eman ta zabal zazu

Universidad del País Vasco
Euskal Herriko Unibertsitatea

SEARCHING FOR THE ORIGIN OF SAMPLES OF GEOLOGICAL, ENVIRONMENTAL AND ARCHAEOLOGICAL INTEREST: ORGANIC BIOMARKERS AS KEY EVIDENCES

LAURA BLANCO ZUBIAGUIRRE

2018

Searching for the origin of samples of geological, environmental and archaeological interest: organic biomarkers as key evidences

Laura Blanco Zubiaguirre

March 2018

A thesis submitted for the international degree of
Philosophiae Doctor in Interdisciplinary Scientific
Strategies in Heritage and Landscape

Supervised by
Dr. Kepa Castro Ortiz de Pinedo
Dr. Maitane Olivares Zabalandikoetxea

Acknowledgments

Laura Blanco Zubiaguirre is grateful to the University of the Basque Country (UPV/EHU) for her pre-doctoral fellowship (from April 2014 to March 2018) as well as to the research group of the Department of Analytical Chemistry, Ikerkuntza eta Berrikuntza Analitikoa (IBeA).

This PhD work has been developed thanks to the financial support received from:

- Punta Begoña project (ref 3171.16REGB) founded by Getxo City Council.
- PRIACE project (Spanish Ministry of Economy and Competetiveness, MINECO, Ref. CTM2012-36612).
- The European Regional Development Funds (FEDER)
- The Special Action (AE14/29) from the University of the Basque Country (UPV/EHU).
- Research Groups of Excellence (Consolidated Groups Program, Government of the Basque Country, ref. IT-742-13)
- Cátedra Territorio, Paisaje y Patrimonio supporting the internationalization of this research work with their action plan for mobility.

Special thanks deserves Dr. Javier García Iñañez together with the Arkeologi Museoa and Dirección de Patrimonio Cultural del Departamento de Cultura del Gobierno Vasco within the project SAI007/13 funded by Bizkaiko Foru Aldundia –Diputación Foral de Bizkaia, for their contribution in this research, providing archaeological samples of great interest for their analysis. I would like to thank as well the Swedish Museum of Natural History (Stockholm, Sweden) for providing me with blubber samples, the Society for the Study and conservation of Marine Fauna AMBAR for the whale oil, Greta Dalle Luche (Griffith University, Brisbane, Australia) and Alessia Andreotti (University of Pisa, Pisa, Italy) for

providing the whale blubber from a humpback whale and Dr. Alex Aguilar, for helping me with the search of reference materials related to whale oil.

Many thanks to Dr. Josefina Pérez-Arantegui, Carlos García-Benito, José Ángel García-Serrano and Julián Pérez-Pérez for giving me the chance to analyze interesting archaeological ceramic samples and organic remains related to wine production.

I would also like to express my sincere gratitude to the research group from the Analytical Chemistry department of the University of Pisa for welcoming me in their laboratories in order to develop a significative part of this PhD work. Especial thanks to Dr. Erika Ribechini, Dr. Jacopo La Nasa and Dr. Ilaria Degano (Department of Analytical Chemistry of the University of Pisa, Italy) for their relevant contribution in this research, through their support, their facilities and their knowledge.

My thankfulness goes also to the research group of the Institute of the Structure of Matter from the Superior Council of Scientific Research (CSIC) for giving me the chance to perform a scientific stay in their laboratories, especially to Dr. Maria Vega Cañamares, for her support and knowledge on Surface Enhanced Raman Spectroscopy and nanoparticle synthesis.

I would like to thank Dr. Maitane Olivares and Dr. Kepa Castro for giving me the chance to perform this PhD Thesis and for their efforts and encouragement all these years.

Finally, I would also like to thank all professors and researchers forming part of the IBeA group, for they help during the development of this research work.

Han pasado 4 años, 5 contando el año del master, desde que me embarqué en la aventura de la investigación, años en los que entre risas y lloros he sacado una tesis adelante. Pero todo esto no habría sido posible sin la gente que me rodea y la gente que he conocido por el camino. Por eso, una tesis no tendría sentido sin dar las gracias a todas estas personas:

A veces parece que fue ayer cuando Luis me presentó a mi directora de master y futura (co)directora de tesis. Maitane, gracias por todo lo que me has enseñado durante estos años y no sólo en el ámbito científico. No ha sido un camino de rosas y hay que reconocer que he pasado momentos duros, pero al final hemos sabido afrontarlos con "optimismo inteligente" y las cosas han salido muy bien. La paciencia hace la ciencia, que nos lo digan a nosotras… Gracias por ayudarme, por preocuparte y sobre todo por animarme. Kepa, gracias a ti también por darme la oportunidad de llevar a cabo esta tesis e introducirme en el mundo de la investigación en el patrimonio cultural. Te agradezco también que me hayas animado a hacer varias estancias desde el principio, con el miedo que me daba marcharme fuera. Y, como no, ¡gracias por el TIRAMISÚ! Cuando todo esto termine haré uno, de momento me estoy entrenando porque tengo un jurado difícil.

Quiero agradecer también al resto de profesores que de un modo u otro han estado presentes en este camino. Luis, gracias por tus aportaciones e ideas, habrá que darle una vuelta al trasplante de árboles de la Gomera a Punta Begoña como futuros sistemas de muestreo (yo me apunto al viaje). Nestor, eskerrik asko arrain eta bale olioa lortzeagatik. Ailette, por hacer que no nos falte de nada en el labo y por estar dispuesta a todo siempre. Iran, beachrock-aren munduan sartzen uzteagatik eta nire tesiko izenburuari aire interesgarria ematen laguntzeagatik. Are, TFG eta TFM bat gidatzeko aukera emateagatik. Y a todos los que formáis parte de este departamento (Gorka, Alberto…etc). Mirari zuri ere eskerrik asko egunero erakusten duzun poztasunagatik eta mintegira heltzen diren barre kutsakorrengatik. Eva, gracias por guiarnos y ayudarnos al dar las prácticas y por dejarnos material siempre que lo hemos necesitado.

A la gente del mintegi, espero que no se me escape nadie… Gracias a todos los que en su día fuisteis los mayores, los ejemplos a seguir y que ya no estáis por aquí: Arantza, Azibar (ay Azibar, dónde quedarán esas grabaciones de media tarde…gracias por tantos momentos de risas), Jone, Julen, Naiara, Oihana, Oier, Oscar, Sandra… Ekhiñe, gracias por tus ánimos y por tu ayuda. Me acuerdo de cuando todavía te sentabas a mi lado y murmurabas así como 5 veces al día, "baina ze ostie..." mientras tratabas datos. Después cambiaste la forma de ver las cosas y me enseñaste a ser más positiva, eskerrik asko! Itsaso, no me creo que tenga que incluirte en este grupo, hace dos días como quien dice estábamos llorando las penas a las 7 de la tarde en el mintegi…te he echado de menos esta última temporada, gracias por tus ánimos doctora, nos vemos pronto! A los demás doctores que seguís por aquí, Olaia (eskerrik asko por la compañía durante todos los descansos de cuando era fumadora) y Ainara, eskerrik asko por los ánimos a los 4 pelados que quedamos. Josean, (te estoy oyendo quejarte por lo que me estoy enrollando…), gracias por ayudarme tanto durante estos últimos años y por hacer más llevaderos los momentos difíciles en los que no sale absolutamente nada bien, siempre sabes sacarle el lado positivo o hacer algún chiste (malo) o broma en la que siempre caigo. Mireia, nahiz eta topera ibili lanean, mila esker beti laguntzeko prest egoteagatik! Zu bai zarela ikerkuntzaren zale amorratua, "más maja que las castañas". Julene, Marco, Ane y Niko, gracias por ayudarme con la parte espectroscópica de la tesis y por dejarme formar parte de la investigación en el beachrock y los biocidas. Olivia, aunque hayas estado poco tiempo en Leioa, gracias por los momentos de café y vinos tan divertidos y por tu milagrosa ayuda con el Photoshop, nos veremos por Irala algún viernes. A Pati y Alfredo gracias también por vuestra ayuda y compañía en los cafés. Leire K., doctoras casi a la par, maldito sea tu propósito de leer antes que yo, me vas a hacer sufrir. Gracias por estar ahí todos estos años siempre dispuesta a aportar ideas para ayudarme cuando las cosas no me han salido. Menuda úlcera de estómago nos va a salir después de tanto café de máquina… Janire, gracias por tu locura, mucha suerte por Cambridge, eres más valiente que ni sé…

A los que todavía os queda un ratito largo, (Dennis, va por ti) mucho ánimo y take it easy, que las cosas poco a poco van saliendo. Bastian, merci beaucoup et bonne chance! Haizea eta Leire M., zuoi eskertu behar dizuedan gauzarik inportanteena ahaztuta neukan euskararen berreskuratzea da. Eskerrik asko egunero nirekin euskaraz egiteagatik, ganbak zuzentzeagatik eta izandako pazientziagatik. Haizea, zorte on eta animo metabolomikarekin! Gutxi falta zaizu eta. Leire, eskerrik asko mintegiko momentuengatik, zenbat barre arratsaldeko musika eta dantza klaseetan, eta noski, txokolateagatik. Asier Cabezas, gracias por tu paciencia y por todo lo que sufriste durante tu año de master con el maldito oxálico, no ha podido con nosotros.

Gracias a toda la gente del CSIC que tanto me enseñó sobre la síntesis de nanopartículas y la técnica SERS. A Mª Vega y Santiago, por darme la oportunidad de hacer la estancia y ayudarme tanto. A Rocío y Diego, espero volver a veros pronto, sois geniales. A Adi, por todo lo que me enseñaste (ay Carlitos, qué mayor te habrás hecho ya) y la alegría que desprendías a diario. A Szuska, Moisés, Francesca, José Vicente, Luis, José Ramón…gracias por recibirme con los brazos abiertos. Aprovecho para dar las gracias también a mis compañeras de piso madrileñas, Irati y Teresa (bueno y Patri, pero te toca más tarde). Esos dos meses me lo pase genial con vosotras y repetiría de cabeza. Teresa, la primera vez que abandonaba Bilbao llena de miedo me hiciste sentir como en casa sin apenas conocernos. Espero que nos reencontremos pronto.

Thanks to the analytical department of the University Of Pisa. Erika, thanks for giving me the chance to work with you twice and for all the facilities and help you offered me. Thanks to the rest of the teaching staff (Ilaria D., Francesca…). Marco, you taught me how to work in your lab and you were the greatest workmate, inside and outside the lab. I wish you all the luck in the world with your PhD and hope to see you again. Diego, thanks for being so nice to me, showing me the city at night and taking me to Firenze, I had a really good time with you and Marc and will always be thankful for including me in your plans. Jacopo, thanks

for teaching me all that I know about TAGs and HPLC-QToF. I really miss your madness, it was great to meet you again in Bilbao, but SO SAD to leave. You all know that you are welcome to my city. Sibila, Arno, Marco Z., Francio… thank you all for your kindness. Ai miei coinquilini, Mateo, Piero, Alfredo and Alice, grazie per tutto. Alice, hola chica! Mi manchi! Ad Alessandro, grazzie per l'hospitalitá e le conversazioni in italiano (or something similar to italian), anche a Massimo, per la sua gentilezza e i dolci.

No me olvido de todos aquellos que han estado a mi lado fuera de la Universidad, porque no todo es trabajo y al final, gracias a vosotros he sido capaz de desconectar, sobre todo en los momentos más difíciles. Gracias a mis amigas, a Irune, por estar conmigo todos estos años, aguantar mis quejas y escucharme, pero sobre todo por hacerme reír. A Bea, por animarme siempre. A Marta, Maite y Nadir, aunque no os vea tan a menudo como me gustaría cuento siempre con vosotras. Gracias a toda la gente del Zulo (Gorka, Igor y Adri gracias por vuestras clases de historia y contexto sobre mis muestras) por lo bien que me lo habéis hecho pasar simplemente tomando una cerveza. A mis amigas de Gorliz, porque sois las mejores e intentáis entender lo que hago, aunque ninguna lo sepa realmente. A Raquel, por su ánimos mañaneros y su alegría y optimismo contagioso. Gracias también a Ainhoa, por escucharme, guiarme y enseñarme a ver las cosas de otra manera y a ser una mejor versión de mi misma.

Adrián, no puedo decirte nada que no sepas. Gracias por estar conmigo durante todos estos años, animándome, interesándote por lo que hago e incluso intentando proponer soluciones a mis problemas en el laboratorio. Pero, sobre todo, gracias por tu cariño, por aguantarme en los malos momentos y ayudarme a mirar hacia delante. Contigo esta última etapa ha sido mucho más fácil (aunque yo a veces haya sido inaguantable). Gracias a Quique, Macu y Leyre por preocuparse por mí y hacerme sentir una más de la familia.

Y, por último, quiero dar las gracias a mi familia por estar siempre ahí. A Jose y Javi, porque me hacéis reír cuando creéis que porque me guste el vino y analice vasijas que contenían vino soy algo así como enóloga… A mis tías Delia y Ana, por el cariño y el apoyo que siempre me habéis dado. A Urko y Olatz, por hacerme reír tanto. A Iñigo, porque me has hecho muchísima compañía estos meses en la biblioteca, me has amenizado las tardes y me lo he pasado genial contigo. A mi hermana Patricia, porque eres la única persona que sabe decir de carrerilla de qué va mi tesis (aunque realmente sólo sabes de que va la mitad, no me atrevo a explicarte la otra mitad) y porque siempre has estado ahí cuando lo he necesitado, aunque vivas lejos con Fer (gracias por los ánimos Fer! Espero que nos veamos pronto y pueda conocerte mejor ahora que voy a tener más tiempo). Patri, gracias por ser la compañera de piso ideal. A aitite y Vicenta, os habéis marchado por el camino, pero siempre os llevaré conmigo, sé que estaríais orgullosísimos de vuestra nieta pequeña.

Ama, no sé ni por dónde empezar…Tu eres la persona a la que más agradecida estoy. Estos cuatro años no habrían sido posibles sin tu apoyo incondicional y tu cariño. Ojalá fuera la mitad de fuerte y positiva de lo que tú eres, me bastaría con eso. Cuando preguntas "algo habré aportado yo también en vuestros logros, verdad?" Sí, mucho más de lo que puedes imaginar. Gracias por ser como eres.

"Happiness only real when shared"

Christopher McCandless

A LYDIA

Y A LOS AITITES

Table of contents

List of abbreviations and acronyms

4,8,12-TMTD	4,8,12-Trimethyl tridecanoic acid
11,12OHC20	11,12-Dihydroxyeicosanoic acid
11,12OHC22	11,12-Dihydroxydocosenoic acid
^{13}C-NMR	Carbon-13 nuclear magnetic resonance

A

–A–	Arachidic acid, eicosanoic acid or ($C_{20:0}$) as acyl chain in TAGs
Ace	Acenaphthene
Ace d-10	Deuterated [$^{2}H_{10}$] Acenaphthene
AcN	Acetonitrile
Acy	Acenaphthylene
Ant	Anthracene
APAA	ω-(*o*-Alkylphenyl) alkanoic acid
ASE	Accelereated solvent extraction
ASF	Asymmetry factor

B

–B–	Behenic acid, docosanoic acid or ($C_{22:0}$) as acyl chain in TAGs
B[a]P	Benzo[a]Pyrene
B[b]F	Benzo[b]Fluoranthene
B[k]F	Benzo[k]Fluoranthene
BaA	Benzo[a]anthracene
BghiP	Benzo[g,h,i]perylene
BNH	28,30-Bisnorhopane
BSTFA	N,O-Bis(trimethylsilyl)trifluoroacetamide

C

–C–	Capric acid, decanoic acid or $C_{10:0}$ as acyl chain in TAGs
C10	Sebacic acid
$C_{8:0}$	Caprylic acid or octadecanoic acid
$C_{10:0}$	Capric acid or decanoic acid
$C_{12:0}$	Lauric acid or dodecanoic acid
$C_{14:0}$	Myristic acido r tetradecanoic acid
$C_{15:0}$	Pentadecanoic acid
$C_{16:0}$	Palmitic acid or hexadecanoic acid
$C_{16:1}$	Palmitoleic acid or 9-cis-hexadenenoic acid

$C_{17:0}$	Heptadecenoic acid
C18	Octadecyl bonded silica based sorbent
$C_{18:0}$	Stearic acid or octadecanoic acid
$C_{18:1}$	Oleic acid or cis-9-octadecenoic acid
$C_{18:2}$	Linoleic acid or 9,12-octadecadienoic acid
$C_{18:3}$	Linolenic acid or 9,12,15-octadecatrienoic acid
$C_{19:0}$	Nonadecanoic acid
C2	Oxalic acid
$C_{20:0}$	Arachidic acid or eicosanoic acid
$C_{20:1}$	Gondoic acid or 11-eicosenoic acid
$C_{20:5}$	5,8,11,14,17-Eicosapentaenoic acid
$C_{22:0}$	Behenic acid or docosanoic acid
$C_{22:1}$	Cetoleic acid or 11-docosenoic acid
$C_{22:6}$	4,7,10,13,16,19-docosahexaenoic acid
$C_{24:0}$	Lignoceric acid or tetracosanoic acid
$C_{24:1}$	Nervonic acid or 9-tetracosenoic acid
C3	Malonic acid
C4	Succinic acid
C4,F	Fumaric acid
C4, M	Maleic acid
C5	Glutaric acid
C6	Adipic acid
C7	Pimelic acid
C8, Ph	Phthalic acid
C9	Azelaic acid
CCD	Central composite design
Chol.	Cholesterol
CIS	Cooled injection system
CPI	Carbon preference index
Cry	Crysene
Cry-d12	Deuterated [$^2H_{12}$] Chrysene
Cu*	Activated copper

D

–D–	Cetoleic acid, 11-docosenoic acid or C22:1 as acyl chain in TAGs
DAG	Diacylglycerol
DahA	Dibenzo[a,h]anthracene
DCM	Dichloromethane
DHA	4,7,10,13,16,19-docosahexaenoic acid or **$C_{22:6}$**
–Dha–	4,7,10,13,16,19-docosahexaenoic acid as acyl chain in TAGs

Diacid	α,ω-dicarboxylic fatty acids
DiC$_x$	α,ω-dicarboxylic fatty acids with x carbon atoms
–Dpa–	5,8,11,14,17-Eicosapentaenoic acid as acyl chain in TAGs
–Dte–	7,10,13,16-Docosatetraenoic acid as acyl chain in TAGs

E

–E–	Gondoic acid or 11-eicosenoic acid as acyl chain in TAGs
–E2–	11,14-Eicosadienoic acid as acyl chain in TAGs
–E3–	11,14,17-Eicosadienoic acid as acyl chain in TAGs
–E4–	Arachidonic acid as acyl chain in TAGs
EPA	5,8,11,14,17-Eicosapentaenoic acid
–Epa–	5,8,11,14,17-Eicosapentaenoic acid as acyl chain in TAGs
ESI	Electrospry ionization
EtOAc	Ethyl acetate
EtOH	Ethanol

F

FA	Fatty acid
FAME	Fatty acid methyl ester
Flr	Fluoranthene
Flu	Fluorene
FT-IR	Fourier transform infrared spectroscopy
FUSLE	Focused ultrasound solid-liquid extraction
FUSLE-LVI-PTV-GC-MS	Focused ultrasound solid-liquid extraction followed by analysis by large volume injection-programmable temperature vaporizer coupled to gas chromatography-mass spectrometry
FUSLE-SPE (MAX)-GC-MS	Focused ultrasound solid-liquid extraction followed by mixed-mode solid phase extraction and gas chromatography-mass spectrometry analysis

G

GC	**Gas chromatography**
GC-C-IRMS	Gas chromatography-combustion isotope ratio-mass spectrometry
GC-MS	Gas chromatography-mass spectrometry

H

HBI	Highly branched isoprenoids
Hex	*n*-Hexane

HLB	Hydrophilic-lipophilic balanced sorbents
HPAH	High molecular weight polycyclic aromatic hydrocarbon
HPLC-APCI-MS	Liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry
HPLC-DAD	Liquid chromatrography with diode array detection
HPLC-ESI-QToF	Liquid chromatography coupled to quadrupole time-of-flight mass spectrometry
HPLC-MS	Liquid chromatography coupled to mass spectrometry
HPLC-MS/MS	Liquid chromatography-tandem mass spectrometry
HRMS	High resolution mass spectrometry
HTGC	High temperature gas chromatography

I

IcdP	Indeno[1,2,3-cd]pyrene
IFA	Isoprenoid fatty acid
IPA	Isopropanol or 2-propanol
IS	Internal standard
IT	Ion trap

K

KOH_{H2O}	Aqueous potassium hydroxide
KOH_{MeOH}	Methanolic potassium hydroxide

L

–L–	Linoleic acid or $C_{18:2}$ as acyl chain in TAGs
–La–	Lauric acid, dodecanoic acid or $C_{12:0}$ as as acyl chain in TAGs
LLE	Liquid-liquid extraction
–Ln–	Linolenic acid or $C_{18:3}$ as acyl chain in TAGs
LOD	Limit of detection
LOD_{instr}	Instrumental limit of detection
LOD_{proc}	Procedural limit of detection
LPAH	Low molecular weight polycyclic aromatic hydrocarbon
LVI	Large volume injection
LVI-PTV	Large volume injection-programmable temperature vaporizer
LVI-PTV-GC-MS	Large volume injection-programmable temperature vaporizer coupled to gas chromatography-mass spectrometry

M

–M–	Myristic acid, tetradecanoic acid or $C_{14:0}$ as acyl chain in TAGs
m/z	Mass-to-charge ratio
MAE	Microwave assisted extraction
MAG	Monoacylglycerol
MAX	Mixed mode sorbent (strong anion exchanger)
MeOH	Methanol
Mixed-mode-SPE-GC-MS	Mixed mode solid phase extraction followed by gas chromatography-mass spectrometry
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MS^1	First mass analyzer in tandem mass spectrometry
MS^2	Second mass analyzer in tandem mass spectrometry
MSn	Tandem mass spectrometry with n mass analyzers
MTBSTFA	N-Methyl-N-tert-butyldimethylsilyltrifluoroacetamide
MUFA	Monounsaturated fatty acid

N

Naph	Naphthalene

O

–O–	Oleic acid, cis-9-octadecenoic acid or $C_{18:1}$ as acyl chain in TAGs
Ø	Diameter
OCP	Organochlorine pesticides
OEP	Odd-to-even preference

P

–P–	Palmitic acid, hexadecanoic acid or $C_{16:0}$ as acyl chain in TAGs
PAH	Polycyclic aromatic hydrocarbon
PCB	Polychlorinated biphenyls
Per-d10	Deuterated [$^2H_{12}$] Perylene
PFC	Perfluorinated compound
Phe	Phenanthrene
Phe-d10	Deuterated [$^2H_{10}$] Phenanthrene
Phy	Phytane
PM_{10}	Particulate matter with ø < 10 µm
$PM_{2.5}$	Particulate matter with ø < 2.5 µm
–Po–	Palmitoleic acid, hexadecenoic acid or $C_{16:1}$ as acyl chain in TAGs

POP	Persistent organic pollutant
Pr	Pristane
Pr/Phy	Pristane-to-phytane ratio
PTFE	Polytetrafluoroethylene or teflon
PTV	Programmable temperature vaporizer
PUFA	Polyunsaturated fatty acid
Py-GC-MS	Pyrolysis-gas chromatography-mass spectrometry
Pyr	Pyrene

Q

Q	Quantifier m/z
q	Qualifier m/Z
QqQ	Triple quadrupole
QToF	quadrupole time-of-flight mass spectrometry

R

R^2	Determination coefficient
RP-SPE	Reversed phase solid phase extraction
RSD	relative standard deviation

S

s	Standard deviation
–S–	Stearic acid, octadecanoic acid or $C_{18:0}$ as acyl chain in TAGs
–S4–	Stearidonic acid, octadecatetraenoic acid or $C_{18:4}$ as acyl chain in TAGs
SAX	Mixed-mode strong anion exchanger
SCX	Mixed-mode strong cation exchanger
SEM	Scanning electron microscopy
SIM	Selected ion monitoring
SPE	Solid phase extraction
SPE-GC-MS	Solid phase extraction followed by gas chromatography-mass spectrometry

T

–T–	tetracosenoic acid or $C_{24:1}$ as acyl chain in TAGs
TAG	Triacylglycerol or triacylglyceride
TAR	Terrigenous/aquatic ratio
TBA	Tetrabutylammonium

T_{cis}	Cooled injection system temperature
TLE	Total lipid extract
TMCS	Trimethylchlorosilane
TMN	Trimethylnaphthalene
TNH	25,28,30-Trisnorhopane
TOC	Total organic carbon
TOF	Time-of-flight mass spectrometer
t_r	Retention time
t_{vent}	Vent time

U

UHPLC	Ultra-high performance liquid chromatography
UMB	Ultrasound micro bath
US EPA	United States Environmental Protection Agency
USB	Ultrasound bath

V

V_{inj}	Injection volume
VOC	Volatile organic compound

W

WAX	Mixed-mode weak anion exchanger
WCX	Mixed-mode weak cation exchanger
WSOC	water soluble organic compound
WWTP	Wastewater treatment plant

Resumen

Los biomarcadores orgánicos se definen como moléculas específicas capaces de proporcionar información valiosa acerca de su fuente, así como de los materiales en los cuales han sido encontrados. La principal característica que diferencia a los biomarcadores del resto de compuestos orgánicos consiste en su elevada estabilidad a lo largo del tiempo (siglos) a pesar de ser expuestos a diversos procedimientos de degradación y envejecimiento tales como la sedimentación o el enterramiento.

De acuerdo con esta definición, los biomarcadores pueden emplearse en múltiples campos de aplicación, siempre y cuando se proporcione una definición adecuada según cuál sea el fin específico de su uso. Desde comienzos de los años 80, los biomarcadores, conocidos también en aquella época como fósiles químicos, comenzaron a emplearse en el campo de la geoquímica para obtener información acerca de la edad y las condiciones de sedimentación de las rocas madre y para conocer el origen de la materia orgánica presente en ellas. En este sentido, una gran cantidad de biomarcadores han sido empleados para proporcionar información sobre el origen, la edad e incluso la madurez de la roca madre, como es el caso de alcanos, esteranos, terpanos, biomarcadores aromáticos y porfirinas.

