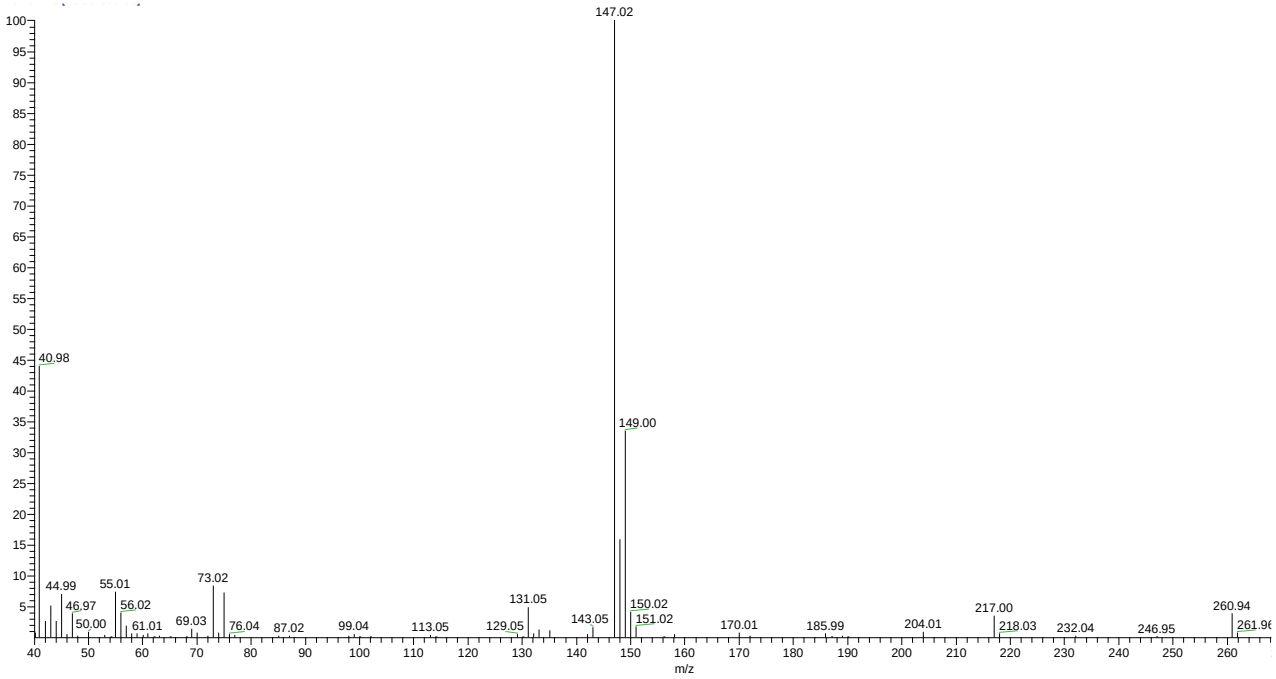
Fumaric acid diTMS

$C_{10}H_{20}O_4Si_2$

Molecular weight: 260.09 g.mol^{-1}

Retention time: 13.82 min



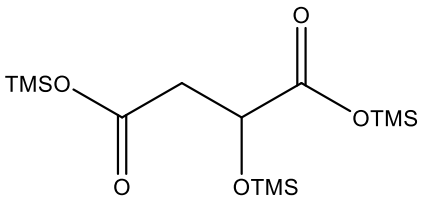


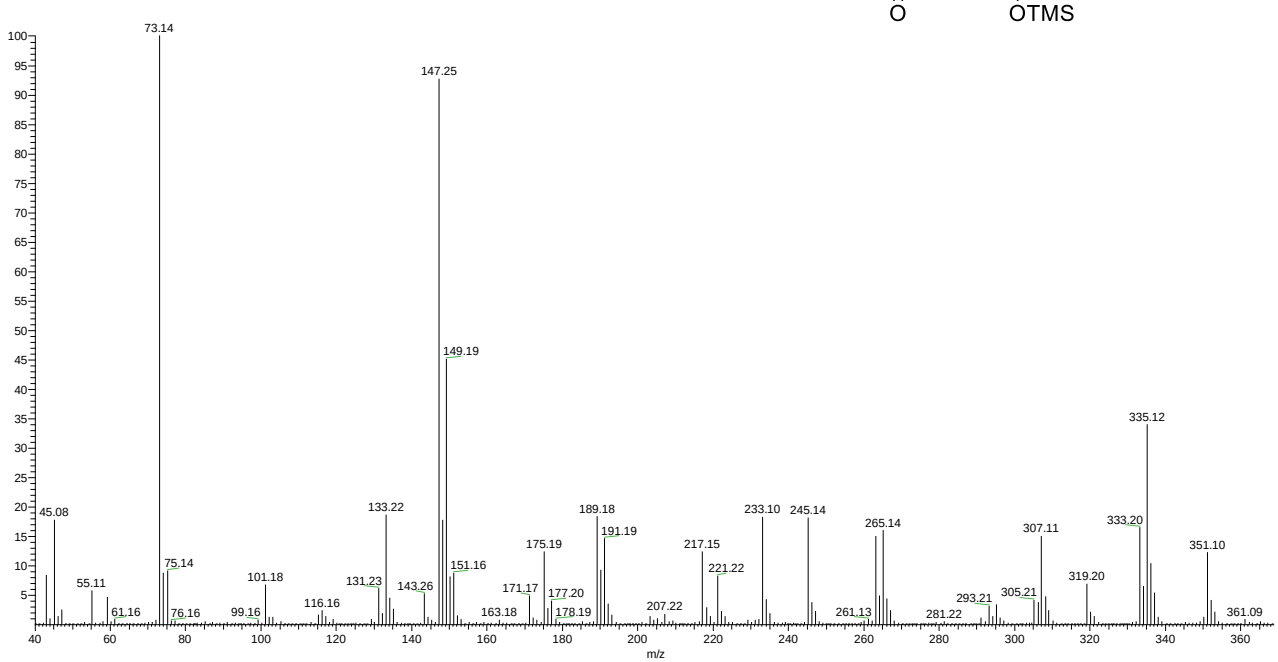
Malic acid triTMS

$C_{13}H_{30}O_5Si_3$

Molecular weight: 350.14 g.mol^{-1}

Retention time: 17.10 min



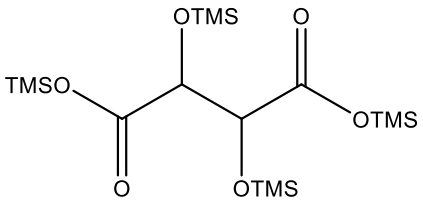


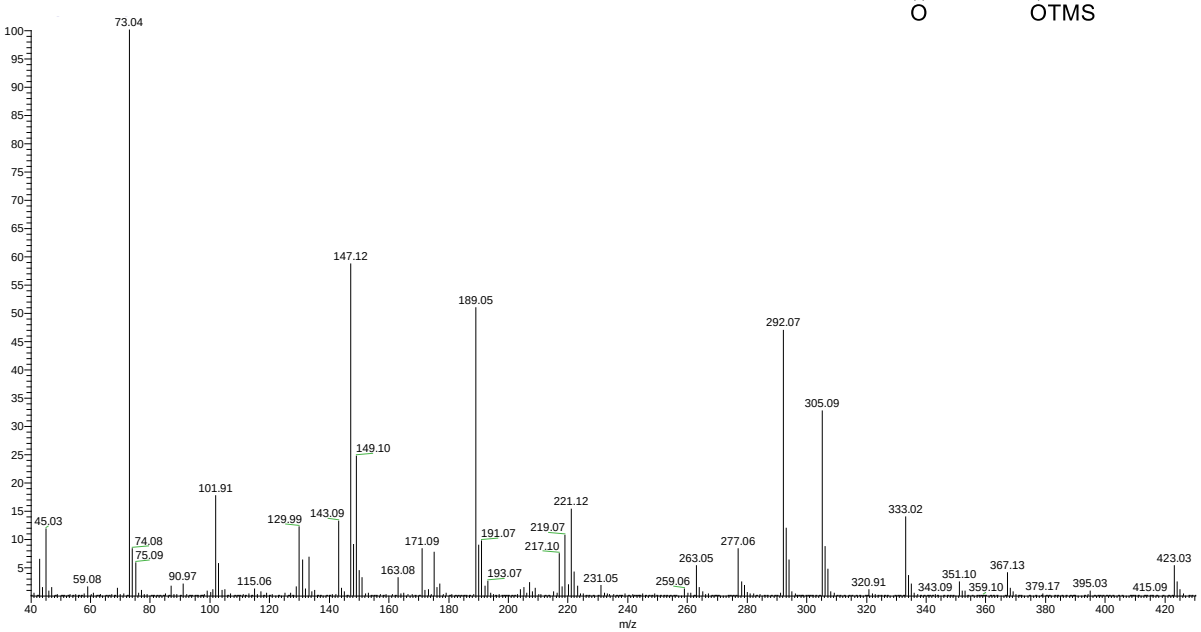
Tartaric acid tetraTMS

$C_{16}H_{38}O_6Si_4$

Molecular weight: 438.17 g.mol^{-1}

Retention time: 21.78 min