Por otro lado, el desarrollo de la instrumentación y de los métodos analíticos han favorecido el uso de los biomarcadores como herramientas esenciales para la detección de fuentes de contaminación que afectan los diversos compartimentos del medio ambiente. Así, compuestos tales como los hidrocarburos policíclicos aromáticos (PAHs), compuestos perfluorados (PFCs), bifenilos policlorados (PCBs) o pesticidas organoclorados (OCPs), han sido empleados como biomarcadores para establecer el origen de su presencia en matrices acuosas (mares, lagos, ríos, etc.), en suelos y sedimentos y en la atmósfera. Además de los compartimentos mencionados y la salud humana, los contaminantes orgánicos también afectan en cierto modo al patrimonio cultural, siendo capaces de deteriorar la superficie de

los monumentos y los edificios históricos, impidiendo así la conservación de los mismos. Como consecuencia, surge la necesidad de estudiar el grado de incidencia de los compuestos orgánicos en el patrimonio construido. Así, estudios previos han detectado los PAHs y los compuestos orgánicos solubles en agua (WSOCs) en las superficies dañadas de monumentos y edificios históricos y han atribuido su presencia a diversas fuentes tales como las primarias (procesos de combustión y emisión directa) o secundarias (formadas a partir de precursores orgánicos gaseosos).

Recientemente, la ciencia multidisciplinar surgida a partir de la colaboración de arqueólogos, químicos analíticos e historiadores se ha centrado en el análisis de los residuos orgánicos preservados en materiales arqueológicos tales como las cerámicas, residuos de plantas, resinas o restos humanos. La aplicación de los biomarcadores en este campo ha ayudado a revelar no sólo el uso de estos materiales en la antigüedad, sino también información sobre la historia y la cultura que los rodea. Los biomarcadores empleados en estudios arqueológicos pueden clasificarse según su naturaleza química, encontrando así los terpenoides (indicadores de resinas), esteroles tales como el colesterol (biomarcador de grasa animal), ácidos grasos y triacilgliceroles y otros biomarcadores específicos que no pertenecen a ninguna de las anteriores familias, como puede ser el ácido tartárico, biomarcador del vino.

Teniendo en cuenta todo esto, el desarrollo de métodos analíticos adecuados para la determinación de los biomarcadores es clave, ya que frecuentemente estos se encuentran a niveles de traza. Por ello, son necesarios métodos rápidos, sensibles, exactos y precisos para la determinación de biomarcadores conocidos y desconocidos. A pesar de la existencia de numerosos métodos establecidos en bibliografía, teniendo en cuenta la información que esconden las muestras de carácter medio ambiental, cultural e histórico en los campos de aplicación mencionados, es necesaria una mayor investigación para desarrollar nuevos métodos. Consecuentemente, los principales objetivos de esta tesis doctoral fueron:

- El desarrollo y validación de una serie de métodos analíticos sensibles, cuantitativos y respetuosos con el medio ambiente para determinar biomarcadores orgánicos de distinta naturaleza en muestras geológicas, medio ambientales y arqueológicas.
- La aplicación de los métodos desarrollados para la determinación de biomarcadores específicos y la obtención de información sobre muestras de carácter geológico, medio ambiental y arqueológico. En este sentido, se analizaron muestras de beachrock (arenas cementadas), morteros de un edificio histórico, aerosol marino y cerámicas arqueológicas.

El primer objetivo se llevó a cabo con el desarrollo de cuatro métodos analíticos para la determinación de biomarcadores en diversas matrices. Todos los métodos se basaron en el uso de cromatografía de gases acoplada a espectrometría de masas (GC-MS) para la detección de biomarcadores conocidos y desconocidos. Tras la derivatización mediante sililación con BSTFA, se obtuvo una sensibilidad adecuada para determinar ácidos dicarboxílicos en muestras de mortero y en cerámicas arqueológicas, así como ácidos grasos y compuestos derivados en cerámicas arqueológicas. Además, el uso de la inyección de grandes volúmenes (LVI-PTV) acoplada a GC-MS permitió cuantificar PAHs en muestras de beachrock. Para extraer los biomarcadores de las matrices sólidas, se hizo uso de ultrasonidos focalizado (FUSLE) en todos los métodos, lo cual permitió la extracción cuantitativa de compuestos específicos haciendo uso de diversos disolventes o mezclas de los mismos. En cuanto a la preconcentración de los biomarcadores, se emplearon técnicas tales como la extracción líquido-líquido (LLE) con hexano para los ácidos grasos y derivados. Sin embargo, esta técnica no fue adecuada para la preconcentración de los ácidos dicarboxílicos (WSOCs y biomarcadores del vino), para los cuales se empleó la extracción en fase sólida haciendo uso de cartuchos de modo mixto. Los cartuchos de modo mixto con intercambio aniónico fuerte permitieron la preconcentración de compuestos ácidos en un rango amplio de polaridades (desde el ácido oxálico C2 hasta el sebácico C10) a través de

un mecanismo dual (fase reversa e intercambio iónico), consiguiendo extracciones cuantitativas, especialmente para los ácidos dicarboxílicos de cadena corta.

Tras determinar las condiciones de trabajo óptimas para la determinación de biomarcadores específicos, los cuatro métodos fueron validados en términos de exactitud, precisión y límites de detección. Como consecuencia, fuimos capaces de proponer nuevos métodos sensibles, exactos y precisos, como alternativa a aquellos establecidos en bibliografía, para determinar biomarcadores orgánicos específicos a niveles de traza en muestras geológicas, medio ambientales y arqueológicas. Una vez adquirido el conocimiento de las propiedades de los biomarcadores a estudiar y una vez desarrolladas las metodologías analíticas necesarias se llevó a cabo el segundo objetivo de la tesis: la identificación de los biomarcadores conocidos y desconocidos en muestras reales para relacionarlos con la "historia" que estas esconden.

Se determinaron los 16 PAHs considerados como prioritarios por la US EPA en muestras de beachrock recogidas en la costa de Bizkaia. La presencia de este fenómeno en esta localidad es algo extraña ya que el beachrock se forma normalmente en climas más cálidos. Por consiguiente, la identificación de biomarcadores orgánicos fue crucial para obtener un mayor conocimiento sobre las condiciones biofísico-químicas de formación del beachrock en este lugar. La identificación del gammacerano, 17α(H),21β(H),28,30-bisnorhopano, 17α(H),21β(H)-norhopano, 22S-17α(H),21β(H)-homohopano, 22R-17α(H),21β(H)-homohopano, 22S-17α(H),21β(H)-bishomohopano y 22R-17α(H),21β(H)-bishomohopano revelaron que la actividad microbiana junto con ambientes sedimentarios subóxicos y un aporte de contaminantes orgánicos elevado, son los responsables de la formación acelerada de este fenómeno en latitudes templadas inusuales. Estos resultados permitieron establecer nuevas líneas de investigación basadas en la caracterización de la fracción orgánica en muestras de beachrock de diversos orígenes con el fin de obtener una visión más amplia acerca de la formación de este fenómeno.

El desarrollo de la metodología basada en la extracción en fase sólida empleando cartuchos de modo mixto acoplada al análisis mediante GC-MS permitió determinar si los compuestos dicarboxílicos de cadena corta (WSOCs) presentes el aerosol marino afectan realmente a los morteros de un edificio histórico del siglo XX (Galerías de Punta Begoña, Getxo) situado en un entorno costero. Tras realizar los análisis pertinentes, se concluyó que las actividades llevadas a cabo en el puerto industrial adyacente podrían ser responsables de las concentraciones de ácidos decarboxílicos determinadas tanto en muestras de aerosol marino como en muestras de mortero. De hecho, los análisis revelaron una mayor concentración de estos compuestos en la zona de las galerías orientada hacia el puerto industrial, mientras que se detectaron concentraciones más bajas (valores por debajo del límite de detección) en el punto de muestreo menos expuesto. Estos compuestos, una vez depositados, reaccionan con los materiales carbonáceos de los edificios. En el caso concreto del ácido oxálico, se da una reacción con el carbonato de calcio para formar el oxalato de calcio, altamente insoluble, dañando así la estructura del material. La detección de ácido oxálico en su forma libre revela un aporte diario de estos compuestos orgánicos solubles en agua. Toda esta información muestra la necesidad de una monitorización a largo plazo de estos compuestos, tanto en aerosol marino como en mortero, para estudiar el impacto que producen en las galerías de Punta Begoña. Además, el método desarrollado puede extrapolarse a otros casos de interés en los que el patrimonio construido se ve afectado por la contaminación atmosférica.

Tras superar el reto analítico basado en la determinación de compuestos orgánicos pequeños en presencia de sales interferentes, se llevó a cabo el análisis de muestras arqueológicas relacionadas con la producción de vino. Se analizaron muestras de fragmentos cerámicos pertenecientes a los siglos II-I AC y muestras de residuos orgánicos recuperados de las paredes de ánforas de origen Romano. A pesar de la antigüedad de las muestras arqueológicas, los ácidos tartárico, málico, succínico, fumárico, cítrico y siríngico fueron detectados en todas las muestras analizadas, confirmando así su uso para almacenar

vino. Además, también se identificaron los ácidos dehidroabiético y 7-oxo-dehidroabiético en las muestras de residuo orgánico, los cuales evidencian la presencia de resina de pino, empleada antiguamente para impermeabilizar la superficie interior de las ánforas y como antioxidante para preservar el vino. Curiosamente, en una de las cerámicas arqueológicas también se detectaron biomarcadores de aceite vegetal (ácido oleico y linoleico). Por lo tanto, estas evidencias químicas sugirieron un posible uso múltiple del ánfora analizada. Si bien es cierto que las conclusiones arqueológicas deben ser interpretadas por especialistas, la detección de todos los compuestos de interés, junto con otros no esperados, mediante el método desarrollado fue satisfactoria.

El análisis de cerámicas arqueológicas sin residuos visibles suele ser el caso más común que se presenta. Teniendo en cuenta que la mayoría de ánforas son lavadas o aclaradas antes de llegar al laboratorio, el reto analítico que supone su análisis es aún mayor. De hecho, nos enfrentamos a estos dos problemas al analizar fragmentos de cerámicas arqueológicas del siglo XVI-XVII relacionadas con el almacenamiento de grasa de ballena. Durante ese período la pesca de la ballena en las costas de Canadá (Terranova y Labrador) fue una de las actividades más importantes para la economía del País Vasco. Los balleneros vascos capturaban las ballenas, extraían el aceite de la grasa y lo transportaban de vuelta a la costa vasca, donde era almacenado en grandes contenedores cerámicos. Para encontrar evidencias de grasa de ballena se desarrolló un método basado en FUSLE, LLE y análisis mediante GC-MS. Se determinaron una serie de compuestos biomarcadores de productos marinos tales como el $C_{14:0}$, $C_{16:0}$, $C_{20:1}$, $C_{22:1}$, $C_{18:1}$ e incluso $C_{20:5}$ y $C_{22:6}$ a nivel de trazas. Como compuestos de degradación, únicamente se detectaron el ácido sebácico (ácido α,ω-dicarboxílico) y el ácido 11,12-dihidroxieicosanoico, procedentes del $C_{20:1}$. Por tanto, se encontraron evidencias para sugerir que los fragmentos bajo estudio pertenecen a contenedores cerámicos destinados a almacenar grasa de ballena.

La metodología desarrollada dio como resultado un procedimiento rápido y aplicable a cualquier cerámica arqueológica para obtener una primera idea sobre la composición lipídica del material. Sin embargo, el uso de técnicas complementarias fueron necesarias para determinar biomarcadores desconocidos específicos de grasa de ballena. Con la colaboración del departamento de Química Analítica de la Universidad de Pisa, tuvimos la oportunidad de complementar los análisis mediante la aplicación de las técnicas cromatografía líquida de alta resolución acoplada a espectrometría de masas de tiempo de vuelo con cuadrupolo e ionización electrospray (HPLC-ESI-QToF) y GC-MS. Además, se emplearon muestras frescas de grasa de ballena de diversas especies, proporcionadas por el Museo de Historia Nacional de Suecia (Estocolmo), como materiales de referencia. Se determinó la distribución de los triacilgliceroles (TAGs) mediante HPLC-ESI-QToF y se encontraron diferentes perfiles dependiendo de la especie. Para aquellas especies pertenecientes al mismo género *(Balaenoptera)*, se obtuvieron perfiles muy similares en las muestras de referencia, mientras que el resto de especies de distinto género presentaron perfiles significativamente diferentes. Además, se establecieron una serie de ratios para cada género bajo estudio. De este modo, al analizar las muestras cerámicas siguiendo el mismo procedimiento, se determinó no sólo la presencia de grasa de ballena, sino también el posible género de la especie de la cual se extrajo el aceite: *Balaenoptera.* A paritr de los análisis llevados a cabo mediante GC-MS en las muestras arqueológicas y los materiales de referencia, se establecieron perfiles de ácidos grasos similares, permitiendo la detección de compuestos de degradación tales como los ácidos α,ω-dicarboxílicos, dihidroxiácidos y compuestos isoprenoides (ácido fitánico, pristánico y 4,8,12-trimetiltridecanoico) altamente relacionados con productos marinos y no detectados con la metodología anterior (FUSLE-LLE-GC-MS).

Los resultados obtenidos fueron muy satisfactorios y de ellos surgió la necesidad de ampliar los análisis a un mayor número de muestras arqueológicas y materiales de referencia. De acuerdo con los datos históricos y arqueológicos, la ballena franca glacial

(género *Eubalaena*), conocida también como ballena de los vascos, y la ballena de Groenlandia (género *Balaena*) fueron las principales especies capturadas por los balleneros vascos en Labrador y Terranova durante ese período (s. XVI-XVII), por lo que deberían estudiarse especies pertenecientes a estos dos géneros en futuros experimentos.

Teniendo en cuenta todos estos resultados, se puede concluir que la presente tesis doctoral cumple los objetivos establecidos. Todos los métodos analíticos fueron satisfactoriamente desarrollados y aplicados a muestras reales obteniendo resultados interesantes y valiosos.

Chapter 1

Introduction

"Knowing where you came from is no less important than knowing where you are going."

Neil deGrasse Tyson

Organic compounds are literally defined as any of a large class of chemical compounds in which one or more atoms of carbon are covalently linked to atoms of other elements, most commonly hydrogen, oxygen, or nitrogen, with the exception of carbides, carbonates, and cyanides, which are not classified as organics [1]. Nevertheless, this definition does not take into account what organic compounds have meant and still mean nowadays.

Life happened. But, what role did organic compounds play during life creation? Exactly how did an inorganic world become organic? Not easily, we should go back 3.5 billion years to try to understand it. Inorganic compounds transmuted to organic ones meaning that somehow non-carbon based substances bonded with carbon to form new compounds. Life on Earth is carbon based; hence, inorganic substances that were abundant on primordial Earth must have turned into organic materials and somehow, after those new organic compounds joined, the very first cell emerged [2].

Many theories about how organic compounds appeared are still under debate. The "organic soup" theory of Oparin and Haldane about how life began, which sounds like a recipe, is based on the premise that the early Earth had a reducing atmosphere, which exposed to energy in various forms produced simple organic compounds ("monomers"). These compounds were accumulated in a "soup", and by further transformations, more complex organic molecules, and ultimately life, were developed [3]. Modern variants arose after Oparin and Haldane's theory and experimentation showed how inorganic processes under primitive Earth conditions could form organic molecules such as amino acids [4]. On the other hand, previous extraterrestrial origin of organic molecules has also been accepted as hypothesis. The theory of panspermia proposes that meteorites or cosmic dust from comets may have brought significant amounts of complex organic molecules to Earth, kicking off this way the evolution of life [5]. These two hypotheses are the only testable hypotheses of organic compounds' and life's origin that are currently available [6].

All the mentioned theories assume that the organic compounds were the building blocks of life. However, where they came from and how they made it is a matter of considerable disagreement. Maybe the building blocks came first. Or maybe it all formed at once. Or maybe the first cell was developed by a completely alien process no one theorized before. We will probably never know. What we know now is that organic compounds are much more than carbon based molecules and we owe them life.

1.1. Chemical fossils, biological markers or biomakers

From mainly biological origins, organic compounds can be incorporated into sedimentary rocks and preserved during geological periods. Then they are returned to the Earth's surface, by natural processes or human action, to participate again in biosynthetic reactions. This is the so-called carbon cycle [7]. Thus, apart from being the base of life during the early Earth, organic compounds also act as molecular fossils, that is, they were originated from biochemicals in formerly living organisms and preserved for geological periods [8]. The term "chemical fossil" was first applied by Eglinton et al. [9], but soon evolved to "biological marker" [10] and was finally contracted to the widely used term "biomarker". The main characteristic of these compounds is that they show little or no change in chemical structure from their parent organic molecules in living organisms.

The usefulness of biomarkers relies on the information that their complex structures give when compared to other simpler molecules. As an example, methane (CH_4), one of the simplest organic compound on Earth, could not be considered as a biomarker since it can be generated when heating almost any organic compound and, consequently, it provides non-specific information. On the contrary, in sedimentary environments, lipids are chemically reduced to hydrocarbon skeletons when they escape the remineralization processes given during metabolic pathways of organisms. These hydrocarbon skeletons act as biomarkers since they can remain intact over hundreds of millions of years in sedimentary rocks, giving valuable information about their precursor structures [8].

According to Petters et al. [8], three are the characteristics that distinguish biomarkers from the rest of the organic compounds:

i) The structures of biomarkers are composed of repeating subunits, indicating that their precursors were components in living organisms.
ii) Each parent biomarker is common in certain organisms. These organisms can be abundant and widespread.
iii) They are chemically stable during sedimentation and early burial, being this structural characteristic the most important one for biomarkers. Biomarkers exist since their structures remain intact during sedimentation and diagenesis processes, being diagenesis the chemical, biological and physical changes that organic matter undergoes.

1.1.1. Other definitions for biomarkers

Before moving on to the classification of biomarkers, it should be highlighted that the information mentioned beforehand refers to biomarkers in the field of geochemistry. However, the term biomarker is also used in other scientific fields such as toxicology and medical sciences. According to Decaprio et al. [11], biological markers or biomarkers reflect molecular and cellular alterations that occur along the temporal and mechanistic pathways connecting exposure to toxic chemicals or physical agents and the presence or risk of clinical disease. Within this context, during the last decades significant efforts have been made to search for potential biomarkers to be used in the emerging discipline of molecular epidemiology [12]. Nevertheless, other authors suggest many other definitions of biomarkers depending on the research field. In this sense, the growth in popularity of the term “biomarker” is reflected in the large number of definitions that appear in the literature [13]. Some of them are summarized in Table 1.1.

Benford et al. [13] highlighted the great difficulties associated with the imprecise use of this word after showing 16 different definitions of the term "biomarker" in the field of toxicology. Hence, despite being recognized that it was originated in the field of geochemistry [8], the term biomarker should be defined before its use within a specific context.

Table 1.1: *Definitions of "biomarker" depending on the field of use.*

Science	Biomarker definition	Ref.
Environmental science	Intact and predominantly functionalized biomolecules found in recent sediments, including ancient DNA, that, together with other environmental markers in groundwater, rivers, lakes, oceans, and the atmosphere, can be used to identify specific source materials.	[8,14,15]
Medicine/Pharmacology	Compounds and genetic markers that are used to diagnose cancerous tumors, the effects of aging and nutritional intake.	[8,11,13]
Toxicology	Components in biological fluids, cells, tissues, or whole organisms that indicate the presence, magnitude, and exposure of an organism to a toxin.	[8,11,13,16]
Cell biology	A molecule that facilitates the detection and isolation of specific cell types.	[13,15]
Genetics	A DNA fragment that causes disease or contains information about susceptibility to a disease.	[15]
Paleobiology	Mineralogical, elemental, isotopic, and morphological indicators for the presence and activity of life in the geological record.	[8,14,15]
Astrobiology	Anything that could yield experimental evidence for the existence, past or present, of life in our solar system and beyond.	[15]

1.1.2. Classification of biomarkers according to their application field

Biomarkers can also be classified according to their use. As they reveal information about their origin, biomarkers are widely used to infer information about many topics in the fields related to organic geochemistry, environmental pollution and archaeology.

The scheme shown in Figure 1.1. summarizes the classification of biomarkers according to the field of application. Organic geochemistry is a discipline that studies all organic compounds in the geosphere and covers a wide range of scopes [17]. Geochemical biomarkers are thoroughly used to understand the origin of petroleum, to analyze the organic matter preserved in rocks and reveal information about the source rocks, or to study biodegradation processes [8,14]. Some other organic biomarkers can also act as key molecules to identify, asses and monitor environmental pollution issues. Therefore, they can be used as indicators to prevent or minimize environmental pollution impact on the environment, human health or even cultural heritage. Last but not least, the characterization of organic biomarkers in archaeological samples can led to the clarification of several aspects of daily past life related to traditions, trade, rituals, etc. [18].

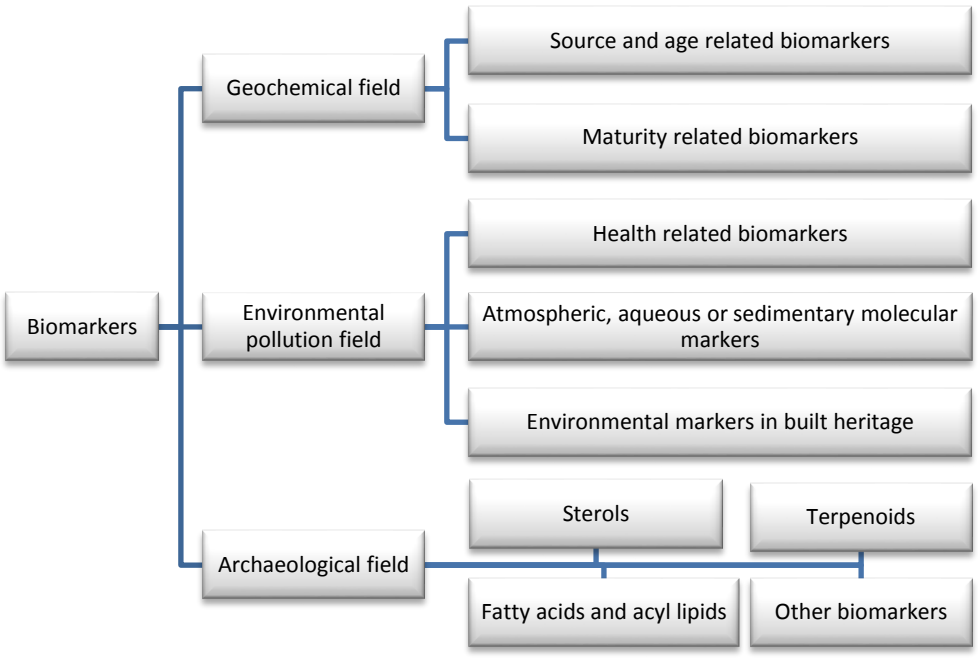


Figure 1.1: *The main classification of biomarkers according to the application field.*

1.2. Biomarkers in geochemical studies

Biomarkers in geological samples are organic compounds that can be structurally related to its precursor molecule, which occurs as a natural product in plant, animal, bacteria, spore, fungi or any other potential source material. Their distributions give information on the source, maturity and biodegradation of organic matter or oils [19]. It is worth noting that during the 1970s the works related to biomarker analysis were focused on the study of recent sediments [20], and thanks to the obtained results and information, their application was expanded to petroleum studies.

Biomarker analysis is required in order to obtain information on the organic matter input and depositional conditions of the sediments and on the lithology, geological age and paleoclimatic conditions in which source rocks were formed. Source and age-related, and maturity-related organic geochemical biomarkers can be sub-classified according to the information that they provide. Some organic biomarkers such as (i) alkanes and acyclic isoprenoids, (ii) steranes and diasteranes, (iii) terpanes and related compounds, (iv) aromatic steroids and hopanoids, and (v) porphyrins provide information about the source and age of the geological material under study (see Table 1.2). Some of these compounds (i.e., terpanes, steranes, aromatic steroids and hopanoids, and porphyrins) together with polycadienes can provide information about the rock maturity as well (see Table 9.1 in the annexes). Examples of the most important organic biomarkers are described in the following lines and some of their structures are shown in Figure 1.2.

1.2.1. Source- and age-related biomarker parameters.

a) Alkanes and acyclic isoprenoids

There are many alkanes and acyclic isoprenoids that act as biomarkers in the field of geology. Ratios involving *n*-alkanes (terrigenous/aquatic ratio or TAR) have been used to

estimate the land-plant or the aquatic input of organic matter in recent sediment extracts [21]. Similarly, the ratio between odd-carbon numbered alkanes and even-numbered *n*-alkanes, known as the odd-to-even preference (OEP) or the carbon preference index (CPI), is widely used in geochemistry works. These values give evidences about the source of the organic matter (marine or land-plant) enclosed in the sedimentary rock, or about the maturity of the sedimentary rocks, as well [22]. Alkyl substituted alkanes act also as biomarkers [14]. As an example, mid-chain monomethyl alkanes have been identified in many oils and source rocks of Precambrian age as potential biomarkers that suggest cyanobacteria in the source-rock setting [23,24].

In the case of isoprenoid compounds, the pristane/phytane ratio (Pr/Phy) provides a measure of redox conditions during diagenesis: values of Pr/Phy > 1 suggest oxic deposition conditions, whereas values Pr/Phy < 1 are generally attributed to anoxic conditions [7]. Squalene is another saturated isoprenoidal alkane that is related with a direct archaebacterial input (see Figure 1.2) [7,14].

b) Steranes and diasteranes

Steranes, which are by-products of steroids or sterols via diagenetic and catagenetic degradation and saturation processes, are potential biomarkers in chemistry. Similarly to the CPI value, the C30/(C27-C30) steranes ratio provides information about the nature of the organic matter input (i.e., marine versus terrigenous). The information can be even more specific when this ratio is plotted against other ratios involving hopanes or 17α-homohopanes (terpanes), giving the latter information about the salinity conditions [25]. Many other examples of biomarkers included in this family are listed in Table 1.2.

c) Terpanes and related compounds

This family of compounds comprises a huge range of biomarkers with different specificities. Tricyclic terpanes are used to: (i) correlate crude oils and source-rock extracts, (ii) predict source-rock characteristics, and (iii) evaluate the extent of thermal maturity and biodegradation [26]. For instance, 28,30-bisnorhopane (BNH) and 25,28,30-trisnorhopane (TNH), found at high concentrations, are typical compounds of petroleum source rocks deposited under clay-poor anoxic conditions [27]. Another important biomarker included in this group is gammacerane. The presence of gammacerane indicates a stratified water column in marine and non-marine source-rock depositional environments, commonly resulting from highly reducing hypersaline conditions during deposition of organic matter [28].

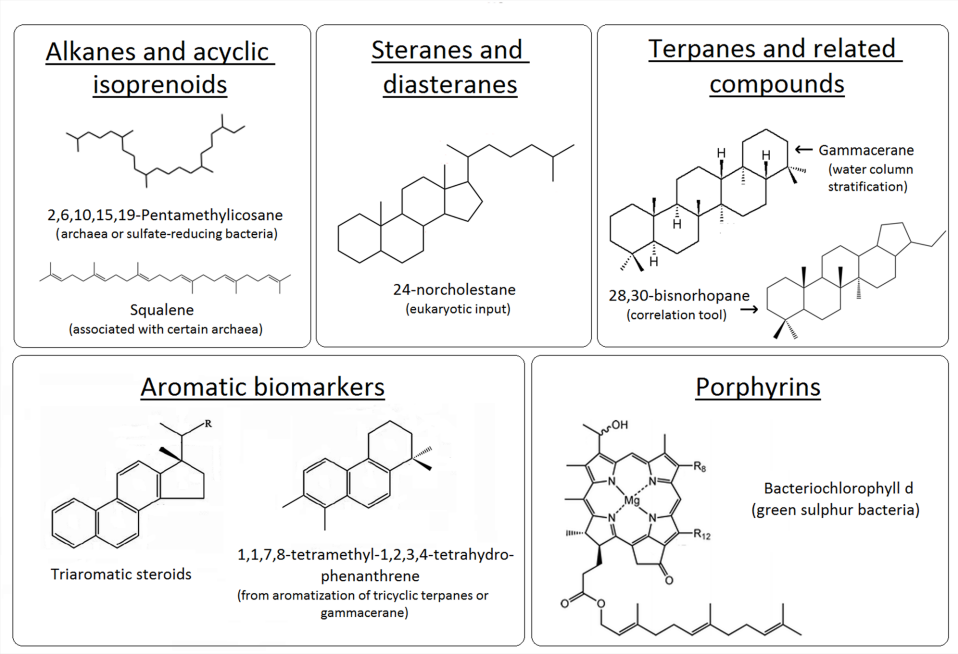


Figure 1.2: *Geochemical biomarkers arranged by families of compounds.*

d) Aromatic biomarkers

Aromatic compounds provide valuable information about the organic matter input. According to Garrigues et al. [29], biogenic polycyclic aromatic hydrocarbons (PAHs) act as molecular markers (further discussion on the term "molecular marker" will be shown on section 1.3.) that provide information about the origin of the sedimentary organic matter and about the geochemical transformations occurring during diagenesis. The ternary diagram involving C27-C28-C29 carbon-ring monoaromatic steroids, used for correlation studies between oil and source rock [30], is another typical example of aromatic organic compounds that can be used as biomarkers.

e) Porphyrins

Porphyrins are tetrapyrrolic compounds occurring naturally as metal complexes or free-base species. They can be isolated from crude oils or rock extracts and demetallated before analysis. These compounds are not often used as biomarkers since they cannot be analyzed by the most frequently employed technique for biomarker analysis (i.e. gas chromatography-mass spectrometry). Nevertheless, ratios of vanadyl and nickel porphyrins are used to establish the redox conditions during source rock deposition [31], whereas porphyrin distributions are used to correlate oils and source rocks [14].

It is worth mentioning that geochemical investigations normally do not use just a specific biomarker but a combination of biomarkers from the previously listed families. In this way, as much information as possible about the characteristics of the source rock can be obtained.

Table 1.2: *Source- and age-related biomarker parameters arranged by families of related compounds.*

Biomarker parameter	Examples	Specificity	References
Alkanes and acyclic isoprenoids			
Acyclic isoprenoids	Phytane, pristane and squalene	Highly specific for certain archaea and for correlation.	[32,33]
Highly branched isoprenoids	C20,C25,C30 and C_{35} hydrocarbons	Diatom markers in Jurassic to Tertiary source rocks and oils.	[34]
Macrocyclic alkanes	Homologous series from ~C15 to C34	Originated from algaenan in the lacustrine alga *B. braunii*.	[35]
β-carotane and related carotenoids	Relation of 20S and 20R at C-14 and C-17 in the C29 regular steranes	Highly specific for lacustrine deposition.	[36]
Steranes and diasteranes			
Regular steranes/17α-hopanes	Relation of C_{27}, C_{28} and C_{29} ααα(20S+20R) and αββ(20S+20R) compounds with C29-C33 pseudohomologs	Moderate specificity for relative input from eukaryotes versus prokaryotes. Maturity may increase this ratio.	[37]
C28/C29	Relation of C28 and C29 steranes	Age-related parameter for oils lacking terrigenous input.	[28]
4-Methylsteranes	C28- C30 analogs of steranes substituted at C-4 and C-24 C30 dinosteranes	Potentially highly specific for marine or non-marine dinoflagellates or bacteria.	[28]
C27, C28 and C29 diasteranes	-	Highly source-specific when plotted in ternary diagrams.	[38]
Terpanes and similar compounds			
Cadinanes	Polycadinene	High specificity for resinous input from higher plants.	[39]
Homohopane distributions	C31-C35 hopanes	Useful in order to assess source-rock redox conditions and for correlation.	[40]
C31/C30 hopane	-	Useful in order to distinguish marine from lacustrine source-rock depositional environments.	[14]
30-Norhopane/hopane	-	Indicator of anoxic carbonate or marl source rocks and oils when values >1.	[14]
Aromatics			
1,1,7,8-tetramehyl-1,2,3,4-tetrahydrophenantrene	-	Originated by aromatization of tricyclic terpanes or gammacerane.	[41]
Trimethylnaphthalenes	1,2,7- and 1,2,5-Trimethylnaphthalene	Specific for angiosperm input.	[42]
Long-chain alkylnaphthalenes	-	Abundant in crude oils and rock extracts where organic matter is dominated by *Botryococcus braunii* or *Gloeocapsomorpha Prisca*.	[43]
Polynuclear aromatic hydrocarbons	Naphthalene and Benzo(*ghi*)perylene	Common in carbonaceous chondrites. Can be specific for paleo-fires.	[44]
Porphyrins			
V / (V+Ni) porphyrins	Relation between vanadyl and nickel porphyrins	Low ratios reflex oxic to suboxic conditions while high ratios reflect anoxic sedimentation.	[31]
Maleimides	1H-pyrrole-2,5-diones	Biomarkers for bacteriochlorophylls c/d/e in green sulfur bacteria and chlorophyll a in phytoplankton.	[42]

1.3. Biomarkers in environmental pollution studies

After World War II, the population increase led to a large-scale production of synthetic organic compounds together with a high combustion of fossil fuels, which meant the appearance of the so-called persistent contaminants in the environment [8]. Thanks to the rapid development of analytical methodologies, the structures and chemical behavior of these compounds were thoroughly studied and, currently, the governments have enough information to regulate the use or production of a large amount of these compounds. They are introduced as pollutants to the atmosphere, sediments, rivers, lakes and oceans because of human's activities together with other natural sources and, eventually, they do not only cause harm to the environment but also to human health and even cultural heritage. Hence, they can act at the same time as markers of environmental quality.

In environmental issues, although many compounds are cataloged as biomarkers and other markers (molecular markers, biogeochemical markers or simply, environmental markers) they do not fulfil the three characteristics that, according to Peters et al. [8], differentiate biomarkers from the rest of organic compounds (see section 1.1.). In fact, the majority of the markers that will be mentioned in this section (e.g. PAHs and WSOCs) are considered non-biomarkers. Nevertheless, as they play a key role to evaluate environmental contamination issues, they cannot be ruled out.

Due to the large variety of anthropogenic organic compounds, it is quite difficult to classify environmental markers according to their chemical nature. Hence, in the following lines, some of the contaminants that will be analyzed in the present PhD thesis work will be described.

1.3.1. Atmospheric, aqueous and sedimentary environmental markers

The uncountable sources of production, use and disposal of numerous chemicals commonly employed in medicine, industry, agriculture and even in common household conveniences, led to the widespread occurrence of organic pollutants. The uncontrolled discharge of such substances into the environment, even at trace concentrations, contributes to the accumulation of some of them in the different compartments, with potentially detrimental effects to both ecosystems and human health. Organic chemicals generated by human activities enter the environment in vast quantities and their release can reach a large temporal and spatial range. The identification of the sources, transport pathways and destination of organic pollutants represent a challenge to the scientific community, but is necessary to solve some environmental issues.

Water is a vital natural resource for humans, being also indispensable for all ecosystems. However, the constant release of organic pollutants by means of landfill leachate, urban runoff, refinery effluent and industrial or wastewater treatment plant (WWTP) discharges into these aquatic bodies promotes their accumulation, endangering this way both the aquatic ecosystems and human health [45]. The concern on the water quality has prompted many case-studies focused on defining the sources and occurrence of persistent and emerging organic pollutants in aquatic environments [46-52] (see Figure 1.3). As it can be observed, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), organochlorine pesticides (OCPs) and perfluorinated compounds (PFCs), to name a few, have numerous sources of discharge to the different aquatic systems, where they can accumulate. The determination of such compounds can provide information of the sources but also they can act as markers in environmental quality assessment studies.

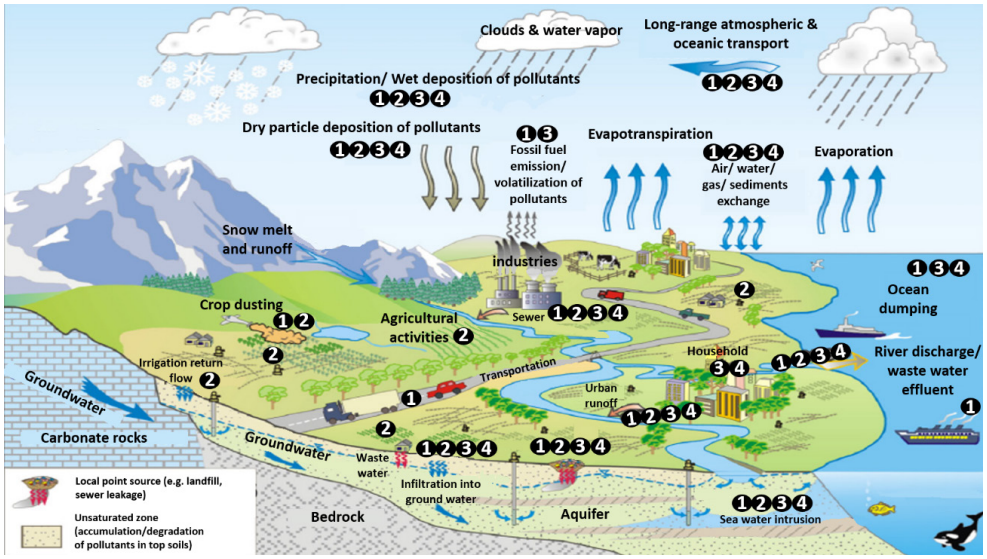


Figure 1.3: *Schematic figure showing sources and transport processes for persistent organic pollutants and their interaction with the hydrological cycle. Numbers indicate classes of pollutants; 1= polycyclic aromatic hydrocarbons (PAHs); 2= organochlorine pesticides (OPCs); 3= polychlorinated biphenyls (PCBs); 4= perfluorinated compounds (PFCs).*

Sediments and soils are also important reservoir for POPs, such as OCPs, PAHs, PCBs, dioxins and dioxin-like compounds (see Figure 1.3). These organic pollutants may have a petrogenic, pyrogenic, biogenic or diagenetic origin and their trends can be used as markers to identify their sources [53]. Similarly to the concern on the quality of water bodies, sediment pollution is a global problem in developed and developing countries. The contamination of agricultural soils with pesticides, fertilizers, plastics, wastewater irrigation or sewage application has been observed during the last years worldwide. The loading of these pollutants means a risk to the ecological functions of soils, plant growth and consequently, human health [54]. On the other hand, sediments are one of the most easily accessed natural archives used to evaluate and reconstruct historical pollution trends in aquatic environments [55].

Finally, all the previously mentioned pollutants (PCBs, PAHs, pesticides, etc.) are released to the atmosphere due to the continued global industrialization (see Figure 1.3). Most of them are present in gaseous phase, while a few sorb onto suspended particles due to their semi-volatile nature [56]. PAHs are an example of markers that are partitioned between gas and particulate phases and then transported to water bodies, vegetation, animal bodies and soils as final sinks. Their use as molecular markers has led to the identification of significant stationary sources such as boilers [57], incinerators [58], coking [59], steel and iron industries [60], paper burning [61], coal fired power plants or asphalt plants [62], together with mobile sources such as gasoline and diesel fueled engines [63,64] and ships [65].

Apart from these pollutants, large quantities of the so-called volatile organic compounds (VOCs) are emitted into the atmosphere from both anthropogenic and natural sources. Several of these compounds have a potential impact on climate due to their properties as greenhouse gases and their ability to form aerosol particles on oxidation. These organic compounds are defined according to Environmental Protection Agency (EPA) as any compound that participates in atmospheric photochemical reactions and has some specific properties such as, vapor pressure >10 Pa at 25 °C, a boiling point of up to 260 °C at atmospheric pressure and 15 or less carbon atoms [66,67]. Alkanes, alkenes, alkynes, aromatic compounds such as, benzene, toluene or xylene and oxygenated compounds (e.g., formaldehyde, acetone or formic acid) are some of the compounds that fulfil these properties. Some of these compounds are not found in the gaseous phase, but in the aerosol particles due to their lower volatility (e.g. dicarboxylic acids such as oxalic, malonic, succinic, maleic or adipic acid) [68]. VOCs are used as markers in order to understand their sources, distributions and chemical transformations and, this way, their impact can be modeled and controlled.

1.3.2. Environmental markers in built heritage

Cultural Heritage encompasses the material and immaterial testimonies of civilization, history and culture developed by communities and passed on from one generation to the next one as an integral part of their identity. Based on international guidelines, buildings, groups of buildings and monumental works of art outstanding for a distinguished artistic or historic value and having indissoluble bond with the territory where they are located are considered *Immovable Cultural Heritage* inside the classification of *Tangible Cultural* Heritage. Here, for simplicity, this type of cultural heritage will be referred as built heritage.

The preservation of built heritage is not merely threatened by environmental degradation pathways, but also by the social, economic and political context. As discussed in section 1.3.1., the fast industrial revolution caused a worrying pollution in the atmosphere and, currently, the concern is still increasing. Such was the impact that, since the beginning of the 19th century, pollution became one of the main causes of built heritage deterioration [69-71]. As Madariaga stated in 2015 [72], there is an analogy between human and built heritage's health, in the sense that a healthy individual, during his life, suffers a progressive and natural ageing. Built heritage, in the same way, remain unaltered when it is located in a controlled atmosphere, without any negative influence from the surrounding environment. However, when it undergoes environmental stress its life might be shortened [72].

Despite the efforts of many institutions to ban or control the release of many organic and inorganic pollutants to the atmosphere, buildings can absorb and accumulate these compounds acting as repositories of past pollution. In fact, significant amounts of pollutants have been found even after the removal of the emission source [70]. Thus, the knowledge of damage is essential to study the atmosphere-materials interaction, identify pollutant sources and take decisions related to prevention, restoration and conservation.

The main inorganic substances that seriously affect built heritage are sulphur and nitrogen oxides (SO_x and NO_x) together with carbonaceous particles [69,73-77]. These pollutants increase the salt content in building materials causing crystallization processes and occasionally generating micro-cracks because of significant changes of salt volume. On the other hand, wet and dry deposition processes combined with gypsum crystal growth result in a dirty, grey-to-black crust formation, in which marine aerosols, dust and particulate matter of various natures, including carbonaceous particles (soot) and heavy metals from atmospheric origin, are entrapped in the mineral matrix [77,78]. These blackish structures, known as black crusts, cause aesthetic damage and make the surfaces of buildings more fragile (see Figure 1.4). The carbon present in black crusts may come from three different origins: i) calcium carbonate, which is mainly derived from the stones and mortars acting as underlying materials, ii) atmospheric particles containing elemental and organic carbon, and iii) biological weathering [70,75].

While the presence of inorganic compounds (mainly atmospheric sulphur and nitrogen compounds) in black crusts, mortars or cementitious materials and stone surfaces is reported in the literature [79-83], the determination of organic molecular markers to assess the influence of pollution is scarce.

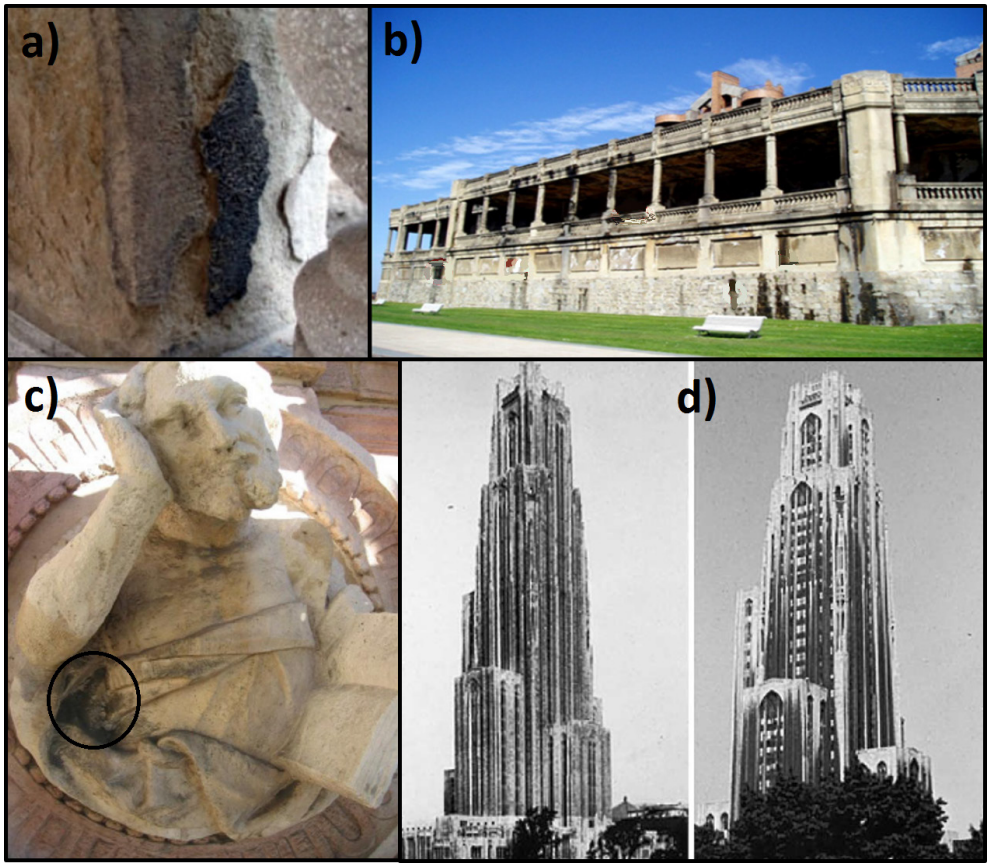


Figure 1.4: *Examples of built heritage affected by environmental pollution. Formation of black crusts in areas protected from the rain in the Cpzaski palace in Kraków (Polonia) [79] (a) and on the Aula Magna facade in Milan (Italy) [75] (c). Deterioration of the facades of two historical buildings with the appearance of black damaged layers due to air pollution in Punta Begoña Galleries in Getxo (Northern Spain) [70] (b) and the cathedral of Learning in Pittsburgh (USA) [84](d).*

In this framework, some recent works analyzed the presence of PAHs in materials of built heritage as marker of their quality status [70,73,85]. When PAHs enter the environment, they are subjected to redistribution and transformation processes, being atmospheric deposition the main input of these compounds to built heritage [73]. Additionally, due to their hydrophobic nature and persistence, they can be retained in

materials such as mortars or black crusts for long periods of time. For example, Orecchio analyzed the organic fraction of the stone of the Temples of Agrigento (Italy), and used the ratios and concentrations of PAHs as molecular markers to identify anthropogenic combustion processes as the main problem of deterioration [73]. PAH ratios were also used by Martínez-Arkarazo et al. to point out the combustion of fuels as the main source of deterioration of sandstones and limestones in the palace house in the metropolitan Bilbao (Basque Country, Northern Spain) [85]. The work performed by Prieto-Taboada et al. [70] is another example of the damage on historical buildings potentially caused by PAHs. According to their observations, PAHs of pyrolytic sources detected in black crusts were one of the responsible of the darkening of a historical building facade located near an industrial harbor [70].

More recently, water-soluble organic compounds (WSOCs) have been used as molecular markers in the stone surfaces of historical monuments [75]. The atmosphere contains WSOCs in both gaseous and liquid phase, and the deposition of these organic compounds in the walls of built heritage can be due to direct emission (primary origin) or may be formed from gaseous organic precursors (secondary origin).

Formate, acetate and oxalate WSOCs, constitute an important fraction of the total carbon that is present in the damaged layers of buildings. Sabbioni et al. [74] reported that primary and secondary atmospheric pollutants must be taken into account as important sources of the mentioned WSOCs, especially in the black crust formation on historic monuments. More concretely, they suggested that the oxalate encountered in black crusts likely originates from the metabolism of microorganisms and protective treatment of surfaces, whereas primary and secondary atmospheric pollutants are likely the main sources of formate and acetate anions [86]. The migration of these compounds through stone porosity causing further damages in the building material was also studied by Fermo

et al. [77], showing the importance of the determination of WSOCs in building materials to stablish the health-status of historical buildings.

Nevertheless, there is still controversy on the role that short chain organic acids play in the determination of deterioration of cement materials due to the fact that they are ubiquitous and conclusions are not so straightforward [76,77,86].

1.4. Biomarkers in archaeological studies

Mankind has always used natural materials around us to produce functional objects or works of art. Objects that are part of our cultural heritage, such as archaeological objects and the organic residues found in association with them (e.g. food, balms or medicines, paintings, textiles, sculptures, furniture, etc.), contain a wide variety of natural and synthetic organic materials [87]. In this respect, there has been a significant interest in the study of amorphous organic materials found at archaeological sites. Human remains and artifacts contain chemical fossils that provide important clues about the past. Critical evidence of humankind's origin, diversification, migration, interactions and cultures can be achieved with the chemical analysis of these materials, providing insights into the use of natural products such as resins, fats and oils from animal or vegetal origin, among others [8,88]. Consequently, organic residue analysis is one of the most established task in archaeology.

1.4.1. The *archaeological biomarker* concept

*Archaeological biomarker*s were first defined in 1993 as those substances occurring in organic residues that provide information relating to human activity in the past [88].There is a high similarity between the use of organic biomarkers in geochemistry and archaeological applications, since in both cases evidences of past activities are discovered. Nevertheless, the *archaeological biomarker* concept is distinctive, since it is mainly focused

on the study of ancient biomolecules that can yield information relating to human activity. It was first defined specifically for lipid biomarkers (i.e., the organic soluble components of living organisms such as fats, waxes or resins) but it can be applied to any class of biomolecules, such as ancient DNA, proteins, carbohydrates, pigments, etc. [89]. Thus, herein, the defined concept of biomarker does not have to fulfil the three characteristics that, according to Peters et al. [8], differentiate biomarkers from the rest of organic compounds.

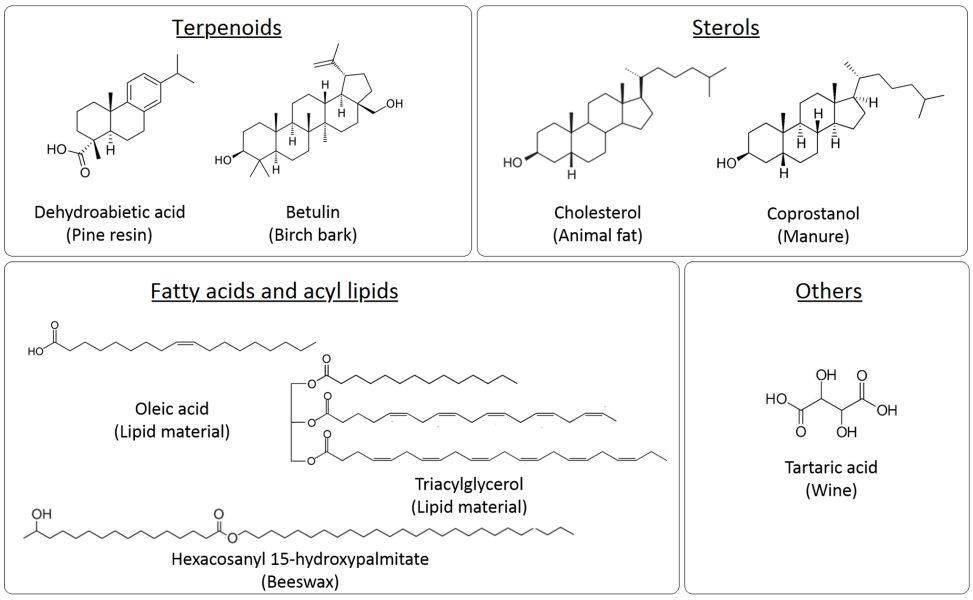


Figure 1.5: *Chemical compounds used as biomarkers of specific sources (in brackets) arranged according to their chemical structures.*

Since biomarkers are diagnostic and specific stable molecules present in the ancient material or formed over the centuries due to ageing, the main goal of this multidisciplinary field is to search for the "chemical fingerprints", compounds or mixtures of compounds that define the origin of an organic residue. In some cases, just a single component or biomarker

can provide insights about the origin of an organic residue. Figure 1.5 shows some examples of compounds used as biomarkers of specific sources arranged according to their chemical structures.

1.4.2. Occurrence of organic residues

Organic residues widely survive under different circumstances and over considerable timescales. Figure 1.6 shows the different locations or artifacts in which, according to the literature, organic residues have been found and for which archaeological biomarker studies have been applied to trace their source [89].