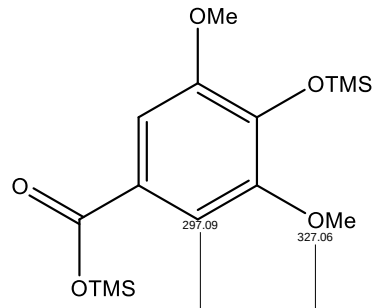


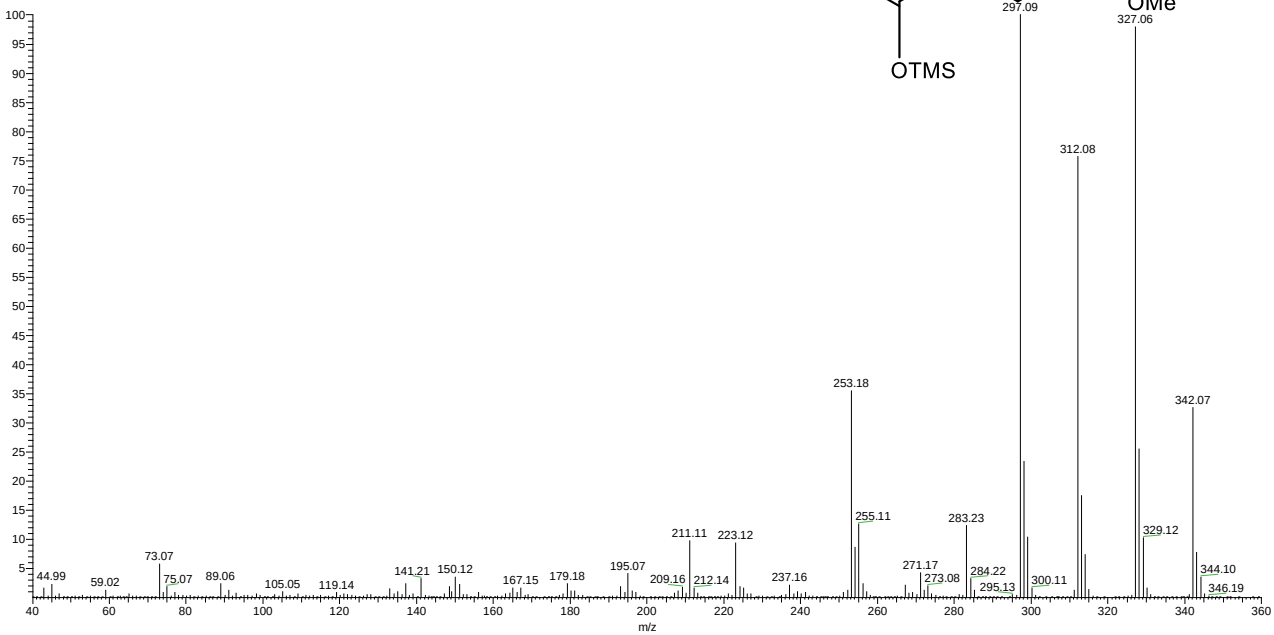
Syringic acid diTMS

$C_{15}H_{26}O_5Si_2$

Molecular weight: 342.13 g.mol^{-1}

Retention time: 30.92 min





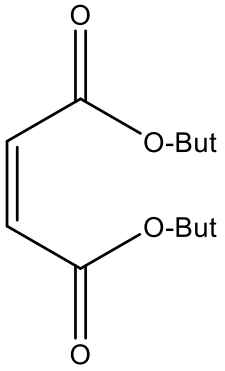
Butylated acids

Dibutyl maleate

$C_{12}H_{20}O_4$

Molecular weight: 228.29 g.mol^{-1}

Retention time: 18.47 min

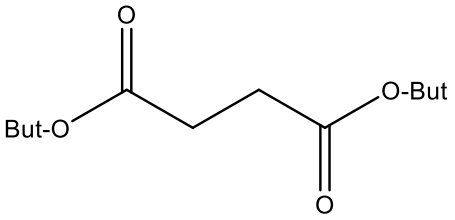


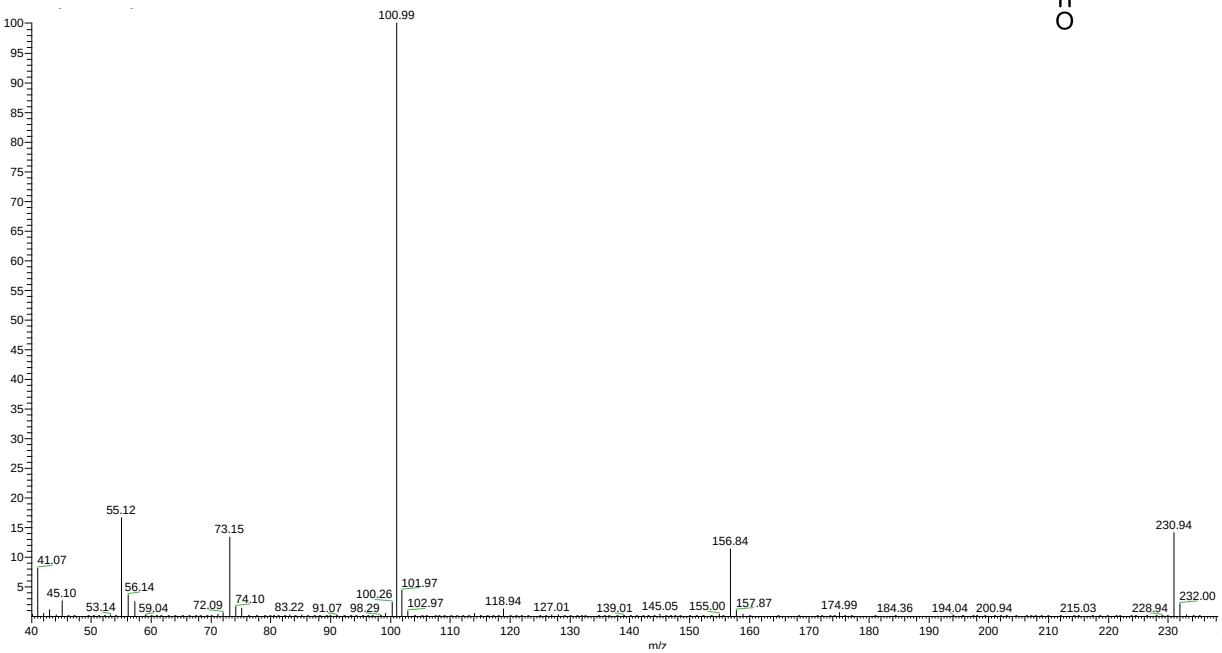
Dibutyl succinate

$C_{10}H_{18}O_4$

Molecular weight: 230.30 g.mol^{-1}

Retention time: 19.51 min