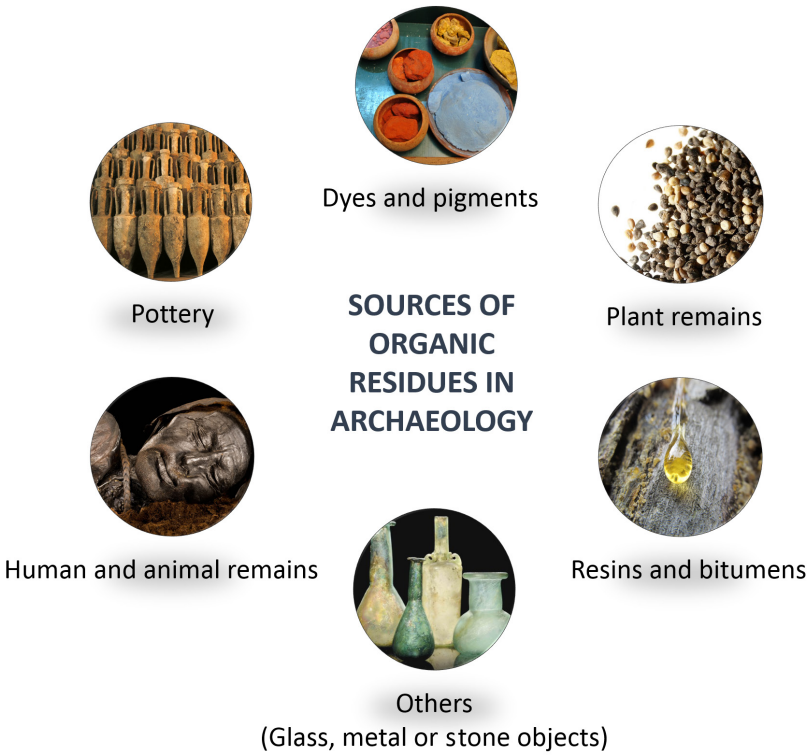


Figure 1.6: *Archaeological matrices where organic residues have been widely found.*

1.4.3. Organic residues absorbed in archaeological ceramics

Amphorae were widely used from at least as early as the Neolithic Period for the transport and storage of solid and liquid products. In this respect, potsherds are one of the most common classes of artefacts recovered from archaeological excavations. Since ceramic containers possess the notable property of absorbing substantial quantities of organic compounds from the commodities processed or stored in them, information on the past daily life related to the vessel function, technologies, diets, trade, medical practices, cosmetics or even religious and ritual activities can be obtained through the analysis of organic residues [18,90-93]. Organic remains can be preserved along the time in the archaeological item in several ways: (i) as intact organic residue preserved inside the vessel; (ii) in the interior or exterior part of the surfaces of the vessels; and (iii) absorbed in the pores of the vessel wall, invisible to the naked eye (see Figure 1.7).

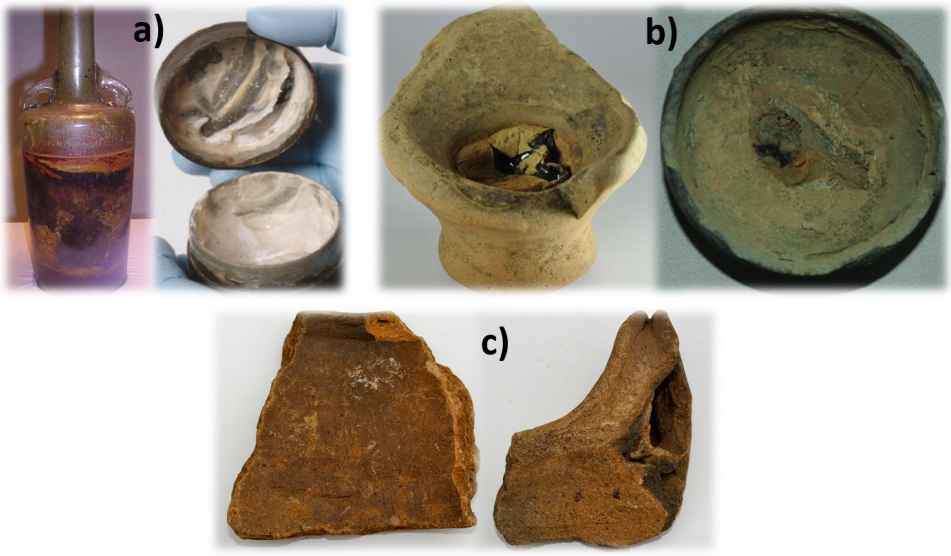


Figure 1.7: *Occurrence of organic residues as: intact organic residues preserved in non-ceramic materials (a), visible organic residues in archaeological ceramic samples (b) and absorbed organic residues in the pores of archaeological potsherds (c).*

It is difficult to find intact organic residues inside the archaeological containers, because the content is often lost or degraded with time and burial. Nevertheless, there are some cases in which sealed pottery has been found containing organic remains, as it is the case of the amphora found in a Roman shipwreck dating back to 100-90 BC which contained 1500 mL of a dark reddish brown liquid suspension that was found to be wine or must [94]. Although is not the most common case, there are some cases in which the organic remains are preserved for long time adsorbed to the surfaces of the archaeological items. For example, carbonized material related to heating during cooking practices is often found both in the interior and exterior surfaces of pottery [95-98] and also as deposits in archaeological lamps using oils as fuels [99,100]. Pharmaceutical ointments found in ceramic apothecary jars [101,102], balms, unguents and other organic residues such as ancient cosmetics have been more commonly found in archaeological glass bottles [18,103] or ancient metallic tools [91]. Resinous materials have also been recovered as organic residues from the bottom of amphorae [92,104-106]. Due to their hydrophobic nature, these materials were frequently used as water-proofing agents, sealing the inside of the amphorae [107,108]. It is worth mentioning that when residues are visible, only few milligrams (1-3 mg) are necessary for the analysis.

The most common scenario is to find organic residues preserved in the pores within the vessel wall. The clay matrix of archaeological pottery acts as a molecular sieve or trap that can preserve organic biomarkers during burial over many millennia. Several works have studied the migration of specific biomarkers through the inner walls of ceramic pottery [109,110]. According to literature, absorbed organic residues survive in over 80% of domestic cooking pottery assemblages worldwide after penetrating the clay matrix through the processing of plant and animal products [89]. Analysis of absorbed residues require larger amounts of sample. In this respect, Evershed established a working-rule that establishes that between 2 and 3 g of clean pottery sherd are necessary to identify organic compounds preserved in this way [111]. Despite many research works have followed this

advice [100,112-117], recently, some authors have achieved promising results even using lower sample amounts (e.g. 0.2-1 g) [118-123].

1.4.4. Lipids as the main biomarkers in archaeological ceramics

It is known that lipids are more resistant than proteins or carbohydrates [124]. This is one of the reasons to monitor lipids, or specifically, their diagenetic (and catagenetic) hydrocarbon products (e.g., steranes and terpanes) in geochemical studies [8]. Since the late 1970s and 1980s, it is known that lipids can be preserved under favorable conditions in archeological artefacts, thus, lipids and their by-products have been the most widely studied organic compounds in archaeology from then on. [102]. The high proportion of saturated hydrocarbon moieties present in lipids confers them hydrophobic properties that tend to reduce their water solubility and thus, limit their loss from the ceramic matrix by water leaching. Consequently, the resistance of lipids to decay, together with their likely persistence at the original site of deposition, makes them excellent candidates as biomarkers in archaeological research. Sterols and derivatives, di- and triterpernoids, fatty acids and acyl lipids are widely characterized in archaeological studies, as it is further described.

a) Sterols and sterol derivatives

Animal and plants can biosynthesize specific sterols and, hence, they can be used as biomarkers of the origin of lipidic organic residue. For example, campesterol and sitosterol are the two major plant sterols, whereas cholesterol is the most abundant animal sterol [88]. Although their differentiation seems easy, their low abundance together with the possible cross-contamination by the handling of artefacts makes their analysis difficult and they are not used as conclusive markers on their own.

When steroidal compounds are detected in soils, sediments and even coprolites, they provide information concerning waste disposal, manuring and other agricultural practices of the past [125]. Cholesterol and derivatives have also been found in animal and human remains such as bones [126,127] and mummies [128]. Cholesterol and its oxidized compounds, such as cholesta-3,5-dien-7-one or cholestanol, have been also detected in archaeological potsherds containing animal fat [95,119,129-132].

b) Diterpenoids and triterpenoids

Many di- and triterpenoid compounds can be found in animals and plants. From an archaeological point of view, these biomarkers are used to determine the nature, origin and possible means of production of resins or resin derivatives such as wood tars and pitches. Although di- and triterpenoids appear in resins, they are never found together in the same resin [88]. Figure 1.4 shows the structures of diterpenoid and triterpenoid compounds that have been widely determined in literature.

Diterpenoid compounds such as dehydroabietic acid and didehydroabietic acid and their oxidation products (e.g. 7-oxo-dehydroabietic, 7-oxo-abietic, 15-hydroxy-dehydroabietic acids) are considered characteristic biomarkers of resins from the *Pinaceae* family [92,103,106,123,133,134]. Moreover, some of these biomarkers can give evidences on heating conditions of the resin. As an example, the presence of methyl dehydroabietate indicates that pine resin was heated in presence of wood at high temperatures [92]. Additionally, these compounds have been frequently detected with wine-related compounds (e.g. tartaric acid) since resins are thought to be used in antiquity as sealing or waterproofing agents in the ceramic containers or to add special taste to wines.

Examples of triterpenoid compounds are oleanonic and oleanolic acids, which, together with other biomarkers, are distinctive of storax resin [105], and betulin, lupeol and derivatives (e.g. lupenone, betulone and betulinic acid), which are characteristic of birch

bark [104]. More recently, miliacin, a pentacyclic triterpene methyl ether that is enriched in grains of millet, has been detected for the first time in prehistoric pottery vessels [135].

c) Fatty acids and acyl lipids

Fatty acids have been the main class of compounds in targeted analysis in archaeological studies. They are stable over long periods, easily recovered from potsherds and their detection through hyphenated analytical techniques is mostly established [136].

Fatty acids are prevalent in the interior walls of ceramic vessels, especially near the neck and rim [96,111]. What is more difficult to demonstrate is that such residues actually represent the unmodified by-products of ancient commodities cooked or stored in the vessels because fatty acids are ubiquitous and produced by nearly every organism, from bacteria to mammals. Hence, they can simply be native to the clays that people use to make pots. However, this fact can be easily ruled out taking into account that during the clay production temperatures between 500 °C and 800 °C are reached, and fatty acids and related compounds do not survive these extremely high firing temperatures. On the other hand, fatty acids could represent post-depositional contamination by, for example, bacteria consuming other organic residues, or free fatty acids leaching into sherds from the surrounding soil. Nevertheless, the entrapment of lipids in the pores of the ceramic matrices will generally lead to a decrease of microbial activity and water penetration. Finally, fatty acids may also be the consequence of cross-contamination during the analysis step, a fact that is easily solved by performing blanks and quality control samples during laboratory analysis [88,136].

Unfortunately, what is inevitable is the impact of any of the normal mechanisms of decay during vessel use (i.e. cooking processes at high temperatures) or under a given burial environment (oxic or anoxic). The low mean concentration of this biomarkers found in archaeological ceramics compared to the capacity of fired ceramic to absorb them is an

evidence of their degradation during burial [124]. When exposed to high temperatures and burial environments, organic compounds can undergo chemical reactions of oxidation, hydrolysis, condensation, etc. [111,136,137]. The design of simulated degradation processes by means of cooking experiments and artificial or natural ageing experiments has been a key procedure in order to determine biomarkers indicative of lipid degradation [100,111,138,139].

1.4.5. Fatty acids and acyl lipids as biomarkers related to specific uses of pottery

The importance of the analysis of fatty acids in archaeological remains deserves to define the most important biomarkers of animal fats, plant lipids and beeswax.

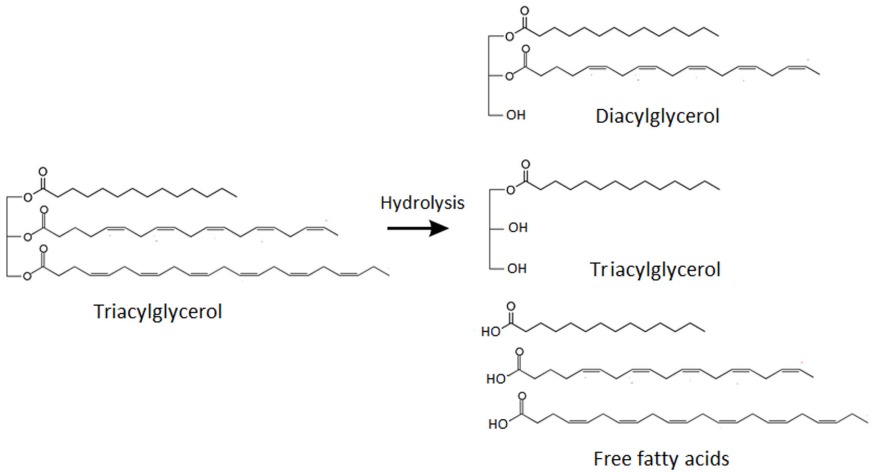


Figure 1.8: *Hydrolysis of a triacylglycerol due to burial or cooking processes and the formation of diacylglicerols, monoacylglycerols and free fatty acid derivatives.*

Animal fat degradation products are the most common compounds detected in archaeological potsherds. Fresh animal fats (e.g., terrestrial mammals and marine mammals) are characterized for containing triacylglycerols (TAGs), a lipid molecule consisting of three long-chain fatty acids joined to a glycerol 'backbone'. During burial

and/or processing, TAGs break down to diacylglycerols (DAGs), monoacylglycerols (MAGs) and, finally, to single chains of free fatty acids (FAs) (from C8 to C24) (see Figure 1.8). A degraded animal fat will typically contain low concentrations of TAG biomarkers, and slightly higher concentrations of DAGs and MAGs, with palmitic ($C_{16:0}$) and stearic ($C_{18:0}$) fatty acids dominating [140]. TAGs, DAGs, MAGs and fatty acids, together with cholesterol, are the most commonly used biomarkers of animal fats [95,109,111,137,140-143].

The determination of fatty acids allows the differentiation between ruminant and non-ruminant animals [138,141,142,144-146]. Ruminant animals' fat and their secondary products (e.g. dairy) contain branched chain fatty alkanoic acids (i.e. $C_{15:0}$ and $C_{17:0}$ branched fatty acids), together with odd-numbered fatty acids (i.e. $C_{15:0}$, $C_{17:0}$ and $C_{19:0}$) in reasonably high abundances due to the specific bacterial synthesis in the gut. These compounds are fairly detected or non-detected in non-ruminant fat. Additionally, mixtures of positional isomers in the case of octadecenoic acid ($C_{18:1}$) with the unsaturation located in 9, 11, 13, 14, 15 and 16-positions arise in the fats of ruminant animals such as sheep and cattle [142].

Similarly, marine and freshwater animal fats and oils can be characterized through the fatty acids profile in each sample. They are characterized by distributions of long-chain polyunsaturated (PUFAs) compounds, such as eicosapentaenoic acid ($C_{20:5}$) and docosahexaenoic acid ($C_{22:6}$), together with monounsaturated fatty acids (MUFAs), such as 11-eicosenoic acid ($C_{20:1}$) and 11-docosenoic acid ($C_{22:1}$). However, these compounds degrade rapidly through oxidation processes and rarely survive as significant components of organic residues during archaeological timescales. Nevertheless, the degradation products are stable and can be detected in lipid residues in prehistoric pots. These by-products preserve the characteristic features of the original fatty acids and can act as reliable biomarkers of the presence of aquatic fats and oils. Examples of these biomarkers are ω-(o-alkylphenyl)alkanoic acids containing 16-20 carbon atoms, which are normally formed from triunsaturated acyl lipids during heating processes at temperatures over

270 ^{o}C [147]. Vicinal dihydroxiacids (DHAs) are another oxidation products of unsaturated fatty acids that indicate the original position of the double bond in the precursor fatty acids. The presence of DHAs with carbon numbers ranging from C16 to C22 in organic residues of pottery vessels found in coastal sites provides an unambiguous evidence of marine origin [148,149].

On the other hand, isoprenoid fatty acids (IFAs) such as pristanic acid, phytanic acid and 4,8,12-trimethyltridecanoic acid (4,8,12-TMTD) are found in particularly high concentrations in marine animals, but are absent, or present at trace levels, in terrestrial animals. The highly branched structure of the IFAs makes them resistant to degradation, allowing them to be transferred along the marine food chain. Phytanic acid can be detected in plasma, tissues, milk and processed butterfat of ruminant animals at low concentrations [150], but 4,8,12-TMTD only occurs in the aquatic environment. Therefore, when phytanic and pristanic acids are identified in association with 4,8,12-TMTD a marine source of the organic remain can be concluded [150].

Due to the wide variety of marine related organic compounds, numerous research works have used isoprenoid compounds and fatty acid by-products as biomarkers to identify marine fats and oils as original contents in ceramic samples [95,98,117,121,129,148,149,151-154].

Overall, the concentration of lipids in plants is lower in comparison to those found in animal fats, and hence, if both types of commodities have been processed in the same pot, animal lipids will dominate the lipid mixture [111].

The most abundant and commonly detected plant lipids are the waxes from leafy vegetables, which are characterized by a distribution of biomarkers with long carbon chains, such as odd-numbered alkanes (C25-C33), even-numbered alcohols and aldehydes (C20-C34 and C24-C28, respectively) and esters (C39-C52) [140,146,155]. Occasionally, some organic compounds can act as specific biomarkers of certain plant oil sources. *Brassicaceae*

(known also as *Cruciferae*) seed oil has been used in ancient times and it presents a very consistent fatty acid profile in which (Z)-11-eicosenoic acid (gondoic acid) and (Z)-13-docosenoic acid (erucic acid) are abundant. They may undergo oxidation processes, and thus, characteristic DHAs (11,12-dihydroxieicosanoic and 13,14-dihydroxydocosanoic acids) could be formed, together with α,ω-dicarboxylic acids (α,ω-undecanedioic and α,ω-tridecanedioic) acting as specific biomarkers of a vegetable oil obtained from *Brassicaceae* seeds [90]. The alkane nonacosane (C29) and its oxygenated derivatives, nonacosan-15-one and nonacosan-15-ol indicate the processing of vegetables that belong to the genus *Brassica,* such as cabbage, turnip, kale and broccoli [140].

In addition to animal and plant lipids, beeswax is another commodity detected in pottery lipid residues. Fresh beeswax is characterized by long-chain odd-numbered alkanes (C23-C33), even-numbered fatty acids and even-numbered wax esters (C40–C54). These last compounds partially degrade over time to form long-chain even-numbered alcohols (C24-C34), fatty acids and alkanes [116,140,156-158]. Apart from to obtain honey, beeswax was also used in various technological, ritual, cosmetic and medicinal applications [158].

1.4.6. Biomarkers of wine residues

Wine is one of the most important beverages that has been produced, consumed and traded in the Mediterranean area. Many ceramic vessels have been used for these purposes, being the amphorae the most common transport container [139]. From an analytical point of view, the detection of wine residues is a great challenge due to the chemical nature and instability of the most wine related organic compounds. Among them we can find tartaric acid, an abundant molecule in grapes used to make wine (approximately 4000 parts per million) [159-161]. Although tartaric acid is very soluble in water, it can be strongly absorbed on silicates by hydrogen bonding and hence, it has been frequently found preserved in the pottery and used as wine biomarker [159].

On the other hand, syringic acid coming from the hydrolysis of malvidin, the anthocyanin responsible of giving grapes and red wines their color [161],is also used as wine biomarker but also occurs naturally in some plant products (e.g., barley or wheat) or in soils due to microbial activity [139,161]. Thus, the use of syringic acid as red wine biomarker is only accurate when its presence is related to the presence of malvidin [162]. Nevertheless, according to the literature, the detection of syringic acid, together with tartaric acid and other less specific biomarkers such as malic (marker of dark grape), succinic and pyruvic acids (fermentation markers) or lactic, fumaric and citric acids has frequently led to the unequivocal identification of the use of archaeological ceramics as wine containers [94,108,120,123,139,161-172]. Interestingly, a recent investigation has shown the earliest biomolecular archaeological evidence for grape wine and viniculture with the analysis of organic residues (i.e. tartaric acid) absorbed into the pottery fabrics from sites in Georgia in the South Caucasus region, dating to the early Neolithic period (ca. 6,000–5,000 BC) [173].

1.5. Analytical methods for the analysis of organic biomarkers

Early applications of chemical analytical methods in search of organic biomarkers demonstrated that scientific investigation is essential to infer information about samples of different scientific fields such as geochemistry, built heritage or archaeology. In this framework, the development of scientific procedures that only need little amount of sample and the increased availability of advanced analytical instrumentation in research labs have led to great research studies related to the identification of biomarkers.

Ideally, non-destructive methodologies are the most suitable techniques to analyze samples belonging to cultural heritage since they do not damage the test surfaces under study. Additionally, they do not require sample pre-treatments and, occasionally, samples can even be measured *in situ* with portable instruments. Techniques such as X-ray fluorescence (XRF) have been used to obtain information about the elemental and mineralogical composition of the samples [174,175]. Fourier transform-infrared

spectroscopy (FT-IR) has also been applied in the archaeological field as a screening tool in order to track information on the functional groups of the organic matter [108,176,177]. Thus, the combination of elemental and molecular non-destructive techniques provide a complete description of the material under study. Nevertheless, spectroscopic techniques require the presence of target compounds at a high concentration level not often found in archaeological remains. Moreover, complex mixtures of organic molecules frequently difficult the interpretation of spectra. Consequently, they are scarcely used to identify the specific biomarkers described in the previous sections.

Technological development has led to the appearance of analytical techniques able to measure organic compounds occurring in the samples under study at both high concentrations and trace levels. These techniques are based on destructive methodologies that require sampling and sampling pre-treatment steps to extract or isolate the biomarkers of interest. Despite being destructive, these analytical methods play an important role in application fields such as archaeology, geochemistry and environmental sciences, because the information inferred in this way gives the clues to understand many of the processes occurred in the past.

During the last decades, research works dealing with the identification of organic compounds, especially in the archaeological field, have used classic analytical procedures for the isolation of biomarkers of interest. However, few are the works in this field aiming to develop new analytical methods that ensure quantitative extraction and preconcentration of specific biomarkers.

1.5.1. Extraction of organic biomarkers from solid matrices

Destructive analytical methods require the organic compounds to be isolated from the archaeological sample and dissolved. Together with sampling, this is one of the critical steps

during the development of new protocols to determine target organic biomarkers in geological and archaeological artifacts.

In geochemical studies, conventional techniques such as Soxhlet extraction have been frequently used (see Table 1.4) regardless of the long extraction periods (8-16 h) and the large amounts of sample required (100-150 g) [178,179]. Additionally, chlorinated solvents (e.g., chloroform, dichloromethane) and azeotrope mixtures of solvents (e.g., chloroform:methanol ($CHCl_3$/MeOH) are often used to isolate a wide range of organic biomarkers. Thus, these traditional techniques require large volumes of non-environmentally friendly solvents [8].

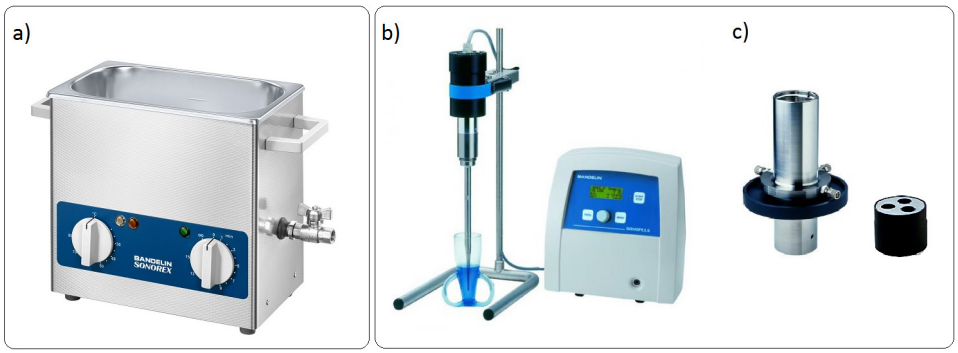


Figure 1.9: *Ultrasound based devices: a) Traditional ultrasound bath (USB), b) Focused ultrasound titanium probe (FUSLE) and c) acrylic cup accessory to perform focused ultrasound extractions with low volumes of solvent.*

Alternatively, ultrasound based solid-liquid extractions are the approaches employed to extract organic compounds from several solid matrices (e.g. soils and sediments, cementitious mortars or pottery). The mechanical effect of ultrasound induces a greater penetration of the solvent into the solid matrix and mechanical erosion of solids, which improves mass transfer leading to enhanced sample extraction efficiency. The most common device is the classic ultrasound bath (USB), which has been successfully applied to

extract lipids from archaeological ceramics (see Figure 1.9a)). In fact, in 1990, Evershed et al. established a protocol to obtain the total lipid extract (TLE) from archaeological samples using USB (see Table 1.4). The protocol consisted in a solvent extraction using 10 mL of a 2:1 *v/v* mixture of $CHCl_3$/MeOH via sonication (2 x 15 min) [180]. From them on, this procedure has been widely used and, nowadays, it continues being the main extraction procedure in the archaeological field [95,121,141,143,155].

Currently, the use of focused ultrasound solid-liquid extraction (FUSLE) has gained interest in many research fields. In contrast to USB, with this extraction technique the ultrasonic waves do not need to cross the wall of the sample container since the ultrasound probe, typically made of titanium, is directly immersed in the solution of the sample to be sonicated (see Figure 1.9b)). In FUSLE, ultrasound radiation generates a disturbance and, due to its periodical repetition, expansion and compression cycles are created [161]. When the ultrasounds reach high intensity, bubbles or cavities are created during the expansion cycle and then, they undergo implosive collapse. This process is called cavitation phenomena [181]. In this way, compared to the poor sonication power of ultrasound baths, FUSLE provides 100 times higher sonication power and the energy is focused on a localized sample area, making cavitation in the liquid more efficient. Moreover, higher reproducibility and extraction efficiency are obtained in shorter times [182,183]. Similarly, when the quantity of extraction solution is limited, alternative cup horns similar to ultrasonic water baths but smaller (12 mL) can be used since they are more intense and suitable for handling low sample volumes (e.g. 1.5 mL) [184]. In cup horns, the titanium probe is held within an acrylic cup filled with water and the samples can be placed in sealed tubes that eliminate cross contamination (see Figure 1.9c)).

Regardless of the extraction technique used, in addition to organic solvents, alkaline saponification and hydrolysis procedures are often required to promote the cleavage of the organic compounds from the sample matrix. In this respect, NaOH or KOH solutions in

hydroalcoholic mixtures of methanol and water have been used to extract biomarkers covalently linked into insoluble residues of potsherds via ester bonds and saponify TAGs [87,148]. These procedures have been performed under sonication (USB) [147,149], using water baths at 60 °C [18,103], as well as assisted by microwave energy (MAE) [185].