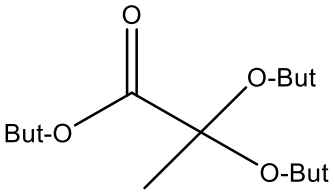


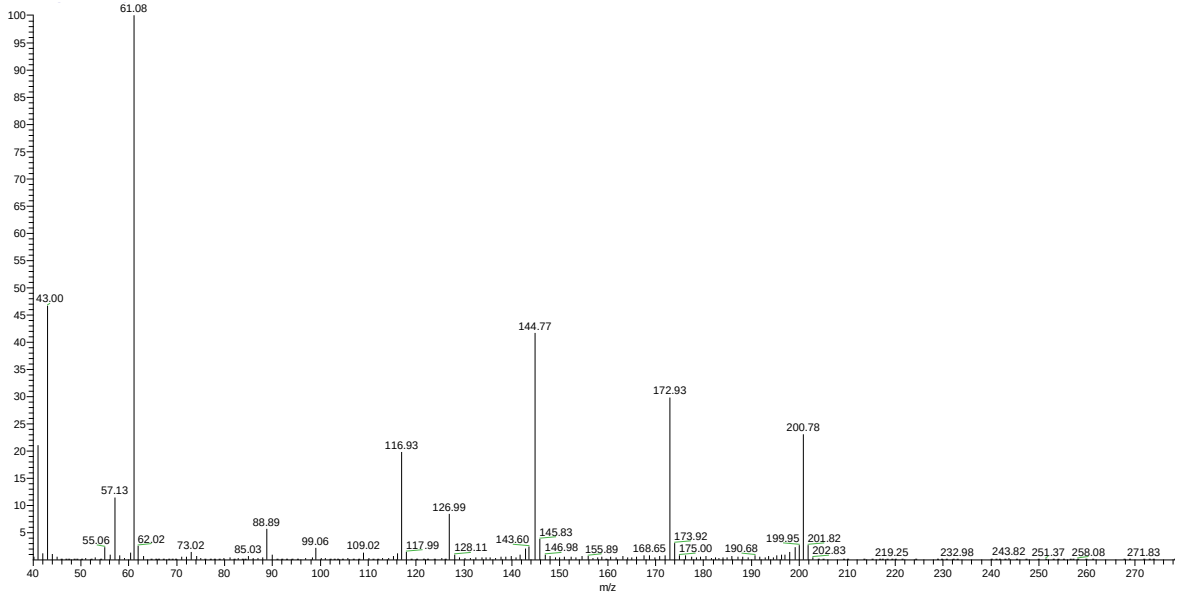
Dibutylacetal pyruvate

$C_{15}H_{30}O_4$

Molecular weight: 274.40 g.mol^{-1}

Retention time: 20.02 min



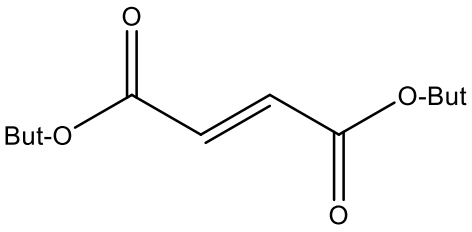


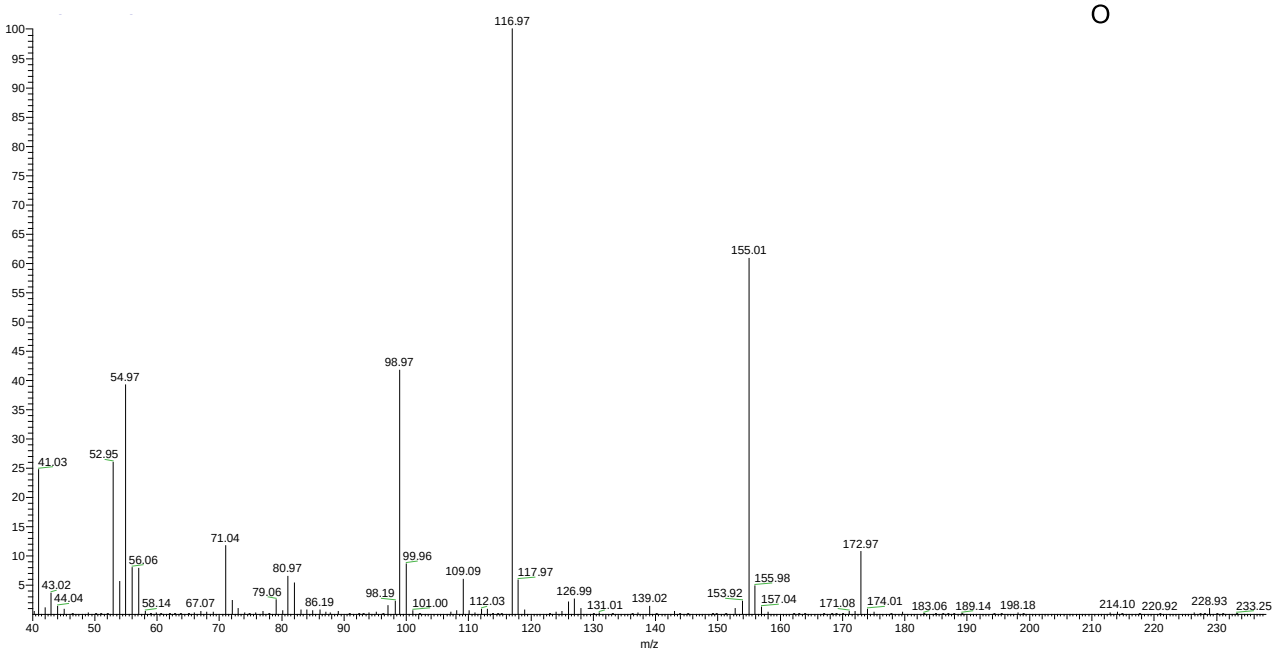
Dibutyl fumarate

$C_{12}H_{20}O_4$

Molecular weight: 228.29 g.mol^{-1}

Retention time: 20.09 min



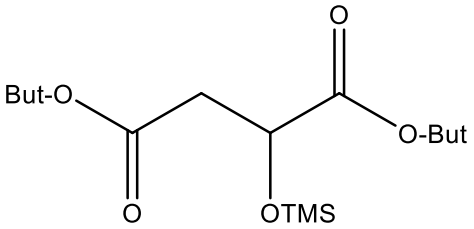


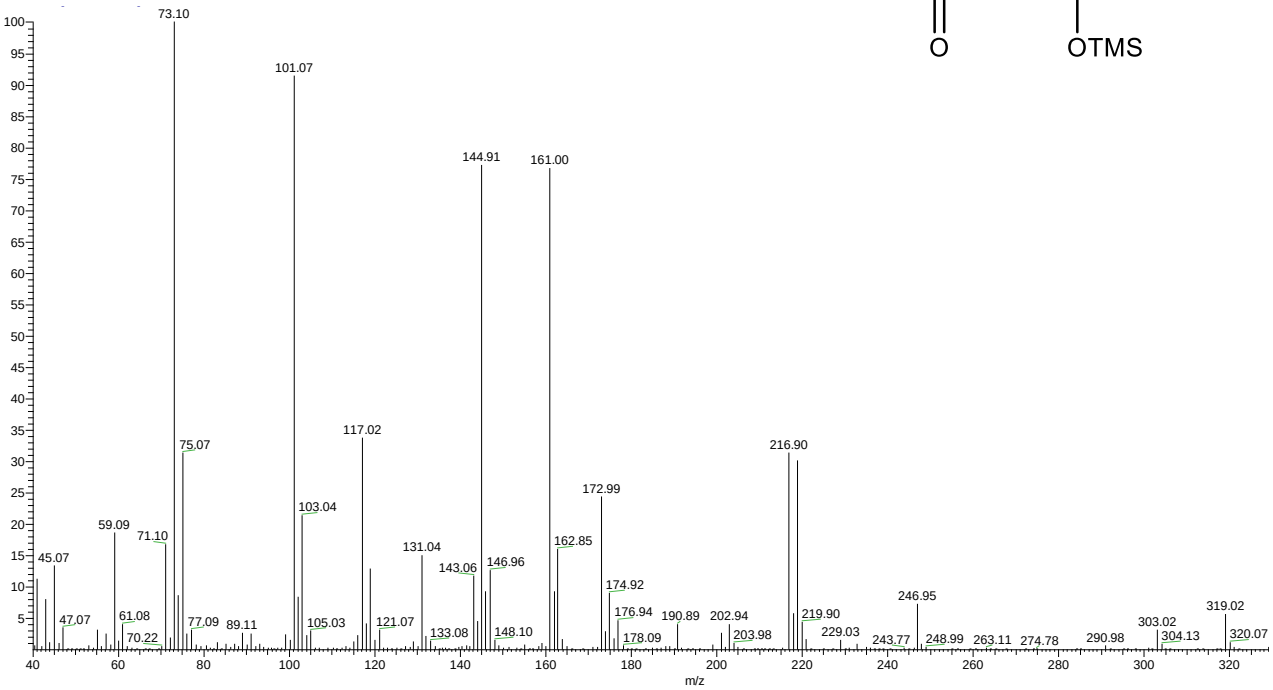
Dibutyl malate TMS

$C_{15}H_{30}O_5Si$

Molecular weight: 318.49 g.mol^{-1}