1.5.2. Transfer and preconcentration of biomarkers into an organic phase

When solid-liquid extractions are performed using aqueous solvents or mixtures of solvents that include water (e.g. hydroalcoholic KOH), a subsequent preconcentration or additional extraction step is mandatory in order to transfer the organic compounds from the aqueous phase to an organic phase compatible with the analytical detection technique. For this purpose, classical liquid-liquid extraction (LLE) has been widely employed [90,105,147,149,185]. The use of large volumes of organic solvents, the addition of ion-pair reagents and performing consecutive extractions have rendered adequate extraction yields for biomarkers in the environmental field [186,187]. However, the separation from interferences with similar characteristics of the target compounds is still not achieved completely. Formation of emulsions and the need for expensive glassware material are some of the other drawbacks connected with the application of classical LLE as extraction technique.

Solid phase extraction (SPE) was initially developed as a complement or replacement for LLE but, currently, is the most preferred technique to isolate a wide variety of organic compounds from aqueous samples or extracts [188,189]. SPE shows many advantages over other traditional preconcentration methods, such as ease analysis throughput, substantial solvent savings, great variety of preconcentration sorbents, large capacities and reduced matrix effect [190]. It is based on the analyte association with a solid sorbent through sorption and is designed to be a quantitative extraction procedure, extracting the analyte exhaustively from the entire sample volume via the sorbent [189]. Moreover, SPE is also

applied in combination with other extraction techniques (even when SPE is used as the extraction technique) as clean-up or purification steps [191]. Figure 1.10 summarizes briefly the main steps involved in SPE extraction: conditioning of the cartridge, sample loading, removal of the interferences and elution of the target analytes.

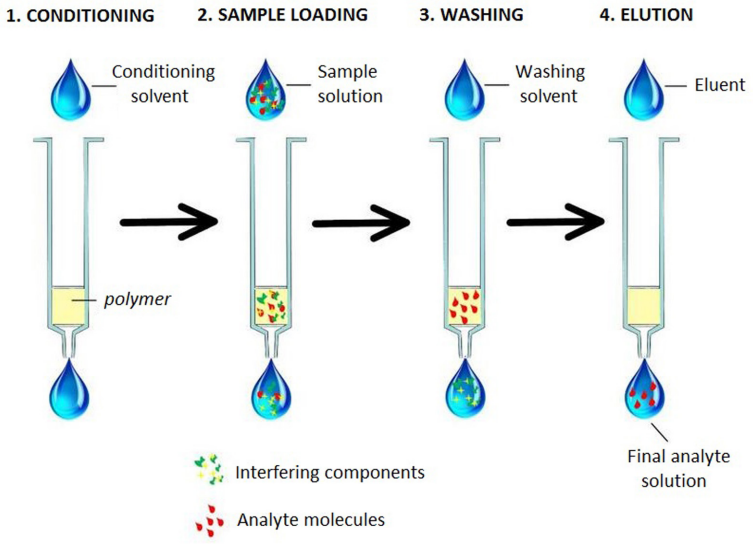


Figure 1.10: *Schematic representation of the main steps in SPE.*

In the case of aqueous samples, reverse phase SPE (RP-SPE) is the most commonly used mode. Among the great variety of reverse phases available in the market for SPE, the most commonly used ones are the traditional silica bonded sorbents (C18, C8, C6, C4, C2, phenyl, cyclohexyl) and the more recently developed hydrophilic polymeric sorbents based on styrene and divinylbenzene [192]. These modern highly cross-linked polymers have larger surface areas, which enhance retention capacity and can be modified with polar functional groups (i.e., acetyl or sulfonate) in order to weaken the interfacial tension between the polymer and aqueous sample, and enhance the interaction between the

analytes and the sorbent materials. As an example, the commercially available Oasis HLB (Waters) based on poly(divinylbenzene-co-N-vinylpyrrolidone) polymer emerged as pre-concentration of compounds from aqueous extracts [193]. Other hydrophilic polymeric sorbents modified with polar functionalities, such as Bond ElutPlexa (Agilent Technologies), Strata-X (Phenomenex) or Lichrolut-EN (Merck Millipore) have been commercialized and used in many applications.

The so-called dual-phase or mixed-mode sorbents have also recently come out in order to isolate target compounds of a wide variety of polarities. These phases allow selective extractions through dual mechanism: ion-exchange and reversed-phase mode, which allow the extraction of both charged and uncharged analytes [194]. The large active surface areas of the sorbent allow loading large sample volumes. Fontanals et al. have reviewed the different types of mixed-mode SPE pinpointing their morphological and chemical characteristics as well as their applications [194]. Briefly, depending on the ion-exchange interactions established between the polymer and the analytes, mixed-mode sorbents can be classified in: strong cation exchange (SCX), weak cation exchange (WCX), strong anion exchange (SAX) and weak anion exchange (WAX). All these ionic interactions can be achieved by modifying the polymer structure with specific functional groups such as sulfonic acid for SCX, carboxylic acid for WCX, quaternary amine for SAX and secondary amine for WAX. In any SPE mode, but especially in mixed-mode SPE, the adequate selection of the solvents used in each step (i.e., loading, washing and elution) is essential if enhanced selectivity is required [189,194].

1.5.3. Identification of organic biomarkers

Nowadays, mass spectrometry (MS) is considered the standard detector due to its universality, selectivity and sensitivity both for gas chromatography (GC) and liquid chromatography (HPLC).

a) Separation by means of gas chromatography (GC) and liquid chromatography (HPLC)

GC has been the most widely used separation technique, especially in the geochemical and archaeological fields, due to its versatility, simplicity, rapidity and reproducibility (see Table 1.3). It is applied for the separation of volatile and thermally stable compounds. In this sense, alkanes, terpanes or persistent organic pollutants such as PAHs have been frequently identified by means of GC-MS.

Lipids, acyl compounds or WSOCs and all the molecules containing functional groups with active hydrogens, such as hydroxyl (-OH), carboxylic acid (-COOH), amine ($-NH_2$) or thiol (-SH), tend to form intermolecular hydrogen bonds that affect their volatility, thermal stability and their interaction with GC column packing materials [195]. Thus, derivatization of these functional groups is necessary prior to their analysis by GC-MS. The derivatization of these compounds increases their volatility and thermal stability, decreasing the polarity of the derivatized compounds. Therefore, analyte adsorption in the GC system is avoided, improving detector response, peak separation and symmetry.

Sylilation has been the most widely used approach for the derivatization of non-volatile organic biomarkers [196], being N,O-bis (trimethylsilyl)trifluoroacetamide (BSTFA) the most commonly used reagent as silyl donor [87]. The enhanced characteristics of silyl derivatives promote, on the one hand, improved GC features (high reproducibility, sensitivity and resolution) and on the other hand, improved mass spectra owing to the more characteristic *m/z* fragment ions of silyl-derivates. Silylation reactions should always be performed under anhydrous conditions since all the silyl-reagents react readily with water, affecting both the reaction speed and efficiency. Besides the absence of humidity, traditional derivatization procedures require high working temperatures to get derivatives quantitatively. Recently, there are some proposals in the literature using other efficient

energies such as microwave energy [193] and ultrasound energy [184] in order to improve derivatization yields, lowering overall analysis time in comparison to conventional methods. Esterification or methylation with alcohols using BF_3 or acetyl chloride as catalysts have also been applied in literature for the derivatization of FAs and dicarboxylic compounds [155,197,198].

The most common injection mode for GC is the splitless mode, which is carried out in a simple and robust split/splitless inlet. However, the application of large volume injection (LVI) in a programmable temperature vaporizer (PTV) inlet has gained importance in the last decades in order to improve sensitivity for the determination of biomarkers at trace levels [199]. Both systems are similar but the main difference is based on the fact that the PTV injector is equipped with a very sophisticated temperature control function. In this sense, the inlet is kept cool during the sample introduction, allowing the analyte to condense inside the liner, whilst the solvent is vented via the split line. On the other hand, the inlet has a very low thermal mass, which allows rapid heating to transfer the analyte to the GC column after solvent venting has taken place. These two important differences in inlet design give the PTV the ability to inject large volumes at controlled speeds, allowing the introduction of very large sample volumes (> 100 µL is possible). In this sense, LVI-PTV can improve the sensitivity in several orders of magnitude in comparison with common split/splitless inlets.

Briefly, LVI-PTV injection consists on several steps [241]:

i) Sample is introduced at a low temperature (below solvent boiling point) once or using a series of small sample aliquots in the inlet.

ii) Solvent is eliminated via the split vent, while the higher-boiling analytes are retained in the liner.

iii) PTV is rapidly heated and the analytes are transferred to the column in the splitless mode. The analytes are focused at the front of the column by keeping the oven temperature below the solvent boiling point.

iv) After the splitless transfer, the split vent is opened in order to remove the residual solvent vapor and the low-volatile matrix components from the inlet.

On the other hand, despite not requiring a derivatization step for the analysis, liquid chromatography (HPLC) has been less used for the separation of organic biomarkers. Nevertheless, it has been catalogued as an attractive technique in order to identify specific molecules such as TAGs [109]. In this sense, high performance liquid chromatography with atmospheric pressure chemical ionization coupled to mass spectrometry (HPLC-APCI-MS) has been used in order to elucidate the structure of TAGs [109,120,200], which cannot be achieved by conventional GC-MS.

In the last years, several strategies have been developed in the field of HPLC in order to maintain good efficiency and reduce analysis time. In this sense, the emergence of ultra-high pressure liquid chromatography (UHPLC) and the use of superficial porous particles (often called core-shell or fused-core) for column packing have to be mentioned. In UHPLC columns are packed with sub 2 μm particles working under high pressure conditions, which implies high efficiency, short analysis time and low solvent consumption [201]. These type of particles provides a higher efficiency at lower backpressures, compared to traditional porous particles [202,203]. In terms of stationary phase, reversed phase chromatography, with columns based on octadecyl (C18), are mostly employed for the analysis of TAGs in the field of archaeology [109,204].

b) Detection

HPLC and GC, in combination with MS continue to be the predominant techniques for the separation, identification and quantification of organic biomarkers in geochemical, environmental and archaeological samples. Table 1.3 summarizes the different hyphenated techniques used for the determination of organic biomarkers in the previously mentioned

fields. As it can be observed, MS coupled to GC (GC-MS) is the most used technique for the analysis of organic biomarkers in specific samples of geochemical, built heritage and archaeological fields. GC-MS is widely used to identify and quantify organic markers such as, alkanes, isoprenoid compounds, steranes, terpanes, WSOCs, PAHs, FAs, wine biomarkers, marine lipid biomarkers, resin biomarkers, to name a few, in complex matrices.

When pyrolysis is coupled to GC-MS (Py-GC-MS), preliminary information on sample composition and on the organic material present in micro-samples can be obtained. This way, the correct sample pretreatment can be chosen before carrying out subsequent analysis by GC-MS [133]. Due to its micro-invasive nature, Py-GC-MS allows the analysis of tiny samples from cultural heritage objects without damaging the original sample, since only a few μg of sample are required [205]. Evidence of lipids, resinous materials, polysaccharides and proteins have been found in organic materials present in ancient artistic objects such as archaeological cosmetics [133] or adhesives used in Roman mosaics [133]. The combination of GC-MS to other detectors can provide complementary information about the target sample. For example, gas chromatography combustion isotope ratio mass spectrometry (GC-C-IRMS) is a highly specialized instrumental technique that can be used to determine the origin of certain lipids in archaeological cooking ceramics (see Table 1.4) [140]. Plots of the $\delta^{13}C$ values obtained for methyl palmitate against those of methyl stearate have been proposed to distinguish different animal species (i.e. ruminant from non-ruminant or terrestrial from marine species) [196].

MS is the most preferred detection technique to be coupled also to HPLC instruments. Triple quadrupole (QqQ or MS/MS), time of flight (ToF) and ion trap (IT) analyzers coupled to HPLC systems have to be underlined in the analysis of organic compounds. In fact, liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS) has become an ideal technique due to its high sensitivity and selectivity [159], and it allows the identification of several biomarkers based on their retention times and fragmentation

patterns. [108,159,161]. The main drawback of QqQ detectors working in the multiple reaction monitoring (MRM) mode is that only selected *m/z* fragments and transitions (parent and product ions) are monitored. Using this strategy selective and sensitive target measurements can be performed, but it is not possible to find out new biomarkers [206]. High resolution mass spectrometry detectors are needed for this last purpose, this is, to perform non-target measurements. In this sense, accurate mass full-scan MS techniques, such as ToF based mass analyzers, are the best tool for multiresidue analysis, since unknown degradation products or previously unidentified biomarkers can be identified with this technique. On the other hand, IT analyzers provide high sensitivity in full scan mode and also provide the possibility to perform multiple-stage fragmentations (MS^n) for structural elucidation of unknown compounds. However, its sensitivity is one order of magnitude lower in comparison with QqQ [206].

Consequently, high resolution mass spectrometry (HRMS) and accurate mass measurements have recently gained popularity due to their great ability to provide more comprehensive information concerning the exact molecular mass, elemental composition and detailed molecular structure of a given compound [207]. HRMS shows many advantages over classical unit-mass-resolution tandem mass spectrometry, such as: (i) differentiation of isobaric compounds (different compounds with the same nominal mass but different elemental composition), (ii) simplification of sample preparation procedures, (iii) information gathered by a single injection that can be used for quantification and screening purposes, including targeted, suspect and non-targeted analysis and (iv) collection of full-scan spectra that can be stored and used in a later stage retrospective analysis, permitting the elucidation of unknown or suspected compounds [208]. Taking all this in mind, during the last years, a combination of the above mentioned mass analyzers has emerged in order to overcome the limitations of both QqQ and ToF: the hybrid quadrupole time of flight (QToF) spectrometers. This mass analyzer could be described as a QqQ in which the last quadrupole has been replaced with a ToF analyzer. The advantage is that it can operate as

a ToF analyzer in the full-scan mode or as a tandem mass spectrometer in the product-ion scan mode. Therefore, the acquisition of full MS spectrum is allowed with high sensitivity, mass accuracy and medium range high resolution which offers the possibility to carry out multiresidue screening, structural elucidation of unknown compounds and accurate mass product-ion spectra. La Nasa et al. applied HPLC-ESI-QToF analysis in the field of cultural heritage in order to identify the TAG profile in modern oil paintings [209].

In any case, regardless of the analytical technique use, the use of multianalytical approaches combining many of the previously mentioned techniques (see Table 1.4) provide information to unequivocally identify the organic markers present in the materials under study.

Table 1.3: *Main parameters related to the analysis of organic biomarkers in different application fields.*

Sample matrix	*Identified biomarker(s)*	*Extraction*	*Preconcentration or clean-up*	*Derivatization*	*Detection method*	*Ref.*
Geochemistry						
Sedimentary rock	Tricyclic and tetracyclic terpanes, gammacerane and Pri/Phy ratio	Soxhlet ($CHCl_3$: MeOH 87:13 v/v, 72 h)	Liquid-solid chromatography of extracts	-	GC-MS	[178]
Sandstone and sediments	n-alkanes, CPI, Phy, diterpenoid compounds and PAHs	Soxhlet (DCM, 8 h)	Evaporation	-	GC-MS (splitless)	[179]
Sedimentary rock	C_{29}-C_{35} hopanes, Pri/Phy ratio, gammacerane	Soxhlet (DCM: Ace 9:1 v/v, 24 h)	Evaporation and liquid-solid chromatography of extracts	-	GC-MS	[210]
Environmental pollution affecting built heritage						
Stone	PAHs	Soxhlet (DCM: Pentane 1:1 v/v, 12 h)	Evaporation	-	GC-MS (splitless)	[73]
Stone, cement and mortars	PAHs and PCBs	MAE (15 mL acetone)	Evaporation and clean-up with SPE (Florisil)	-	GC-MS	[70]
Black crusts	WSOCs	Sonication with 10 mL of H_20		-	TOC analyzer	[75]
$PM_{2.5}$	WSOCs	USB (5-20 mL Milli-Q H_2O:MeOH:DCM, 10-30 min)	Evaporation	Sylilation with BSTFA	GC-MS (splitless)	[211]
Seawater	WSOCs	SPE (active charcoal)	Evaporation	Methylation with BF_3/butanol	GC-MS (splitless)	[212]

CPI: carbon preference index; Pri: pristane; Phy: phytane; GC-MS: gas chromatography-mass spectrometry; PAH: polycyclic aromatic hydrocarbon; DCM: dichloromethane; Ace: acetone; PCB: polychlorinated biphenyl; MAE: microwave assisted extraction; WSOCs: water soluble organic compounds;.TOC: total organic carbon; BSTFA: N,O-bis (trimethylsilyl)trifluoroacetamide; $PM_{2.5}$: particulate matter with ø < 2.5 µm; SPE: solid phase extraction.

Table 1.3: *Continuation*

Sample matrix	*Identified biomarker(s)*	*Extraction*	*Preconcentration or clean-up*	*Derivatization*	*Detection method*	*Ref.*
Archaeology						
Potsherds	FAs, DHAs, MAGs, DAGs, TAGs, Phy, Pri, chol. and chol. derivatives	USB (2 x 10 mL $CHCl_3$: MeOH 2:1 v/v, 20 min) for TLE Hydrolysis of TLE with $NaOH_{MeOH}$	LLE for NaOH extracts (extraction of neutral and acid fractions)	Sylilation of TLE and neutral fraction with BSTFA. Methylation of acid fraction	GC-MS (splitless)	[95]
Potsherds	FAs, DAGs, TAGs, ketones and $\delta C_{16:0}$ and $\delta C_{18:0}$ values.	USB (2 x 10 mL $CHCl_3$: MeOH 2:1 v/v, 20 min) for TLE	Evaporation	Sylilation with BSTFA	HTGC, GC-MS and GC-C-IRMS	[143]
Organic residue	FAs, DHAs, diacids, diterpenoid acids, n-alcohols, n-alkanes, β-sitosterol, long chained esters	Alkaline hydrolysis (1 ml of KOH_{MeOH} (10% weight)/KOH_{H2O} (10% weight), 2:3 v/v) at 60 ^{o} C, 3 h	LLE of neutral fraction with n-hexane and LLE of acid fraction with diethyl ether	Sylilation with BSTFA	HTGC, DE-MA and GC-MS	[103]
Potsherds	FAs, Phy, Pri, 4,8,12-TMTD, APAAs, DHAs and diacids	Alkaline hydrolysis (5 ml of NaOH 0.5M (MeOH:H2O, 9:1 v/v) at 70 ^{o} C, 1 h)	Liquid-solid chromatography of methylated extracts	Methylation with BF_3/ MeOH and sylilation with BSTFA	GC-MS	[149]
Charred surface deposits in potsherds	MUFAs, Phy, Pri, 4,8,12-TMTD, APAAs and $\delta C_{16:0}$ and $\delta C_{18:0}$ values.	Alkaline hydrolysis (NaOH 5% in MeOH, w/v) at 70 ^{o} C, 2 h)	LLE of acid fraction with n-hexane	Methylation with BF_3/ MeOH	GC-MS and GC-C-IRMS	[98]
Ceramic vessels	FA, TAGs, ketones, triterpenoids, APAAs and diacids	Acid extraction (H_2SO_4/MeOH 2% v/v) at 70 ^{o} C, 1 h for TLE	LLE of acid fraction with n-hexane	Sylilation with BSTFA	GC-MS and GC-C-IRMS	[114]
Potsherd	Tartaric acid and syringic acid	USB (H_2O/methanol/HCl (150/1.5/0.1, v/v/v) for tartaric acid Alkaline hydrolysis (KOH 5.9 M in MeOH, w/v) at 50 ^{o} C, 5 min) for syringic acid	SPE (C18) LLE with ethyl acetate	-	HPLC-MS/MS	[161]

FA: fatty acid; DHA: dihydroxy fatty acid; TAG: triacylglycerol; DAG: diacylglycerol; MAG: monoacylglycerol; Pri: pristane; Phy: phytane; Chol.: cholesterol; USB: ultrasound bath; TLE: total lipid extract; LLE: liquid-liquid extraction; BSTFA: N,O-bis (trimethylsilyl)trifluoroacetamide; HTGC: high temperature gas chromatography; GC-C-IRMS: gas chromatography combustion isotope ratio mass spectrometry; diacids: α,ω-dicarboxilic acids; 4,8,12-TMTD: 4,8,12-trimethyltridecanoic acid; APAAs: ω-(o-alkylphenyl)alkanoic acids; MUFA:monounsaturated fatty acid; SPE: solid phase extraction; HPLC-MS/MS: liquid chromatography coupled to tandem mass spectrometry.

1.6. References

[1] The Editors of Encyclopædia Britannica, Organic compound, Encyclopædia Britannica (Online version), Encyclopædia Britannica, inc., 2018. https://www.britannica.com/science/organic-compound (Lat updated: January, 2018)

[2] L. Shiel, The Evolution Conspiracy Vol 1. Exposing Life's Inexplicable Origins & the Cult of Darwin, 1st ed., Slipdown Mountain Publications, United States, 2009.

[3] F. Borgia, The World of Man, 1st ed., Page Publishing, Inc., New York, 2016.

[4] S.L. Miller, H.C. Urey, J. Oró, Origin of organic compounds on the primitive earth and in meteorites, J. Mol. Evol. 9 (1976) 59-72.

[5] T. Fenchel, The Origin and Early Evolution of Life, 1st ed., Oxford University Press, New York, 2002.

[6] C. Wickramasinghe, The Search for our Cosmic Ancestry, World Scientific Publishing Co. Pte. Ltd. ed., Singapore, 1939.

[7] S. Killops, V. Killops, An Introduction to Organic Geochemistry, Longman Scientific & Technical, New York, 1993.

[8] K. Peters, C. Walters, J. Moldovan, The Biomarker Guide Volume 1: Biomarkers and Isotopes in the Environment and Human History. 2nd ed., Cambridge University Press, United Kingdom, 2005.

[9] G. Eglinton, M. Calvin, Chemical fossils, Scientific American. 216 (1967) 32-43.

[10] J. Hunt, Petroleum Geochemistry and Geology, WH Freeman & Co., San Francisco, 1980.

[11] A.P. Decaprio, Biomarkers: Coming of age for environmental health and risk assessment, Environ. Sci. Technol. 31 (1997) 1837-1848.

[12] P.A. Schulte, 1 - A Conceptual and Historical Framework for Molecular Epidemiology, in: P.A. Schulte, F.P. Perera (Eds.), Molecular Epidemiology, Academic Press, San Diego, 1993.

[13] D.J. Benford, A.B. Hanley, K. Bottrill, S. Oehlschlager, M. Balls, F. Branca, J.J. Castegnaro, J. Descotes, K. Hemminiki, D. Lindsay, B. Schilter, Biomarkers as predictive tools in toxicity testing, ATLA Altern. Lab. Anim. 28 (2000) 119-131.

[14] K. Peters, C. Walters, J. Moldovan, The Biomarker Guide Volume 2: Biomarkers and Isotopes in Petroleum Exploration and Earth History, 2nd ed., Cambridge University Press, United Kingdom, 2005.

[15] J. Reitner, V. Thiel, Encyclopedia of Geobiology, Springer, Dordrecht, The Netherlands, 2011.

[16] T. Schettino, R. Caricato, A. Calisi, M.E. Giordano, M.G. Lionetto, Biomarker approach in marine monitoring and assessment: New insights and perspectives, Open Environ. Sci. 6 (2012) 20-27.

[17] F. Albarède, Geochemistry: An Introduction, 2nd ed., Cambridge University Press, New York, 2009.

[18] E. Ribechini, F. Modugno, C. Baraldi, P. Baraldi, M.P. Colombini, An integrated analytical approach for characterizing an organic residue from an archaeological glass bottle recovered in Pompeii (Naples, Italy), Talanta. 74 (2008) 555-561.

[19] R.P. Philp, C.A. Lewis, Organic geochemistry of biomarkers. Annual review of earth and planetary sciences. 15 (1987) 363-395.

[20] R.P. Philp, E. Yang, Alkaline potassium permanganate degradation of insouble organic residues (kerogen) isolated from recently-deposited algal mats, Energ. Source. 3 (1977) 149-161.

[21] R.A. Bourbonniere, P.A. Meyers, Anthropogenic influences on hydrocarbon contents of sediments deposited in eastern Lake Ontario since 1800, Environ. Geol. 28 (1996) 22-28.

[22] E.S. Scalan, J.E. Smith, An improved measure of the odd-even predominance in the normal alkanes of sediment extracts and petroleum, Geochim. Cosmochim. Acta. 34 (1970) 611-620.

[23] E. Gelpi, H. Schneider, J. Mann, J. Oró, Hydrocarbons of geochemical significance in microscopic algae, Phytochemistry. 9 (1970) 603-612.

[24] I.M. Höld, S. Schouten, J. Jellema, J.S. Sinninghe Damsté, Origin of free and bound mid-chain methyl alkanes in oils, bitumens and kerogens of the marine, Infracambrian Huqf Formation (Oman), Org. Geochem. 30 (1999) 1411-1428.

[25] J.M. Moldowan, C.Y. Lee, P. Sundararaman, T. Salvatori, A. Alajbeg, B. Gjukic, G.J. Demaison, Source correlation and maturity assessment of select oils and rocks from the Central Adriatic Basin (Italy and Yugoslavia), Prepr. Div. Pet. Chem. Am. Chem. Soc. 34 (1989) 112-121.

[26] K.E. Peters, Petroleum tricyclic terpanes: predicted physicochemical behavior from molecular mechanics calculations, Org. Geochem. 31 (2000) 497-507.

[27] J.A. Curiale, J.R. Odermatt, Short-term biomarker variability in the Monterey formation, Santa Maria Basin, Org. Geochem. 14 (1989) 1-13.

[28] J.M. Moldowan, W.K. Seifert, E.J. Gallegos, Relationship between petroleum composition and depositional environment of petroleum source rocks. AAPG Bull. 69 (1985) 1255-1268.

[29] P. Garrigues, A. Saptorahardjo, C. González, P. Wehrung, P. Albrecht, A. Saliot, M. Ewald, Biogeochemical aromatic markers in the sediments from mahakam delta (Indonesia), Org. Geochem. 10 (1986) 959-964.