Retention time: 24.89 min



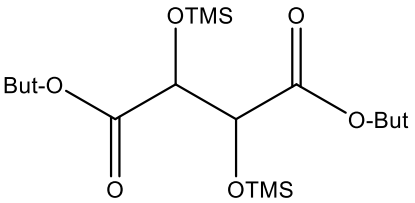


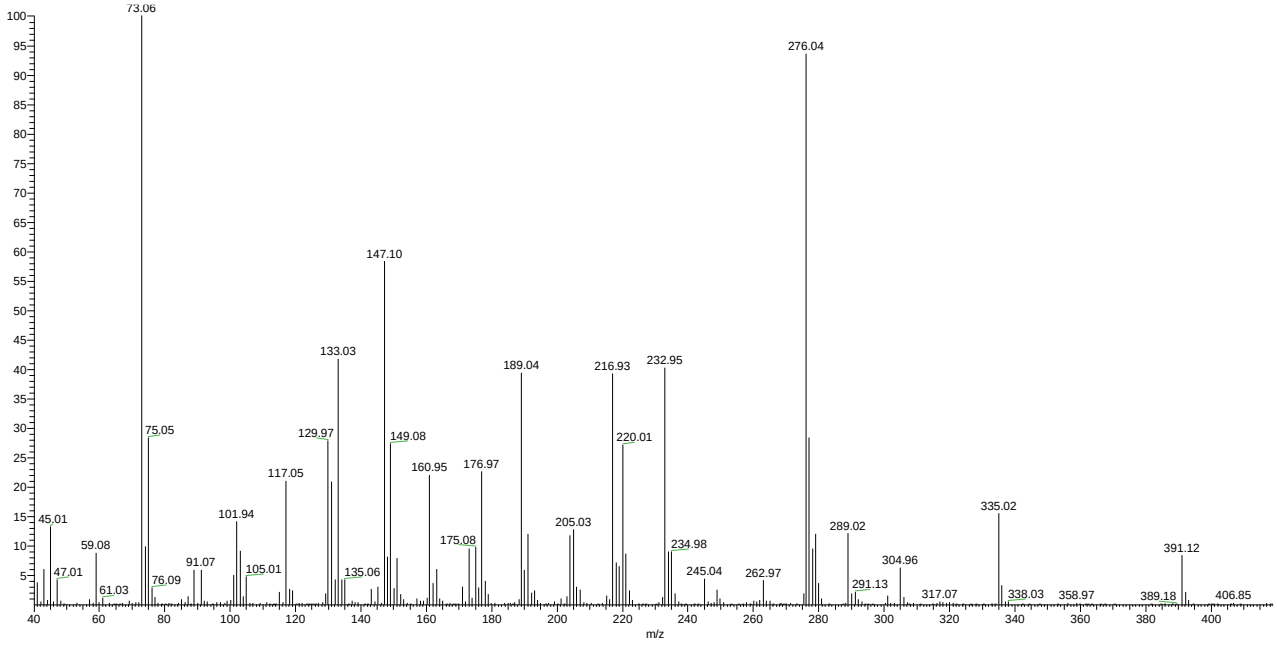
Dibutyl tartrate diTMS

$C_{18}H_{38}O_6Si_2$

Molecular weight: 406.67 g.mol^{-1}

Retention time: 29.89 min



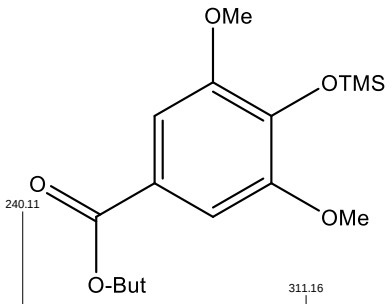


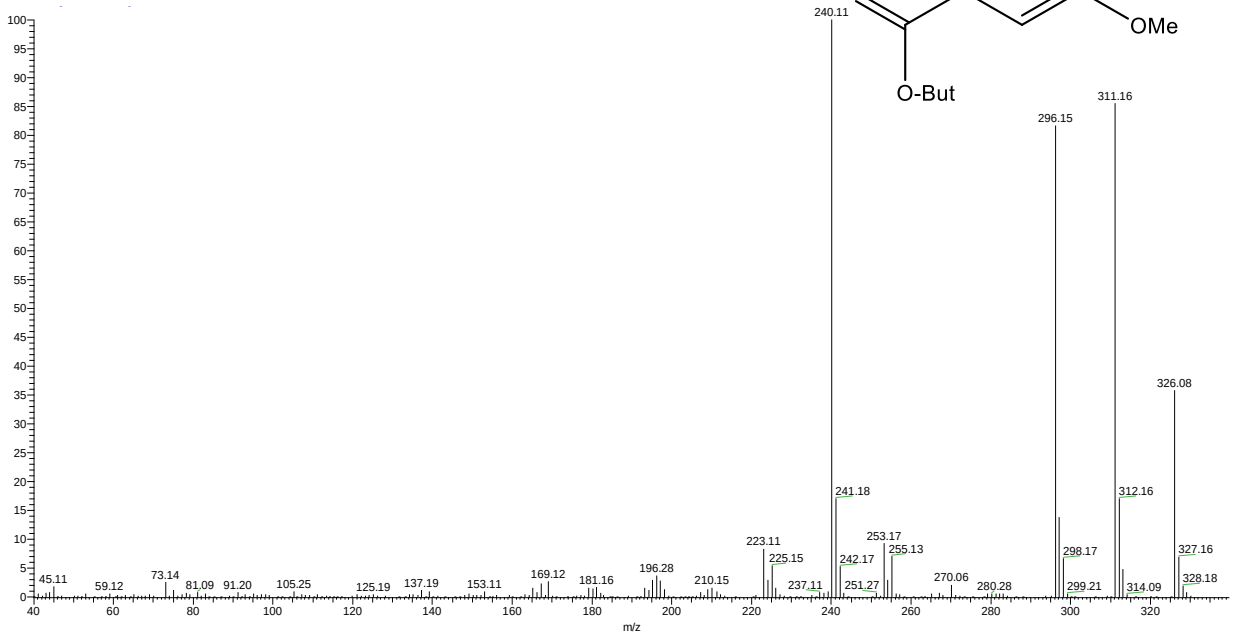
Butyl syringate TMS

$C_{16}H_{26}O_5Si$

Molecular weight: 326.46 g.mol^{-1}

Retention time: 36.50 min



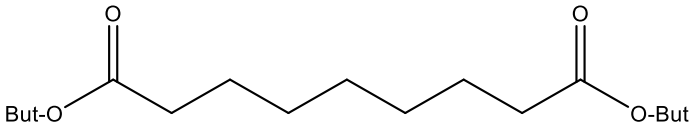


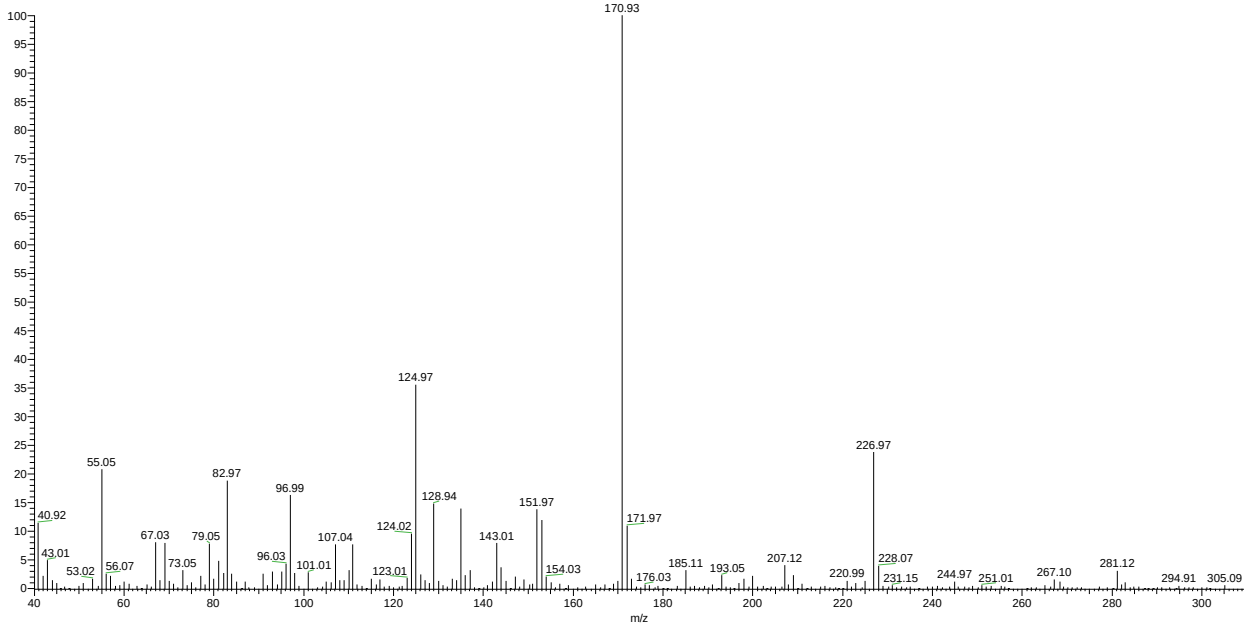
Dibutyl azelaic acid