[30] K.E. Peters, J.M. Moldowan, A.R. Driscole, G.J. Demaison, Origin of Beatrice oil by co-sourcing from Devonian and Middle Jurassic source rocks, Inner Moray Firth, United Kingdom, AAPG Bull. 73 (1989) 454-471.

[31] J.M. Moldowan, P. Sundararaman, M. Schoell, Sensitivity of biomarker properties to depositional environment and/or source input in the Lower Toarcian of SW-Germany, Org. Geochem. 10 (1986) 915-926.

[32] A.A. Petrov, N.S. Vorobyova, Z.K. Zemskova, Isoprenoid alkanes with irregular "head-to-head" linkages, Org. Geochem. 16 (1990) 1001-1005.

[33] H.L. Ten Haven, J.W. de Leeuw, J.S. Sinninghe Damsté, Application of biological markers in the recognition of paleohypersaline environments, Geol. Soc. Spec. Publ. 40 (1988) 123-130.

[34] R.N. Erlich, A. Astorga, Z. Sofer, L.M. Pratt, S.E. Palmer, Palaeoceanography of organic-rich rocks of the Loma Chumico Formation of Costa Rica, Late Cretaceous, eastern Pacific, Sedimentology. 43 (1996) 691-718.

[35] M. Audino, K. Grice, R. Alexander, R.I. Kagi, Macrocyclic-alkanes: A new class of biomarker, Org. Geochem. 32 (2001) 759-763.

[36] J. Fu, G. Sheng, P. Peng, Organic geochemical characteristics of major types of terrestrial source rocks in China. Geol. Soc. Spec. Publ. 40 (1988) 279-289.

[37] W.K. Seifert, J.M. Moldowan, G.W. Smith, E.V. Whitehead, First proof of structure of a C28-pentacyclic triterpane in petroleum, Nature. 271 (1978) 436-437.

[38] K.E. Peters, A.E. Kontorovich, B.J. Huizinga, J.M. Moldowan, C.Y. Lee, Multiple oil families in the West Siberian Basin, Am. Assoc. Pet. Geol. Bull. 78 (1994) 893-909.

[39] B.G.K. van Aarssen, C. Kruk, J.K.C. Hessels, J.W. de Leeuw, Cis-cis-trans-bicadinane, a novel member of an uncommon triterpane family isolated from crude oils, Tetrahedron Lett. 31 (1990) 4645-4648.

[40] F. Picha, K.E. Peters, Biomarker oil-to-source rock correlation in the Western Carpathians and their foreland, Czech Republic, Petr. Geosci. 4 (1998) 289-302.

[41] D.A. Azevedo, F.R. Aquino Neto, B.R.T. Simoneit, A.C. Pinto, Novel series of tricyclic aromatic terpanes characterized in Tasmanian tasmanite, Org. Geochem. 18 (1992) 9-16.

[42] K. Grice, M. Audino, C.J. Boreham, R. Alexander, R.I. Kagi, Distributions and stable carbon isotopic compositions of biomarkers in torbanites from different palaeogeographical locations, Org. Geochem. 32 (2001) 1195-1210.

[43] L. Ellis, S.J. Fisher, R.K. Singh, R. Alexander, R.I. Kagi, Identification of alkenylbenzenes in pyrolyzates using GC-MS and GC-FTIR techniques: Evidence for kerogen aromatic moieties with various binding sites, Org. Geochem. 30 (1999) 651-665.

[44] H. Naraoka, A. Shimoyama, K. Harada, Isotopic evidence from an Antarctic carbonaceous chondrite for two reaction pathways of extraterrestrial PAH formation, Earth Plan. Sci. Lett. 184 (2000) 1-7.

[45] J.C.G. Sousa, A.R. Ribeiro, M.O. Barbosa, M.F.R. Pereira, A.M.T. Silva, A review on environmental monitoring of water organic pollutants identified by EU guidelines, J. Hazard. Mater. 344 (2018) 146-162.

[46] S. Cerar, N. Mali, Assessment of presence, origin and seasonal variations of persistent organic pollutants in groundwater by means of passive sampling and multivariate statistical analysis, J. Geochem. Explor. 170 (2016) 78-93.

[47] D.P. Samsonov, A.I. Kochetkov, E.M. Pasynkova, M.A. Zapevalov, Levels of persistent organic pollutants in the components of the Lake Baikal unique ecosystem, Russ. Meteorol. Hydrol. 42 (2017) 345-352.

[48] J.L. Wilkinson, P.S. Hooda, J. Swinden, J. Barker, S. Barton, Spatial distribution of organic contaminants in three rivers of Southern England bound to suspended particulate material and dissolved in water, Sci. Total Environ. 593-594 (2017) 487-497.

[49] E. Herrero-Hernández, M.S. Rodríguez-Cruz, E. Pose-Juan, S. Sánchez-González, M.S. Andrades, M.J. Sánchez-Martín, Seasonal distribution of herbicide and insecticide residues in the water resources of the vineyard region of La Rioja (Spain), Sci. Total Environ. 609 (2017) 161-171.

[50] J. Robles-Molina, B. Gilbert-López, J.F. García-Reyes, A. Molina-Díaz, Monitoring of selected priority and emerging contaminants in the Guadalquivir River and other related surface waters in the province of Jaén, South East Spain, Sci. Total Environ. 479-480 (2014) 247-257.

[51] A.S. Nagy, G. Simon, J. Szabó, I. Vass, Polycyclic aromatic hydrocarbons in surface water and bed sediments of the Hungarian upper section of the Danube River, Environ. Monit. Assess. 185 (2013) 4619-4631.

[52] S.Y. Wee, A.Z. Aris, Endocrine disrupting compounds in drinking water supply system and human health risk implication, Environ. Int. 106 (2017) 207-233.

[53] P. Bigus, M. Tobiszewski, J. Namiesnik, Historical records of organic pollutants in sediment cores, Mar. Pollut. Bull. 78 (2014) 26-42.

[54] J. Sun, L. Pan, D.C.W. Tsang, Y. Zhan, L. Zhu, X. Li, Organic contamination and remediation in the agricultural soils of China: A critical review, Sci. Total Environ. 615 (2018) 724-740.

[55] H.B. Moon, M. Choi, H.G. Choi, G. Ok, K. Kannan, Historical trends of PCDDs, PCDFs, dioxin-like PCBs and nonylphenols in dated sediment cores from a semi-enclosed bay in Korea: Tracking the sources, Chemosphere. 75 (2009) 565-571.

[56] J. Annamalai, V. Namasivayam, Endocrine disrupting chemicals in the atmosphere: Their effects on humans and wildlife, Environ. Int. 76 (2015) 78-97.

[57] S.L. Lin, W.J. Lee, S.S. Chang, C.F. Lee, L.F. Lee, C.S. Lin, H. Loong, Energy savings and emission reduction of traditional pollutants, particulate matter, and polycyclic aromatic hydrocarbon using solvent-containing water emulsified heavy fuel oil in boilers, Energy Fuels. 25 (2011) 1537-1546.

[58] J.M. Park, S.B. Lee, J.P. Kim, M.J. Kim, O.S. Kwon, D.I. Jung, Behavior of PAHs from sewage sludge incinerators in Korea, Waste Manage. 29 (2009) 690-695.

[59] L. Mu, L. Peng, J. Cao, Q. He, F. Li, J. Zhang, X. Liu, H. Bai, Emissions of polycyclic aromatic hydrocarbons from coking industries in China, Particuology. 11 (2013) 86-93.

[60] H.H. Yang, S.O. Lai, L.T. Hsieh, H.J. Hsueh, T.W. Chi, Profiles of PAH emission from steel and iron industries, Chemosphere. 48 (2002) 1061-1074.

[61] J.Y. Rau, H.H. Tseng, M.D. Lin, M.Y. Wey, Y.H. Lin, C.W. Chu, C.H. Lee, Characterization of polycyclic aromatic hydrocarbon emission from open burning of joss paper, Atmos. Environ. 42 (2008) 1692-1701.

[62] W.F. Donahue, E.W. Allen, D.W. Schindler, Impacts of coal-fired power plants on trace metals and polycyclic aromatic hydrocarbons (PAHs) in lake sediments in central Alberta, Canada, J. Paleolimnol. 35 (2006) 111-128.

[63] E. Borrás, L.A. Tortajada-Genaro, M. Vázquez, B. Zielinska, Polycyclic aromatic hydrocarbon exhaust emissions from different reformulated diesel fuels and engine operating conditions, Atmos. Environ. 43 (2009) 5944-5952.

[64] O. Devos, E. Combet, P. Tassel, L. Paturel, Exhaust emissions of pahs of passenger cars, Polycyclic Aromat. Compd. 26 (2006) 69-78.

[65] D. Contini, A. Gambaro, F. Belosi, S. De Pieri, W.R.L. Cairns, A. Donateo, E. Zanotto, M. Citron, The direct influence of ship traffic on atmospheric PM 2.5, PM 10 and PAH in Venice, J. Environ. Manage. 92 (2011) 2119-2129.

[66] R. Koppmann, Volatile Organic Compounds in the Atmosphere, Blackwell Publishing, Oxford, 2007.

[67] US Environmental Protection Acengy, Volatile organic compounds, (2017).

[68] A. Chebbi, P. Carlier, Carboxylic acids in the troposphere, occurrence, sources, and sinks: A review, Atmos. Environ. 30 (1996) 4233-4249.

[69] N.M. Puica, E. Ardelean, The industrial pollution impact on religious heritage in Romania, Eur. J. Sci. Tech. 4 (2008) 51-59.

[70] N. Prieto-Taboada, I. Ibarrondo, O. Gómez-Laserna, I. Martínez-Arkarazo, M.A. Olazabal, J.M. Madariaga, Buildings as repositories of hazardous pollutants of anthropogenic origin, J. Hazard. Mater. 248-249 (2013) 451-460.

[71] C. Saiz-Jiménez, Deposition of anthropogenic compounds on monuments and their effect on airborne microorganisms, Aerobiologia. 11 (1995) 161-175.

[72] J.M. Madariaga, Analytical chemistry in the field of cultural heritage, Anal. Methods. 7 (2015) 4848-4876.

[73] S. Orecchio, Analytical method, pattern and sources of polycyclic aromatic hydrocarbons (PAHs) in the stone of the Temples of Agrigento (Italy), J. Hazard. Mater. 176 (2010) 339-347.

[74] C. Sabbioni, N. Ghedini, A. Bonazza, G. Gobbi, M.A. Raggi, M. Gigli, G. Zappia, Formate, acetate and oxalate in black crusts on historic monuments, Adv. Air Pollut. 10 (2001) 273-282.

[75] P. Fermo, R.G. Turrion, M. Rosa, A. Omegna, A new approach to assess the chemical composition of powder deposits damaging the stone surfaces of historical monuments, Environ. Sci. Pollut. Res. 22 (2015) 6262-6270.

[76] I. Ozga, A. Bonazza, E. Bernardi, F. Tittarelli, O. Favoni, N. Ghedini, L. Morselli, C. Sabbioni, Diagnosis of surface damage induced by air pollution on 20th-century concrete buildings, Atmos. Environ. 45 (2011) 4986-4995.

[77] C. Saiz-Jiménez, Deposition of airborne organic pollutants on historic buildings, Atmos. Environ. Part B Urban Atmos. 27 (1993) 77-85.

[78] N. Prieto-Taboada, M. Maguregui, I. Martínez-Arkarazo, M.A. Olazabal, G. Arana, J.M. Madariaga, Spectroscopic evaluation of the environmental impact on black crusted modern mortars in urban-industrial areas, Anal. Bioanal. Chem. 399 (2011) 2949-2959.

[79] S. Kramar, M. Urosevic, H. Pristacz, B. Mirtic, Assessment of limestone deterioration due to salt formation by micro-Ramanspectroscopy: Application to architectural heritage, J. Raman Spectrosc. 41 (2010) 1441-1448.

[80] M. Maguregui, A. Sarmiento, I. Martínez-Arkarazo, M. Angulo, K. Castro, G. Arana, N. Etxebarria, J.M. Madariaga, Analytical diagnosis methodology to evaluate nitrate impact on historical building materials, Anal. Bioanal. Chem. 391 (2008) 1361-1370.

[81] A. Sarmiento, M. Maguregui, I. Martínez-Arkarazo, M. Angulo, K. Castro, M.A. Olazábal, L.A. Fernández, M.D. Rodríguez-Laso, A.M. Mujika, J. Gómez, J.M. Madariaga, Raman spectroscopy as a tool to diagnose the impacts of combustion and greenhouse acid gases on properties of Built Heritage, J. Raman Spectrosc. 39 (2008) 1042-1049.

[82] C. García-Florentino, M. Maguregui, H. Morillas, U. Balziskueta, A. Azcarate, G. Arana, J.M. Madariaga, Portable and Raman imaging usefulness to detect decaying on mortars from Punta Begoña Galleries (Getxo, North of Spain), J. Raman Spectrosc. 47 (2016) 1458-1466.

[83] H. Morillas, I. Marcaida, C. García-Florentino, M. Maguregui, G. Arana, J.M. Madariaga, Micro-Raman and SEM-EDS analyses to evaluate the nature of salt clusters present in secondary marine aerosol, Sci. Total Environ. 615 (2018) 691-697.

[84] M.F. Striegel, E.B. Guin, K. Hallett, D. Sandoval, R. Swingle, K. Knox, F. Best, S. Fornea, Air pollution, coatings, and cultural resources, Prog. Org. Coatings. 48 (2003) 281-288.

[85] I. Martínez-Arkarazo, M. Angulo, L. Bartolomé, N. Etxebarria, M.A. Olazabal, J.M. Madariaga, An integrated analytical approach to diagnose the conservation state of building materials of a palace house in the metropolitan Bilbao (Basque Country, North of Spain), Anal. Chim. Acta. 584 (2007) 350-359.

[86] C. Sabbioni, N. Ghedini, A. Bonazza, Organic anions in damage layers on monuments and buildings, Atmos. Environ. 37 (2003) 1261-1269.

[87] M.P. Colombini, F. Modugno, Organic Mass Spectrometry in Art and Archaeology, John Wiley & Sons, Ltd, Pisa, 2009.

[88] R.P. Evershed, Biomolecular Archaeology and Lipids, World Archaeol. 25 (1993) 74-93.

[89] R.P. Evershed, Organic residue analysis in archaeology: The archaeological biomarker revolution, Archaeometry. 50 (2008) 895-924.

[90] M.P. Colombini, F. Modugno, E. Ribechini, Organic mass spectrometry in archaeology: Evidence for Brassicaceae seed oil in Egyptian ceramic lamps, J. Mass Spectrom. 40 (2005) 890-898.

[91] J. Pérez-Arantegui, G. Cepriá, E. Ribechini, I. Degano, M.P. Colombini, J. Paz-Peralta, E. Ortiz-Palomar, Colorants and oils in Roman make-ups-an eye witness account, TrAC Trends Anal. Chem. 28 (2009) 1019-1028.

[92] M.P. Colombini, G. Giachi, F. Modugno, E. Ribechini, Characterisation of organic residues in pottery vessels of the Roman age from Antinoe (Egypt), Microchem. J. 79 (2005) 83-90.

[93] E. Ribechini, F. Modugno, J. Pérez-Arantegui, M.P. Colombini, Discovering the composition of ancient cosmetics and remedies: Analytical techniques and materials, Anal. Bioanal. Chem. 401 (2011) 1727-1738.

[94] D. Arobba, F. Bulgarelli, F. Camin, R. Caramiello, R. Larcher, L. Martinelli, Palaeobotanical, chemical and physical investigation of the content of an ancient wine amphora from the northern Tyrrhenian sea in Italy, J. Archaeol. Sci. 45 (2014) 226-233.

[95] A. Horiuchi, Y. Miyata, N. Kamijo, L. Cramp, R.P. Evershed, A dietary study of the kamegaoka culture population during the final jomon period, Japan, using stable isotope and lipid analyses of ceramic residues, Radiocarbon. 57 (2015) 721-736.

[96] S. Charters, R.P. Evershed, L.J. Goad, A. Leyden, P.W. Blonkhorn, V. Denham, Quantification and distribution of lipid in archaeological ceramics: implications for sampling potsherds for organic residue analysis and the classification of vessel use. Archaeometry. 35 (1993) 211-223.

[97] T.F.M. Oudemans, G.B. Eijkel, J.J. Boon, Identifying biomolecular origins of solid organic residues preserved in Iron Age Pottery using DTMS and MVA, J. Archaeol. Sci. 34 (2007) 173-193.

[98] O.E. Craig, H. Saul, A. Lucquin, Y. Nishida, K. Taché, L. Clarke, A. Thompson, D.T. Altoft, J. Uchiyama, M. Ajimoto, K. Gibbs, S. Isaksson, C.P. Heron, P. Jordan, Earliest evidence for the use of pottery, Nature. 496 (2013) 351-354.

[99] K. Romanus, W. Van Neer, E. Marinova, K. Verbeke, A. Luypaerts, S. Accardo, I. Hermans, P. Jacobs, D. De Vos, M. Waelkens, Brassicaceae seed oil identified as illuminant in Nilotic shells from a first millennium AD Coptic church in Bawit, Egypt, Anal. Bioanal. Chem. 390 (2008) 783-793.

[100] M.S. Copley, H.A. Bland, P. Rose, M. Horton, R.P. Evershed, Gas chromatographic, mass spectrometric and stable carbon isotopic investigations of organic residues of plant oils and animal fats employed as illuminants in archaeological lamps from Egypt, Analyst. 130 (2005) 860-871.

[101] F. Saliu, F. Modugno, M. Orlandi, M.P. Colombini, HPLC-APCI-MS analysis of triacylglycerols (TAGs) in historical pharmaceutical ointments from the eighteenth century, Anal. Bioanal. Chem. 401 (2011) 1785-1800.

[102] F. Saliu, I. Degano, M.P. Colombini, Identification of triacylglycerols in archaelogical organic residues by core-shell reversed phase liquid chromatography coupled to electrospray ionization-quadrupole-time of flight mass spectrometry, J. Chromatogr. A. 1346 (2014) 78-87.

[103] E. Ribechini, F. Modugno, M.P. Colombini, R.P. Evershed, Gas chromatographic and mass spectrometric investigations of organic residues from Roman glass unguentaria, J. Chromatogr. A. 1183 (2008) 158-169.

[104] F. Modugno, E. Ribechini, M.P. Colombini, Chemical study of triterpenoid resinous materials in archaeological findings by means of direct exposure electron ionisation mass spectrometry and gas chromatography/mass spectrometry, Rapid Commun. Mass Spectrom. 20 (2006) 1787-1800.

[105] F. Modugno, E. Ribechini, M.P. Colombini, Aromatic resin characterisation by gas chromatography-mass spectrometry. Raw and archaeological materials, J. Chromatogr. A. 1134 (2006) 298-304.

[106] M.P. Colombini, F. Modugno, E. Ribechini, Direct exposure electron ionization mass spectrometry and gas chromatography/mass spectrometry techniques to study organic coatings on archaeological amphorae, J. Mass Spectrom. 40 (2005) 675-687.

[107] I. Jerkovic, Z. Marijanovic, M. Gugic, M. Roje, Chemical profile of the organic residue from ancient amphora found in the adriatic sea determined by direct GC and GC-MS analysis, Molecules. 16 (2011) 7936-7948.

[108] B. Stern, C. Heron, T. Tellefsen, M. Serpico, New investigations into the Uluburun resin cargo, J. Archaeol. Sci. 35 (2008) 2188-2203.

[109] K. Romanus, J. Poblome, K. Verbeke, A. Luypaerts, P. Jacobs, D. De Vos, M. Waelkens, An evaluation of analytical and interpretative methodologies for the extraction and identification of lipids associated with pottery sherds from the site of Sagalossos, Turkey, Archaeometry. 49 (2007) 729-747.

[110] E.D. Teodor, G.I. Badea, A. Alecu, L. Calu, G.L. Radu, Interdisciplinary study on pottery experimentally impregnated with wine, Chem. Pap. 68 (2014) 1022-1029.

[111] R.P. Evershed, Experimental approaches to the interpretation of absorbed organic residues in archaeological ceramics, World Archaeol. 40 (2008) 26-47.

[112] M.S. Copley, P.J. Rose, A. Clapham, D.N. Edwards, M.C. Horton, R.P. Evershed, Detection of palm fruit lipids in archaeological pottery from Qasr Ibrim, Egyptian Nubia, Proc. R. Soc. B Biol. Sci. 268 (2001) 593-597.

[113] M.S. Copley, R. Berstan, S.N. Dudd, G. Docherty, A.J. Mukherjee, V. Straker, S. Payne, R.P. Evershed, Direct chemical evidence for widespread dairying in prehistoric Britain, Proc. Natl. Acad. Sci. U. S. A. 100 (2003) 1524-1529.

[114] M. Correa-Ascencio, R.P. Evershed, High throughput screening of organic residues in archaeological potsherds using direct acidified methanol extraction, Anal. Methods. 6 (2014) 1330-1340.

[115] A. Lucquin, A.C. Colonese, T.F.G. Farrell, O.E. Craig, Utilising phytanic acid diastereomers for the characterisation of archaeological lipid residues in pottery samples, Tetrahedron Lett. 57 (2016) 703-707.

[116] R.P. Evershed, S.N. Dudd, V.R. Anderson-Stojanovic, E.R. Gebhard, New chemical evidence for the use of combed ware pottery vessels as beehives in ancient Greece, J. Archaeol. Sci. 30 (2003) 1-12.

[117] M. Roffet-Salque, J. Dunne, D.T. Altoft, E. Casanova, L.J.E. Cramp, J. Smyth, H.L. Whelton, R.P. Evershed, From the inside out: Upscaling organic residue analyses of archaeological ceramics, J. Archaeol. Sci.: Reports. (2016).

[118] A. Pecci, E. Degl'Innocenti, G. Giorgi, M.Á Cau Ontiveros, F. Cantini, E. Solanes Potrony, C. Alós, D. Miriello, Organic residue analysis of experimental, medieval, and post-medieval glazed ceramics, Archaeol. Anthropol. Sci. 8 (2016) 879-890.

[119] A.C. Colonese, A. Lucquin, E.P. Guedes, R. Thomas, J. Best, B.T. Fothergill, N. Sykes, A. Foster, H. Miller, K. Poole, M. Maltby, M. Von Tersch, O.E. Craig, The identification of poultry processing in archaeological ceramic vessels using in-situ isotope references for organic residue analysis, J. Archaeol. Sci. 78 (2017) 179-192.

[120] K. Romanus, J. Baeten, J. Poblome, S. Accardo, P. Degryse, P. Jacobs, D. De Vos, M. Waelkens, Wine and olive oil permeation in pitched and non-pitched ceramics: relation with results from archaeological amphorae from Sagalassos, Turkey, J. Archaeol. Sci. 36 (2009) 900-909.

[121] S. Isaksson, F. Hallgren, Lipid residue analyses of Early Neolithic funnel-beaker pottery from Skogsmossen, eastern Central Sweden, and the earliest evidence of dairying in Sweden, J. Archaeol. Sci. 39 (2012) 3600-3609.

[122] F. Inserra, A. Pecci, M.A.C. Ontiveros, J.R. Buxó, Organic residues analysis of Late Antique pottery from Plaça Major-Horts de Can Torras (Castellar del Vallés, Catalonia, Spain), Period. Mineral. 84 (2015) 123-138.

[123] E. Manzano, S. Cantarero, A. García, A. Adroher, J.L. Vílchez, A multi-analytical approach applied to the archaeological residues in Iberian glasses. Earliest evidences on the consumption of fermented beverages in votive rituals, Microchem. J. 129 (2016) 286-292.

[124] R.P. Evershed, S.N. Dudd, S. Charters, H. Mottram, A.W. Stott, A. Raven, P.F. Van Bergen, H.A. Bland, Lipids as carriers of anthropogenic signals from prehistory, Philos. Trans. R. Soc. B Biol. Sci. 354 (1999) 19-31.

[125] J. Gea, M.C. Sampedro, A. Vallejo, A. Polo-Di´az, M.A. Goicolea, J. Fernández-Eraso, R.J. Barrio, Characterization of ancient lipids in prehistoric organic residues: Chemical evidence of livestock-pens in rock-shelters since early neolithic to bronze age, J. Sep. Sci. 40 (2017) 4549-4562.

[126] R.P. Evershed, G. Turner-Walker, R.E.M. Hedges, N. Tuross, A. Leyden, Preliminary Results for the Analysis of Lipids in Ancient Bone, J. Archaeol. Sci. 22 (1995) 277-290.

[127] A.C. Colonese, T. Farrell, A. Lucquin, D. Firth, S. Charlton, H.K. Robson, M. Alexander, O.E. Craig, Archaeological bone lipids as palaeodietary markers, Rapid Commun. Mass Spectrom. 29 (2015) 611-618.

[128] D.S. Lin, W.E. Connor, Fecal steroids of the coprolite of a Greenland Eskimo mummy, AD 1475: A clue to dietary sterol intake, Am. J. Clin. Nutr. 74 (2001) 44-49.

[129] C. Heron, S. Andersen, A. Fischer, A. Glykou, S. Hartz, H. Saul, V. Steele, O. Craig, Illuminating the Late Mesolithic: Residue analysis of 'blubber' lamps from Northern Europe, Antiquity. 87 (2013) 178-188.

[130] K. Kimpe, C. Drybooms, E. Schrevens, P.A. Jacobs, R. Degeest, M. Waelkens, Assessing the relationship between form and use of different kinds of pottery from the archaeological site Sagalassos (southwest Turkey) with lipid analysis, J. Archaeol. Sci. 31 (2004) 1503-1510.

[131] Ogrinc N., Petrisic M. G., Zigon D., A.Z. Gasparic, M. Budja, Pots and lipids: molecular and isotope evidence of food processing at Maharski prekop, Documenta Praehistorica. XXXIX (2012) 339-347.

[132] Y. Miyata, A. Horiuchi, T. Nishimoto, Traces of sea mammals on pottery from the Hamanaka 2 archaeological site, Rebun Island, Japan: Implications from sterol analysis, stable isotopes, and radiocarbon dating, Org. Geochem. 25 (2009) 15-27.

[133] E. Ribechini, S. Orsini, F. Silvano, M.P. Colombini, Py-GC/MS, GC/MS and FTIR investigations on LATE Roman-Egyptian adhesives from opus sectile: New insights into ancient recipes and technologies, Anal. Chim. Acta. 638 (2009) 79-87.

[134] R. Stacey, C. Cartwright, S. Tanimoto, Villing A., Coatings and contents: investigations of residues on four fragmentary sixth-century bc vessels from Naukratis (Egypt). The British Museum Technical Research Bulletin. 4 (2010) 19-26.

[135] C. Heron, S. Shoda, A. Breu Barcons, J. Czebreszuk, Y. Eley, M. Gorton, W. Kirleis, J. Kneisel, A. Lucquin, J. Müller, Y. Nishida, J.-. Son, O.E. Craig, First molecular and isotopic evidence of millet processing in prehistoric pottery vessels, Sci. Rep. 6 (2016) 1-9.

[136] J.W. Eerkens, Organic Residue Analysis and the Decomposition of Fatty Acids in Ancient Potsherds, in: H. Barnard, J.W. Eerkens (Eds.), Theory and Practice of Archaeological Residue Analysis, Archaeopress, Oxford, 2007.

[137] J.M. Skibo, M. Malainey, Chapter 5: Residue analysis, in: Understanding Pottery Function, Springer Science and Business Media, New York, 2013.

[138] S.N. Dudd, R.P. Evershed, A.M. Gibson, Evidence for varying patterns of exploitation of animal products in different prehistoric pottery traditions based on lipids preserved in surface and absorbed residues, J. Archaeol. Sci. 26 (1999) 1473-1482.

[139] A. Pecci, G. Giorgi, L. Salvini, M.Á Cau Ontiveros, Identifying wine markers in ceramics and plasters using gas chromatography-mass spectrometry. Experimental and archaeological materials, J. Archaeol. Sci. 40 (2013) 109-115.

[140] J. Smyth, R.P. Evershed, The molecules of meals: New insight into Neolithic foodways, Proc. R. Ir. Acad. Sect. C Archaeol. Celtic Stud. Hist. Linguist. Lit. 115 (2015) 27-46.

[141] F. Kherbouche, J. Dunne, S. Merzoug, S. Hachi, R.P. Evershed, Middle Holocene hunting and herding at Gueldaman Cave, Algeria: An integrated study of the vertebrate fauna and pottery lipid residues, Quat. Int. 410 (2016) 50-60.

[142] R.P. Evershed, H.R. Mottram, S.N. Dudd, S. Charters, A.W. Stott, G.J. Lawrence, A.M. Gibson, A. Conner, P.W. Blinkhorn, V. Reeves, New criteria for the identification of animal fats preserved in archaeological pottery, Naturwissenschaften. 84 (1997) 402-406.

[143] O.P. Nieuwenhuyse, M. Roffet-Salque, R.P. Evershed, P.M.M.G. Akkermans, A. Russell, Tracing pottery use and the emergence of secondary product exploitation through lipid residue analysis at Late Neolithic Tell Sabi Abyad (Syria), J. Archaeol. Sci. 64 (2015) 54-66.

[144] M.S. Copley, R. Berstan, V. Straker, S. Payne, R.P. Evershed, Dairying in antiquity. II. Evidence from absorbed lipid residues dating to the British Bronze Age, J. Archaeol. Sci. 32 (2005) 505-521.

[145] M.S. Copley, R. Berstan, A.J. Mukherjee, S.N. Dudd, V. Straker, S. Payne, R.P. Evershed, Dairying in antiquity. III. Evidence from absorbed lipid residues dating to the British Neolithic, J. Archaeol. Sci. 32 (2005) 523-546.

[146] A.J. Mukherjee, A.M. Gibson, R.P. Evershed, Trends in pig product processing at British Neolithic Grooved Ware sites traced through organic residues in potsherds, J. Archaeol. Sci. 35 (2008) 2059-2073.

[147] F.A. Hansel, M.S. Copley, L.A.S. Madureira, R.P. Evershed, Thermally produced ω-(o-alkylphenyl)alkanoic acids provide evidence for the processing of marine products in archaeological pottery vessels, Tetrahedron Lett. 45 (2004) 2999-3002.

[148] L. Cramp, R.P. Evershed, Reconstructing Aquatic Resource Exploitation in Human Prehistory Using Lipid Biomarkers and Stable Isotopes, in: Treatise on Geochemistry: Second Edition, Oxford, 2013.

[149] F.A. Hansel, R.P. Evershed, Formation of dihydroxy acids from Z-monounsaturated alkenoic acids and their use as biomarkers for the processing of marine commodities in archaeological pottery vessels, Tetrahedron Lett. 50 (2009) 5562-5564.

[150] L.T. Corr, M.P. Richards, S. Jim, S.H. Ambrose, A. Mackie, O. Beattie, R.P. Evershed, Probing dietary change of the Kwäday Dän Ts'ìnchi individual, an ancient glacier body from British Columbia: I. Complementary use of marine lipid biomarker and carbon isotope signatures as novel indicators of a marine diet, J. Archaeol. Sci. 35 (2008) 2102-2110.

[151] M.S. Copley, F.A. Hansel, K. Sadr, R.P. Evershed, Organic residue evidence for the processing of marine animal products in pottery vessels from the pre-colonial archaeological site of Kasteelberg D east, South Africa, S. Afr. J. Sci. 100 (2004) 279-283.

[152] M.S. Copley, H.A. Bland, P. Rose, M. Horton, R.P. Evershed, Gas chromatographic, mass spectrometric and stable carbon isotopic investigations of organic residues of plant oils and animal fats employed as illuminants in archaeological lamps from Egypt, Analyst. 130 (2005) 860-871.

[153] O.E. Craig, M. Forster, S.H. Andersen, E. Koch, P. Crombé, N.J. Milner, B. Stern, G.N. Bailey, C.P. Heron, Molecular and isotopic demonstration of the processing of aquatic prooducts in northern European prehistoric pottery, Archaeometry. 49 (2007) 135-152.

[154] J. Baeten, B. Jervis, D. De Vos, M. Waelkens, Molecular evidence for the mixing of Meat, Fish and Vegetables in Anglo-Saxon coarseware from Hamwic, UK, Archaeometry. 55 (2013) 1150-1174.

[155] S.E. Fraser, T. Insoll, A. Thompson, B.E. van Dongen, Organic geochemical analysis of archaeological medicine pots from Northern Ghana. The multi-functionality of pottery, J. Archaeol. Sci. 39 (2012) 2506-2514.

[156] S. Charters, R.P. Evershed, P.W. Blinkhorn, V. Denham, Evidence for the mixing of fats and waxes in archaeological ceramics, Archaeometry. 37 (1995) 113-127.

[157] M. Roffet-Salque, M. Regert, R.P. Evershed, A.K. Outram, L.J.E. Cramp, O. Decavallas, J. Dunne, P. Gerbault, S. Mileto, S. Mirabaud, M. Pääkkönen, J. Smyth, L. Šoberl, H.L. Whelton, A. Alday-Ruiz, H. Asplund, M. Bartkowiak, E. Bayer-Niemeier, L. Belhouchet, F. Bernardini, M. Budja, G. Cooney, M. Cubas, E.M. Danaher, M. Diniz, L. Domboróczki, C. Fabbri, J.E. González-Urquijo, J. Guilaine, S. Hachi, B.N. Hartwell, D. Hofmann, I. Hohle, J.J. Ibáñez, N. Karul, F. Kherbouche, J. Kiely, K. Kotsakis, F. Lueth, J.P. Mallory, C. Manen, A. Marciniak, B. Maurice-Chabard, M.A. Mc Gonigle, S. Mulazzani, M. Özdogəan, O.S. Perić, S.R. Perić, J. Petrasch, A.-. Pétrequin, P. Pétrequin, U. Poensgen, C. Joshua Pollard, F. Poplin, G. Radi, P. Stadler, H. Stäuble, N. Tasić, D. Urem-Kotsou, J.B. Vuković, F. Walsh, A. Whittle, S. Wolfram, L. Zapata-Peña, J. Zoughlami, Widespread exploitation of the honeybee by early Neolithic farmers, Nature. 527 (2015) 226-230.

[158] A.S. Mayyas, M.A. Al-Qudah, K.A. Douglas, F.K. Al-Ajlouny, Beeswax Preserved in Archaeological Ceramics: Function and Use, Annals of the Faculty of Arts, Ain Shams University. 40 (2012) 343-371.

[159] M.R. Guasch-Jané, M. Ibern-Gómez, C. Andrés-Lacueva, O. Jáuregui, R.M. Lamuela-Raventós, Liquid Chromatography with Mass Spectrometry in Tandem Mode Applied for the Identification of Wine Markers in Residues from Ancient Egyptian Vessels, Anal. Chem. 76 (2004) 1672-1677.

[160] P.E. McGovern, A. Mirzoian, G.R. Hall, Ancient Egyptian herbal wines, Proc. Natl. Acad. Sci. U. S. A. 106 (2009) 7361-7366.

[161] H. Barnard, A.N. Dooley, G. Areshian, B. Gasparyan, K.F. Faull, Chemical evidence for wine production around 4000 BCE in the Late Chalcolithic Near Eastern highlands, J. Archaeol. Sci. 38 (2011) 977-984.

[162] I. Jerkovic, Z. Marijanovic, M. Gugic, M. Roje, Chemical profile of the organic residue from ancient amphora found in the adriatic sea determined by direct GC and GC-MS analysis, Molecules. 16 (2011) 7936-7948.

[163] N. Garnier, P. Richardin, V. Cheynier, M. Regert, Characterization of thermally assisted hydrolysis and methylation products of polyphenols from modern and archaeological vine derivatives using gas chromatography-mass spectrometry, Anal. Chim. Acta. 493 (2003) 137-157.

[164] N. Garnier, S.M. Valamoti, Prehistoric wine-making at Dikili Tash (Northern Greece): Integrating residue analysis and archaeobotany, J. Archaeol. Sci. 74 (2016) 195-206.

[165] Y. Hamilakis, Wine, oil and the dialectics of power in Bronze Age Crete: A review of the evidence, Oxf. J. Archaeol. 15 (1996) 1-32.

[166] J. Hopkins, R.A. Armitage, Characterizing organic residues on ceramics by direct analysis in real time time-of-flight mass spectrometry, ACS Symp. Ser. 1103 (2012) 131-142.

[167] A. Pecci, M.T. Cau Ontiveros, N. Garnier, Identifying wine and oil production: Analysis of residues from Roman and Late Antique plastered vats, J. Archaeol. Sci. 40 (2013) 4491-4498.

[168] C. Milanesi, I. Bigliazzi, C. Faleri, B. Caterina, M. Cresti, Microscope observations and DNA analysis of wine residues from Roman amphorae found in Ukraine and from bottles of recent Tuscan wines, J. Archaeol. Sci. 38 (2011) 3675-3680.

[169] P.E. McGovern, G.R. Hall, Charting a Future Course for Organic Residue Analysis in Archaeology, J. Archaeol. Method and Theory. (2015).

[170] E. Allevato, M. Buonincontri, M. Vairo, A. Pecci, M.A. Cau, M. Yoneda, G.F. De Simone, M. Aoyagi, C. Angelelli, S. Matsuyama, K. Takeuchi, G. Di Pasquale, Persistence of the cultural landscape in Campania (Southern Italy) before the AD 472 Vesuvius eruption: archaeoenvironmental data, J. Archaeol. Sci. 39 (2012) 399-406.

[171] M.R. Guasch-Jané, The meaning of wine in Egyptian tombs: The three amphorae from Tutankhamun's burial chamber, Antiquity. 85 (2011) 851-858.

[172] E.D. Teodor, G.I. Badea, A. Alecu, L. Calu, G.L. Radu, Interdisciplinary study on pottery experimentally impregnated with wine, Chem. Pap. 68 (2014) 1022-1029.

[173] P. McGovern, M. Jalabadze, S. Batiuk, M.P. Callahan, K.E. Smith, G.R. Hall, E. Kvavadze, D. Maghradze, N. Rusishvili, L. Bouby, O. Failla, G. Cola, L. Mariani, E. Boaretto, R. Bacilieri, P. This, N. Wales, D. Lordkipanidze, Early Neolithic wine of Georgia in the South Caucasus, Proc. Natl. Acad. Sci. U. S. A. 114 (2017) E10309-E10318.

[174] M.D. Petit-Domínguez, R. García-Giménez, M.I. Rucandio, Chemical characterization of Iberian amphorae and tannin determination as indicative of amphora contents, Microchim. Acta. 141 (2003) 63-68.

[175] K.L. Rasmussen, J. Gunneweg, J. Van Der Plicht, I. Kralj Cigic, A.D. Bond, B. Svensmark, M. Balla, M. Strlic, G. Doudna, On the age and content of Jar-35-A sealed and intact storage jar found on the southern plateau of Qumran, Archaeometry. 53 (2011) 791-808.

[176] A.K. Styring, H. Manning, R.A. Fraser, M. Wallace, G. Jones, M. Charles, T.H.E. Heaton, A. Bogaard, R.P. Evershed, The effect of charring and burial on the biochemical composition of cereal grains: Investigating the integrity of archaeological plant material, J. Archaeol. Sci. 40 (2013) 4767-4779.

[177] L.M. Shillito, M.J. Almond, K. Wicks, L.-.R. Marshall, W. Matthews, The use of FT-IR as a screening technique for organic residue analysis of archaeological samples, Spectrochim. Acta Part A Mol. Biomol. Spectrosc. 72 (2009) 120-125.

[178] F. Hao, X. Zhou, Y. Zhu, Y. Yang, Lacustrine source rock deposition in response to co-evolution of environments and organisms controlled by tectonic subsidence and climate, Bohai Bay Basin, China, Org. Geochem. 42 (2011) 323-339.

[179] V. Suchý, I. Sýkorová, M. Havelcová, V. Machovic, A. Zeman, H. Trejtnarová, Cementation and blackening of Holocene sands by peat-derived humates: A case study from the Great Dune of Pilat, Landes des Gascogne, Southwestern France, Int. J. Coal Geol. 114 (2013) 19-32.

[180] R.P. Evershed, C. Heron, L. John Goad, Analysis of organic residues of archaeological origin by high-temperature gas chromatography and gas chromatography - Mass spectrometry, Analyst. 115 (1990) 1339-1342.

[181] J.L. Luque-García, M.D. Luque de Castro, Ultrasound: a powerful tool for leaching, Trends Anal. Chem. 22 (2003) 41-47.

[182] M. Olivares, A. Vallejo, M. Irazola, X. Murelaga, J.I. Baceta, A. Tarriño, N. Etxebarria, Optimisation of focused ultrasound extraction (FUSE) and microwave-assisted extraction (MAE) of hydrocarbons in geological chert samples, Talanta. 83 (2010) 605-612.

[183] N. Prieto-Taboada, O. Gómez-Laserna, I. Martínez-Arkarazo, M.A. Olazabal, J.M. Madariaga, Optimization of two methods based on ultrasound energy as alternative to European standards for soluble salts extraction from building materials, Ultrason. Sonochem. 19 (2012) 1260-1265.

[184] A. Vallejo, A. Usobiaga, I. Martínez-Arkarazo, A. Prieto, N. Etxebarria, O. Zuloaga, L.A. Fernández, Ultrasonic-assisted derivatization of estrogenic compounds in a cup horn booster and determination by GC-MS, J. Sep. Sci. 33 (2010) 104-111.

[185] A. Lluveras, I. Bonaduce, A. Andreotti, M.P. Colombini, GC/MS analytical procedure for the characterization of glycerolipids, natural waxes, terpenoid resins, proteinaceous and polysaccharide materials in the same paint microsample avoiding interferences from inorganic media, Anal. Chem. 82 (2010) 376-386.

[186] M. Rezaee, Y. Assadi, M.R. Milani Hosseini, E. Aghaee, F. Ahmadi, S. Berijani, Determination of organic compounds in water using dispersive liquid-liquid microextraction, J. Chromatogr. A. 1116 (2006) 1-9.

[187] J.G. Huddleston, H.D. Willauer, R.P. Swatloski, A.E. Visser, R.D. Rogers, Room temperature ionic liquids as novel media for 'clean' liquid-liquid extraction, Chem. Commun. 16 (1998) 1765-1766.

[188] R. Loos, G. Locoro, S. Contini, Occurrence of polar organic contaminants in the dissolved water phase of the Danube River and its major tributaries using SPE-LC-MS2 analysis, Water Res. 44 (2010) 2325-2335.

[189] S. Mitra, Sample Preparation Techniques in Analytical Chemistry, John Wiley & Sons, New Jersey, 2003.

[190] C.F. Poole, New trends in solid-phase extraction, TrAC Trends Anal. Chem. 22 (2003) 362-373.

[191] A. Vallejo, L.A. Fernández, M. Olivares, A. Prieto, N. Etxebarria, A. Usobiaga, O. Zuloaga, Optimization of large volume injection-programmable temperature vaporization-gas chromatography–mass spectrometry analysis for the determination of estrogenic compounds in environmental samples, J. Chromatogr. A. 1217 (2010) 8327-8333.

[192] N. Fontanals, R.M. Marcé, F. Borrull, New hydrophilic materials for solid-phase extraction, TrAC Trends Anal. Chem. 24 (2005) 394-406.

[193] B. Jurado-Sánchez, E. Ballesteros, M. Gallego, Determination of carboxylic acids in water by gas chromatography–mass spectrometry after continuous extraction and derivatisation, Talanta. 93 (2012) 224-232.

[194] N. Fontanals, R.M. Marcé, F. Borrull, P.A.G. Cormack, Mixed-mode ion-exchange polymeric sorbents: dual-phase materials that improve selectivity and capacity, TrAC Trends Anal. Chem. 29 (2010) 765-779.

[195] F. Orata, Derivatization Reactions and Reagents for Gas Chromatography Analysis , in: M.A. Mohd (Ed.), InTech, Croatia, 2012, pp. 83.

[196] J. Kaluzna-Czaplinska, A. Rosiak, M. Kwapinska, W. Kwapinski, Different Analytical Procedures for the Study of Organic Residues in Archeological Ceramic Samples with the Use of Gas Chromatography-mass Spectrometry, Crit. Rev. Anal. Chem. 46 (2016) 67-81.

[197] S.N. Dudd, R.P. Evershed, Direct demonstration of milk as an element of archaeological economies, Science. 282 (1998) 1478-1481.

[198] M.C. Pietrogrande, D. Bacco, M. Mercuriali, GC-MS analysis of low-molecular-weight dicarboxylic acids in atmospheric aerosol: Comparison between silylation and esterification derivatization procedures, Anal. Bioanal. Chem. 396 (2010) 877-885.

[199] E. Hoh, K. Mastovska, Large volume injection techniques in capillary gas chromatography, Journal of Chromatography A. 1186 (2008) 2-15.

[200] K. Kimpe, P.A. Jacobs, M. Waelkens, Analysis of oil used in late Roman oil lamps with different mass spectrometric techniques revealed the presence of predominantly olive oil together with traces of animal fat, J. Chromatogr. A. 937 (2001) 87-95.

[201] D.T.T. Nguyen, D. Guillarme, S. Rudaz, J.L. Veuthey, Fast analysis in liquid chromatography using small particle size and high pressure, J. Sep. Sci. 29 (2006) 1836-1848.

[202] E. Lesellier, Extension of the carotenoid test to superficially porous C18 bonded phases, aromatic ligand types and new classical C18 bonded phases, J. Chromatogr. A. 1266 (2012) 34-42.

[203] R. Hayes, A. Ahmed, T. Edge, H. Zhang, Core–shell particles: Preparation, fundamentals and applications in high performance liquid chromatography, J. Chromatogr. A. 1357 (2014) 36-52.

[204] J. La Nasa, E. Ghelardi, I. Degano, F. Modugno, M.P. Colombini, Core shell stationary phases for a novel separation of triglycerides in plant oils by high performance liquid chromatography with electrospray-quadrupole-time of flight mass spectrometer, J. Chromatogr. A. 1308 (2013) 114-124.

[205] J. Pérez-Arantegui, G. Cepriá, E. Ribechini, I. Degano, M.P. Colombini, J. Paz-Peralta, E. Ortiz-Palomar, Colorants and oils in Roman make-ups-an eye witness account, Trends Anal. Chem. 28 (2009) 1019-1028.

[206] M.C. Moreno-Bondi, M.D. Marazuela, S. Herranz, E. Rodriguez, An overview of sample preparation procedures for LC-MS multiclass antibiotic determination in environmental and food samples, Anal. Bioanal. Chem. 395 (2009) 921-946.

[207] P. Lucci, C.P.B. Martins, Liquid chromatography-high-resolution mass spectrometry in environmental and food analysis, in: Fast Liquid Chromatography-Mass Spectrometry Methods in Food and Environmental Analysis, London, 2015.

[208] P. Lucci, J. Saurina, O. Núñez, Trends in LC-MS and LC-HRMS analysis and characterization of polyphenols in food, Trends Anal. Chem. 88 (2017) 1-24.

[209] J. La Nasa, M. Zanaboni, D. Uldanck, I. Degano, F. Modugno, H. Kutzke, E.S. Tveit, B. Topalova-Casadiego, M.P. Colombini, Novel application of liquid chromatography/mass spectrometry for the characterization of drying oils in art: Elucidation on the composition of original paint materials used by Edvard Munch (1863–1944), Anal. Chim. Acta. 896 (2015) 177-189.

[210] M. Slowakiewicz, Z. Mikolajewski, Upper Permian Main Dolomite microbial carbonates as potential source rocks for hydrocarbons (W Poland), Mar. Pet. Geol. 28 (2011) 1572-1591.

[211] M.C. Pietrogrande, D. Bacco, S. Chiereghin, GC/MS analysis of water-soluble organics in atmospheric aerosol: Optimization of a solvent extraction procedure for simultaneous analysis of carboxylic acids and sugars, Anal. Bioanal. Chem. 405 (2013) 1095-1104.

[212] M. Tedetti, K. Kawamura, B. Charrièra, N. Chevalier, R. Sempéré, Determination of low molecular weight dicarboxylic and ketocarboxylic acids in seawater samples, Anal. Chem. 78 (2006) 6012-6018.

Chapter 2

Aims and objectives

"Somewhere, something incredible is waiting to be known."

Carl Sagan, Cosmos

Taking in consideration the stage-of-the-art regarding the use of biomarkers in many different fields of application, in the present PhD work we focused our attention on the analysis of the organic residues present in the field of landscape and heritage (fields that come together with those mentioned in the Doctorate Programme in which this project has been developed called "Interdisciplinary Scientific Strategies in Heritage and Landscape").

Landscape, according to the European Landscape Convention, is an area whose character is the result of the action and interaction of natural and/or human factors. Inside the landscape we find the cultural heritage, but also how human beings have altered it and its surroundings, including pollution issues. This novel and wide definition of landscape allows the interaction of many area of knowledge in order to protect and manage natural, rural, urban and peri-urban landscapes. In this sense, the analysis of biomarkers is a key action in order to understand the evolution of the different landscapes.

To be more precise, during the last decades, applications of biomarker technology in the geologic, environmental, and archeological fields have been continuously growing. From the early 80s, chemical fossils have been used in the field of geochemistry in order to provide information about the age, sedimentary conditions and the source of organic matter inputs in source rocks. From then on, new developments in analytical methods and instrumentation have turned out the use of biomarkers into an essential tool in order to search for pollution sources affecting the different compartments of the environment. If it was not enough, more recently, the multidisciplinary science that emerged from the collaboration between archaeologists, historians and scientists (i.e. analytical chemists) has focused its attention on the analysis of the organic residues preserved in

archaeological artifacts. The application of biomarker studies to these materials has helped revealing their use in the past together with the encompassing history.

In the framework of the analysis of organic compounds at trace levels in the field of landscape and heritage, there is a call for the development of analytical methods that enable rapid, sensitive and efficient determination of a wide variety of known and unknown organic biomarkers. Despite the suitability of several extraction, preconcentration and detection techniques largely established in the literature, the search of new analytical methods is a matter of further research. On the other hand, attending to the needs of several application fields (i.e., geological, environmental, archaeological sciences), concerning the information that is hidden in plenty of cultural and historical samples, analytical methods that provide as many chemical evidences as possible is required. Consequently, the main objectives considered in this PhD Thesis were:

1. To develop and validate a set of sensitive, quantitative and environmentally friendly analytical procedures to allow the determination of plenty of organic markers in several scientific fields concerning geological, environmental and archaeological issues.

2. For each special case, we aimed to apply the developed analytical methods in order to identify the specific organic markers and to find out information about the samples of environmental, cultural and historical interest.

In order to accomplish these main goals, several operative and specific objectives were considered in each case, as it is following described:

i) To develop, optimize and validate an analytical method for the determination of polycyclic aromatic hydrocarbons (PAHs) in beachrock samples

using FUSLE followed by LVI-PTV-GC-MS. This goal would be achieved after both extraction and analysis optimization and validation in order to determine the most important conditions that could affect the extraction efficiency of PAHs as well as other organic biomarkers related to the conditions of sedimentation. After the application of the developed method to real beachrock samples from temperate latitudes, more accurate information on the formation conditions of this unusual cemented beach grains and the influence of the organic pollutants (PAHs) during the cementation process would be studied.

ii) To develop, optimize and validate an analytical method to determine a wide range of short-chain dicarboxylic acids (C2 – C10) in marine aerosols using mixed-mode SPE-GC-MS approach. These compounds can react with carbonaceous building materials causing damages in the surface and, consequently, the method was extended to their analysis in mortar samples. The method was thought to be applied to marine aerosol water samples affecting the facade of a 20th century historic building (Punta Begoña Galleries, Getxo), located in a coastal area with an important industrial harbor (Biscay Gulf, Basque Country, Northern Spain), and to the mortars from the affected columns of the building.

iii) Similarly to the previous approach, to develop, optimize and validate an alternative analytical procedure to determine wine related organic compounds (e.g. tartaric and syringic acids) at ultra-trace level in archaeological ceramic samples. To this aim, a new method based on FUSLE-mixed-mode SPE-GC-MS would be compared with a previously established approach (i.e., ultrasound extraction followed by LLE-GC-MS). The new proposed method would be quantitative enough to identify wine-related organic compounds not only in conserved residues but also in ceramic remains. Archaeological ceramic samples dating back to II-I BCE and collected in an archaeological site from Zaragoza,

suspected to be related to wine production, would be analyzed with the developed methodology. Additionally, visible organic residues would be also studied in search of wine biomarkers due to the classification of the original amphorae (Roman origin) from which they were extracted as possible wine containers.

iv) Trying to find answers to other questions related to archaeological samples, we proposed an alternative analytical procedure to characterize lipidic organic residues that have remained in archaeological ceramic samples. The method would consist on the extraction of fatty acids (C8-C18) and related biomarkers from ceramic samples by means of FUSLE-LLE-GC-MS. The applicability of the method would be tested by a preliminary analysis of ceramic fragments that were suspected to have been used by the Basque whalers in the period from 16th to 17th century to store whale oil.

v) A multianalytical approach is often required to fully characterize organic residues of archaeological remains. Thus, in order to find not only fatty acids, but also the triacylglicerols and other organic biomarkers related to whale oil, this objective consisted on applying a method based on MAE-HPLC-QToF. We aimed to complement the results obtained by GC and HPLC approaches to find out fingerprints in archaeological ceramics where whale oil was stored after Basque whalers came back from their expeditions to Newfoundland and Labrador, in which the main objective was to capture whales.