$C_{17}H_{32}O_4$

Molecular weight: 300.44 g.mol^{-1}

Retention time: 37.40 min





Appendix 4. Retention time and main mass fragments of the molecules

Molecule TMS	Retention time (t_r)	Main mass fragments
Octanoic acid	12’34	75; 201
Maleic acid	12'96	147; 215; 245
Succinic acid	13'16	147; 172; 247; 262
Pyruvic acid	13’57	45; 66; 147; 217
Fumaric acid	13'82	147; 217; 261
Nonanoic acid	14’07	75; 215
Glutaric acid	15’01	147; 261
Decanoic acid	16’34	75; 229
Citramalic acid	16’52	147; 321
Mandelic acid	16’69	147; 296
Malic acid	17'10	147; 189; 233; 245; 335; 351
Adipic acid	17’65	149; 207
Cinnemic acid	18’83	161; 205; 220
m-benzoic acid	19’41	205; 267
Pimelic acid	20’70	155; 239
p-benzoic acid	21’39	223; 267
Tartaric acid	21'78	147; 189; 292; 305; 333; 423
Dodecanoic acid	22’32	75; 257
Napthalene	23’00	183; 198
Suberic acid	23’92	149; 309
Vannilic acid	26’14	267; 312
Isovannilic acid	26’3	297; 312
Azelaic acid	27’48	149; 317
Myristic acid	29’36	117; 285; 300
Syringic acid	30'92	253; 297; 312; 327; 342
Sebacic acid	31’05	331; 346
18-Norabiéta-8,11,13-triene	33’70	159; 241
19-Norabiéta-8,11,13-triene	34’99	159; 241
10,18-Bisnorabieta-5,7,9(10),11,13-triene	35’85	181; 223; 238
Palmitic acid	36’51	117; 313; 328
Tetrahydroretene	36’66	223; 314
10,18-Bisnorabieta-5,7,9(10),11,13-pentaene	37’63	223; 238
Retene	41’5	219; 234
Dehydroabietane	41’77	173; 255; 270
Oleic acid	42’32	117; 128; 198; 338; 356
Stearic acid	43’32	117; 341; 356
Pimaric acid	44’46	121; 241; 257; 359
Sandaracopimaric acid	45’04	121; 241; 257; 359
Isopimaric acid	45’62	241; 256; 359
DHAM	45’79	239; 314
Palustric acid	46’28	241; 359; 374

DHA	47’46	239; 372
Abietic acid	48’06	185; 241; 256; 359
3-hydroxy-DHAM	49’44	191; 402
7-hydroxy-DHAM	50’41	237; 370
3-hydroxy-DHA	50’93	191; 460
7-hydroxy-DHA	51’47	237; 460
15-hydroxy-DHAM	52’65	327; 445; 460
7-oxo-DHAM	53’50	253; 328
15-hydroxy-DHA	53’56	445; 460
7-oxo-DHA	53’71	253; 268; 386
7,15-dihydroxy-DHAM	55’44	7475; 490
15-hydroxy-7-oxo-DHAM	58’9	341; 401; 459
15-hydroxy-7-oxo-DHA	59’03	341; 401; 459

Table A2. Retention time and main mass fragments of the principal molecules identified by GC-MS