In the following chapters, the development and achievement of the mentioned aims and objectives are described

Chapter 3

Determination of organic biomarkers in beachrocks

Ultrasonics Sonochemistry 27 (2015) 430–439

"Geologists have a saying—rocks remember"

Neil Armstrong

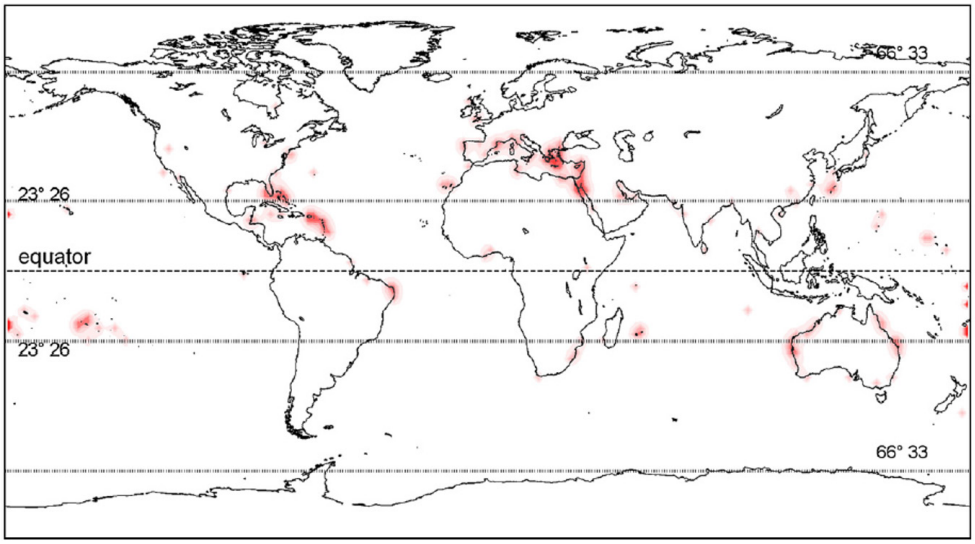
Sedimentary rocks can be formed by a series of chemical, physical and biological transformations, known as diagenesis, in environments such as, beaches, rivers, oceans and wherever sand, mud and other types of sediments are accumulated. In this respect, during the diagenetic process of lithification, the sediment is transformed into solid rock through compactation and cementation. At the latter stage of the diagenetic process, minerals are precipitated in the pore spaces of sediments binding together the primary particles [1]. Within this context, beachrocks come as a result of the arrangement of sedimentary rocks. Hence, in the specific case of beachrocks, lithification must involve beach sediments.

The origin of beachrock has been considered enigmatic and exceptional and, nowadays, is still not fully understood how it is formed [2]. Different authors have used countless definitions to define these peculiar structures, but all of them converge to a common point. In general, beachrock can be defined as consolidated coastal sedimentary formations resulting from relatively rapid indurations of beach sediments by early in situ intergranular calcium carbonate cement precipitations in the intertidal zones [3]. The mechanisms proposed for beachrock formation include biological and physicochemical processes. Recent literature has been dominated by those relating to CO_2 degassing owing to agitated marine waters and increasing temperature, mixing of alkaline fresh waters with seawater or chemical changes associated to decay of organic matter [4-8].

Until the early 60s, this sedimentary process was mainly considered characteristic of, or even limited to, tropical and subtropical coasts [9]. However, subsequent research showed that the phenomenon occurs in many diverse places, even at higher latitudes [2,10]. The principal zones where the formation of beachrock occurs are: i) tropical or subtropical zones with humid climate such as the Pacific Ocean, the Caribbean area or Australia, ii) tropical or subtropical zones with arid or semiarid climate such as the Persian Gulf and, iii) Mediterranean climate zones around the Mediterranean sea. Most reported beachrock occurrences are associated with coasts with latitudes between 0° and 40° and, in particular, between 20° and 40° as can be observed in Figure 3.1, suggesting that colder climates do

not favour beachrock formation [11]. Nevertheless, beachrock formation was detected in colder areas in the Black Sea, the Caspian Sea, the Atlantic coast of Portugal and Spain or even at higher cold climate latitudes such as Scotland, Ireland and United Kingdom [2].

Figure 3.1: *'Hot spots' of beachrock occurrence, in which the shading changes according to the*



number of occurrences in each cell.

The predominant distributional evidence identifies warm climates with pore waters rich in calcium carbonate as essential cement precipitation prerequisites for beachrock formation. Nevertheless, the mechanisms for the formation of these sedimentary rocks in cold-temperate climates, where the seawater tends to be subsaturated with respect to $CaCO_3$ and low rates of evaporation in the intertidal zone are registered, are less well known than in warmer climates.

Previous works related to beachrocks have generally focused their attention on the characterization of the mineral phases composing the cements of the beachrocks. As a consequence, several hypotheses about the implications of the formation of such deposits

in the coastal zones have been described enabling the construction of different diagenetic models. To achieve this goal, scanning electron microscopy (SEM) [4], X-Ray spectrometry and radiocarbon dating [12] have commonly been used, although in more recent studies analyses by Raman spectroscopy have also been performed [2,13]. Recently, the outstanding improvements in resolution of chromatographic techniques have opened new research lines in beachrock characterization, being possible the analysis of the organic fraction preserved during cementation. In fact, high resolution chromatographic techniques allow performing organic biomarker analyses in order to get complementary information to that given by traditional techniques [14]. Besides the improvement in chromatographic resolution, the use of analytical techniques with good sensitivity and selectivity is essential in order to detect organic biomarker compounds at trace levels (often present at sub $ng \cdot g^{-1}$ quantities). The use of large volume injection (LVI) in a programmable temperature vaporizer (PTV) (as injection system) or the use of high sensitivity mass spectrometers (as detection system) allows us to fulfill the objective.

Biomarkers are strongly useful as they can provide information about the organic matter present in the source rock (source), environmental conditions during its deposition and burial (diagenesis), the thermal maturity experienced by a rock or oil (catagenesis), the degree of biodegradation, as well as some aspects related to the source rock mineralogy (lithology) and age [15]. Among other organic biomarkers, polycyclic aromatic hydrocarbons (PAHs) are interesting compounds to be determined in sedimentary rocks. Although PAHs can be originated from natural (i.e., biomass burning, volcanic eruptions and diagenesis [16]) and from anthropogenic (i.e., coal and wood burning, petrol and diesel oil combustion, and industrial processes [17]) processes, they are always emitted as a mixture. The relative molecular concentration ratios of PAHs are considered to be characteristic of a given petrogenic or pyrogenic origin [18]. Other biomarkers such as C27-C35 pentacyclic triterpanes (hopanes) can indicate the conditions during the formation of sedimentary rocks, as for example, salinity, presence or absence of oxygen, microbial activity, etc. [15].

For instance, the presence of gammacerane ($C_{30}H_{52}$) indicates hypersaline environments and water column stratification in marine deposits. It has been found that gammacerane is also a component of bacteria and other primitive organisms [19]. Thus, the presence of such compounds within the cements could also confirm the influence of biologically mediated processes in the first stages of cementation.

Notwithstanding the remarkable information that could be obtained from the organic residues preserved in geological samples, few authors have studied the presence of organic compounds within the beachrock cements. Although Marshall et al. [20] determined biomarkers in other matrices such as Precambrian glacial sediments by means of accelerated microwave extraction, there is a lack of analytical methodologies developed to determine organic biomarkers in beachrock samples. Moreover, most of the works dealing with the analysis of such organic compounds in similar matrices such as sediments, sands, soils and rocks often use very time-consuming extraction techniques. In this framework, conventional Soxhlet extraction is the most referenced technique, regardless of the long extraction periods (i.e., 8-16 h), large amount of sample (i.e., 100-150 g) and large extractant volumes (i.e., 150 mL) required [21-24]. Other extraction methods such as microwave assisted extraction (MAE) [25] have shown to be adequate extraction techniques for several solid matrices allowing the simultaneous extraction of multiple samples, but it is still time consuming and high-priced. Accelerated solvent extraction (ASE) has also been used to extract organic compounds from similar matrices [26], but it is more expensive than the classical techniques. As a cheaper alternative, ultrasound based solid-liquid pretreatments drive effective extraction processes in shorter time, with less analyte loss during the sample pre-treatment and using a, safe, low-cost and eco-friendly methodology [27]. Currently, among the different ultrasonic processors, ultrasound bath (USB) is one of the most common instruments to accelerate the extraction of both organic and inorganic compounds from several matrices [28,29]. However, the poor sonication power of ultrasound baths has made the focused ultrasound solid-liquid extraction (FUSLE)

systems to be increasingly popular. The use of focused ultrasound energy provides a short, reproducible and quantitative extraction from different matrices [30-32], including organic compounds as target analytes [30].

The main objective of the research was to develop a fast, cost-effective, eco-friendly and sensitive analytical method based on focused ultrasound solid liquid extraction in combination with LVI-PTV-GC-MS analysis. As far as we know, this methodology has never been applied to the analysis of organic compounds (PAHs and organic biomarkers) from beachrock samples. Finally, the developed method was applied to beachrock samples with the purpose of finding PAHs and biomarkers to identify probable sources of organic matter and shed some light on the diagenetic process of these sedimentary outcrops.

3.1. Materials and methods

3.1.1. Studied material

The beachrock samples used in the optimization of the analytical procedure and its assessment belong to Tunelboka cove, which is located at 43^{o}N latitude (Bay of Biscay, Northern Spain). The beachrock is placed at an unusual temperate latitude setting, in contrast to the most documented beachrocks at tropical and subtropical emplacements [2,11]. More exactly, the cove is located in the surroundings of the Nerbioi-Ibaizabal estuary, an area marked by a strong industrialization background. This context induces a considerable heterogeneous character to the beachrocks, being the granulometric separation a helpful tool to, somehow, differentiate the cements between the framework grains [13]. Indeed, this finest particle size related to the cements is the target one in order to determine biomarkers, which will help to conclude whether microorganisms were involved in the formation of the beachrock outcrops and to understand the specific formation conditions at this latitude. Figure 3.2 shows the location of Tunelboka cove and the beachrock found in that location.

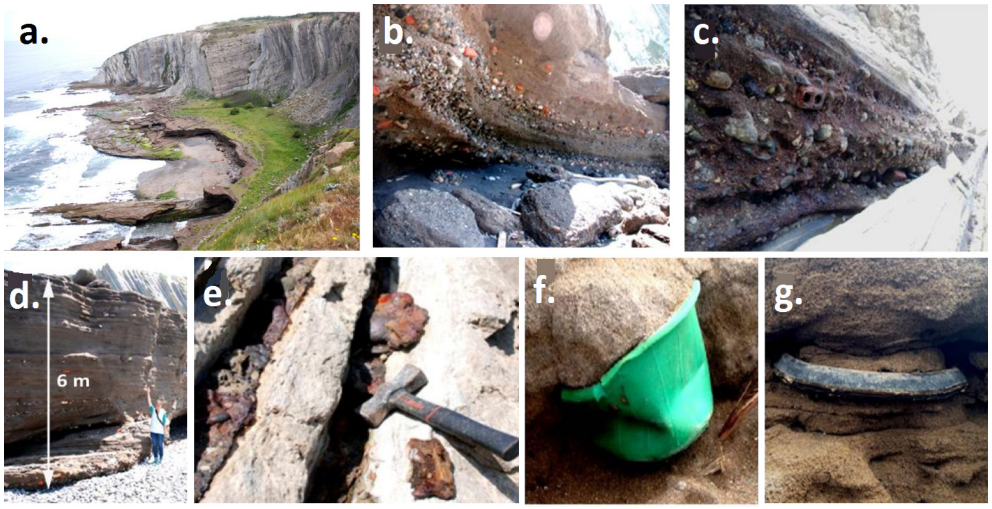


Figure 3.2: *a) General view of beachrock from Tunelboka cove; b-d) vertical sections of Tunelboka cove showing beachrock formation; e) example of a sampling point and f-g) anthropogenic artifacts cemented within the beachrock outcrops. Source: a-g) from Arrieta (2014) [9].*

3.1.2. Reagents and materials

Laboratory material was carefully cleaned with abundant pure water (<0.2 $\mu S \cdot cm^{-1}$, Millipore, USA) and without using detergent to avoid possible contamination produced by detergent residues. The material was sonicated under clean acetone (Q.P., Panreac Química, Spain) for an hour and then rinsed with ultrapure water (<0.057 $\mu S \cdot cm^{-1}$, Milli-Q model, Millipore, USA). Finally, the glass material was dried in an oven at 400 °C for 4 h.

PAH mix containing naphthalene (Naph), acenaphthylene (Acy), acenaphthene (Ace), fluorene (Flu), phenanthrene (Phe), anthracene (Ant), fluoranthene (Flr), pyrene (Pyr), benzo[a]anthracene (BaA), crysene (Cry), benzo[b]fluoranthene (B[b]F), benzo[k]fluoranthene (B[k]F), benzo[a]pyrene (B[a]P), indeno[1,2,3-cd]pyrene (IcdP), dibenzo[a,h]anthracene (DahA) and benzo[g,h,i]perylene (BghiP) at 2000 $\mu g \cdot mL^{-1}$ was purchased from Supelco (Sigma-Aldrich, Germany). A mix of five deuterated PAHs ([$^2H_2$]

Naphthalene, [$^2H_8$] Acenaphthene, [$^2H_{10}$] Phenanthrene, [$^2H_{12}$] Chrysene and [$^2H_{12}$] Perylene) supplied by Supelco (Sigma-Aldrich, Germany) was used as surrogate. Mixed fresh solutions with ≈50 $\mu g \cdot g^{-1}$ of each target compound were monthly prepared and stored in amber vials at -20 ^oC. Dilutions of these stock solutions were daily prepared in *n*-hexane according to the experimentation.

The used solvents, *n*-hexane (Hex), dichloromethane (DCM) and methanol (MeOH) (all HPLC grade, 99.8%) were purchased from LabScan (Dublin, Ireland). Copper (powder Cu), used in order to eliminate the high amounts of sulfur present in samples, was acquired from Merck (Darmstadt, Germany).

3.1.3. Extraction of PAHs and organic biomarkers from beachrock samples

3.1.3.1. Sample preparation

Special care was taken in all analytical steps (including sample crushing, fractionation and extraction) in order to minimize cross-contamination sources. Prior to the extraction, samples were air dried in a fume hood during 24 h until constant weigh and introduced in an octagon digital sieve shaker (Endecotts, London, UK).

In order to achieve the organic fraction preserved in the carbonaceous cements, and considering the anthropogenic nature of the high amount of slag and the rubble trapped within the bulk of the stratified beds, samples were subjected to a granulometric separation. Based on the grain size examinations, the finest sediment fraction (Ø < 75 μm) yielded the major part related to the carbonaceous cements, while the higher fractions were related to the particulate metallic materials (75 μm < Ø < 250 μm) and to slag and natural carbonaceous grains (i.e., gastropod shells) (250 μm < Ø < 2 mm). This fact was observed in previous studies related to beachrock matrices [13] and in other geochemical studies [33]. Indeed, during the sieving, particles suffer a grain-to-grain superficial abrasion

and thus, the cements covering the sediment surfaces could be first powdered and transferred to the fraction with the smallest grain size. The resulting Ø <75 µm grain size (mainly related with the cements) was homogenized with an agate ball mill (Pulverisette 6, Fritsch, Germany). 0.25 g of the homogenized sample were exactly weighed to be extracted by means of FUSLE and the two traditional extraction techniques (i.e., Soxhlet and ultrasound bath extractions), as detailed below.

3.1.3.2. Focused Ultrasound Solid-Liquid Extraction (FUSLE)

The isolation of the organic fraction from beachrock samples was performed by means of FUSLE. These extractions were carried out using a Bandelin Sonoplus HD 3100 sonifier ultrasonic cell disruptor/homogenizer (100 W, 20 kHz; Bandelin Electronic, Berlin, Germany) equipped with a 3 mm titanium micro-tip. The efficiency of several organic solvents was firstly evaluated and then, the extraction process variables were thoroughly optimized by means of an experimental design approach (i.e., sonication time, sonication amplitude and extraction time). Under optimum conditions, 0.25 g of the samples were accurately weighed in a glass vessel and mixed with 0.50 g of powdered activated copper and 250 $ng \cdot mL^{-1}$ of deuterated PAHs were added as surrogate before the extraction. The mixture was treated with 15 mL of DCM, immersed in an ice-water bath, and extracted for 5 minutes using a duty cycle of 0.5 $s \cdot s^{-1}$ and a sonication amplitude of 20%.

Once the extraction step was completed, and after decantation, the liquid fraction was separated from the residual solid and it was passed through glass cartridges (Supelco, Bellefonte, PA, USA) containing 0.75 g of activated copper in order to eliminate the interferences from sulfides in the chromatographic detection. The clean eluate was concentrated to dryness under a gentle stream of nitrogen at 25 ˚C (Turbovap® LV, Caliper, Life sciences, USA) and reconstituted in 200 µL of *n*-hexane. Finally, the reconstituted extract was filtered through a 0.2 µm polypropylene filter before LVI-PTV-GC-MS analysis. Figure 3.3 shows the flow chart that summarizes the whole analytical procedure.

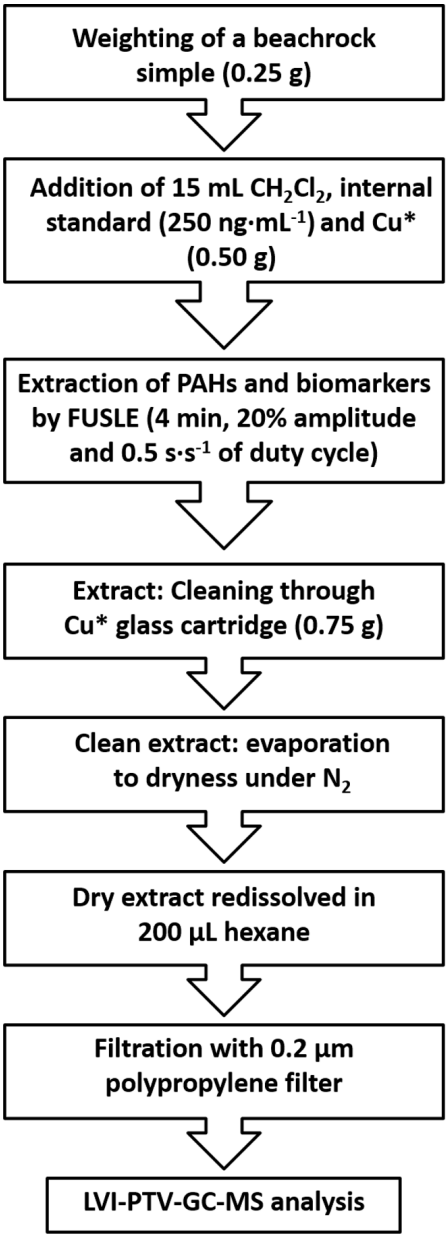


Figure 3.3: *Flow diagram of the procedure for organic matter determination in beachrock samples by FUSLE extraction and LVI-PTV-GC-MS analysis.*

3.1.3.3. Comparison with traditional extraction techniques

Beachrock samples were also extracted with traditional extraction techniques used in geochemistry applications in order to compare their extraction capabilities and to assess the suitability of the optimized method (i.e. Soxhlet and ultrasound bath).

The Soxhlet method was based on a standard method described in the literature [24]. In brief, 0.25 g of the sample were weighed and extracted with 280 mL of DCM for 8 h in the Soxhlet apparatus (in duplicate). After the extraction, the solvent was removed in a rotatory evaporator (Laborota 4000 Heidolph). The extracts were subsequently passed through glass cartridges with 0.75 g of activated copper and concentrated to dryness under a gentle stream of nitrogen at 25 °C. Finally, the concentrated extract was reconstituted in 200 µL of *n*-hexane, filtered through a 0.2 µm polypropylene filter, and analyzed by LVI-PTV-GC-MS.

In the case of ultrasound bath (USB) extraction, 0.25 g of sample were weighed and mixed with 0.5 g of activated copper. Three replicates were extracted with 15 mL of DCM for 30 min by an Ultrasons-H bath from P-Selecta with a working frequency of 40 kHz (1000 W). Once the extraction time was over, the same procedure used for FUSLE extracts was applied (see section 3.1.3.2.).

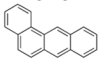
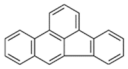
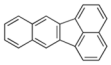
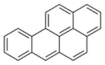
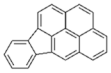
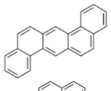
3.1.4. LVI-PTV-GC-MS analysis

The analysis was performed in a 6890N gas chromatograph (Agilent Technologies, Avondale, PA, USA) equipped with a large volume injection (LVI) system and an Agilent 5975N electron impact ionization mass spectrometer. A 20-µL aliquot of sample extract was injected using a 100 µL syringe in a cooled injection system (CIS) which consisted of a septum-less head and an empty baffled deactivated gas liner cooled with liquid nitrogen. LVI-PTV injection parameters were optimized by means of an experimental design. Under

optimum conditions, the inlet temperature was held at 20 °C while the column head pressure was fixed to 2.7 psi and the flow rate through the split vent was set at 75 mL·min^{-1} to eliminate most of the solvent. At a vent time of 0.8 min the analytes were focused onto the column in splitless mode for 1.5 min while the temperature of the PTV injection port was increased at 12 °C·s^{-1} to 300 °C and held for 5 min. Finally, the inlet was cleaned at a purge flow of 50 mL·min^{-1} before further injections.

Analytes were separated on a HP-5MS capillary column (30 m × 0.25 mm, 0.25 µm) from Agilent Technologies (Agilent Technologies, PA, USA). The oven temperature was programmed as follows: start at 55 °C for 5 min, increase at 45 °C·min^{-1} to 90 °C and a final increase at 6 °C·min^{-1} to 300 °C which was held for 15 min. Hydrogen (AD-1020 Hydrogen Generator, Cinel Strumenti Scientifici, Padova, Italy) was used as carrier gas at a constant flow of 1.3 mL·min^{-1}. The transfer line temperature was maintained at 310 °C and the ion source and the quadrupole at 230 °C and 150 °C, respectively. Detection was carried out both in the scan (50-450 *m/z*) and in the selected ion monitoring (SIM) modes simultaneously. Table 3.1 shows the m/z values of the fragment ions monitored in the SIM together with the abbreviation, structure and the deuterated surrogate used for each PAH.

Table 3.1: *Name, abbreviature, structure, formula, monitored m/z ion fragments (quantitative, qualitative) and surrogate for each PAH congener studied.*

Compound *(Abbrev.)*	Structure	Formula	m/z	Surrogate
Acenaphthene *Ace*		$C_{12}H_{10}$	**153**,154	Ace-d10
Acenaphthylene *Acy*		$C_{12}H_8$	**152**,153	Ace-d10
Fluorene *Flu*		$C_{13}H_{10}$	**165**,166	Phe-d10
Phenantrene *Phe*		$C_{14}H_{10}$	**178**,179	Phe-d10
Anthracene *Ant*		$C_{14}H_{10}$	**178**,179	Phe-d10
Fluoranthene *Flr*		$C_{16}H_{10}$	**202**,203	Cry-d12
Pyrene *Pyr*		$C_{16}H_{10}$	**202**,203	Cry-d12
Benzo[a]Anthracene *BaA*		$C_{18}H_{12}$	**228**,229	Cry-d12
Chrysene *Cry*		$C_{18}H_{12}$	**228**,229	Cry-d12
Benzo[b]Fluoranthene *B[b]F*		$C_{20}H_{12}$	**252**,253	Per-d10
Benzo[k]Fluoranthene *B[k]F*		$C_{20}H_{12}$	**252**,253	Per-d10
Benzo[a]Pyrene *B[a]P*		$C_{20}H_{12}$	**252**,253	Per-d10
Indeno [1,2,3-cd]Pyrene *IcdP*		$C_{22}H_{12}$	**276**	Per-d10
Dibenzo [a,h]Anthracene *DahA*		$C_{22}H_{14}$	**276**	Per-d10
Benzo[ghi]Perylene *BghiP*		$C_{22}H_{12}$	**276**	Per-d10

Ace-d10: [$^2H_{10}$] Acenaphthene; Phe-d10: [$^2H_{10}$] Phenanthrene; Cry-d12: [$^2H_{12}$] Chrysene; Per-d10: [$^2H_{12}$] Perylene.

3.2. Results and discussion

3.2.1. Optimization of LVI-PTV-GC-MS

In order to achieve higher sensitivity in the analysis of real samples, LVI in the solvent-vent mode was optimized and applied afterwards. Although the maximization of the chromatographic peak area is often one of the most important response variables to be considered, chromatographic peak symmetry can also be assessed when, under several chromatographic conditions, high peak tailing is observed. The former can be estimated from the asymmetry factor (ASF) parameter, which ideally should be close to the unit. ASF value is calculated as the ratio of the distance between the center of the peak and the trailing edge to the distance between the center of the peak and the leading edge, with both measurements made at 10% of the maximum peak height (IUPAC and American Society for Testing and Materials, ASTM).

To achieve the optimum working conditions, an experimental design approach was performed. Despite LVI efficiency could be affected by several variables, some of them were fixed according to a previous work [34]: vent flow: 75 $mL \cdot min^{-1}$, purge flow: 75 $mL \cdot min^{-1}$, splitless time: 1.5 min and injection volume: 20 µL. Hence, in the present work the evaluation of three instrumental parameters was considered: vent time (t_{vent},), cryo-focusing temperature (T_{cis}) and injection speed (v_{inj}) in the ranges of 0.12-3.98 min for t_{vent}, 20-80 °C for T_{cis} and 60-360 $\mu L \cdot s^{-1}$ for v_{inj}. A Central Composite Design (CCD) was carried out using the Statgraphics software (Centurion XV, Statpoint Tech. Inc., USA) and using a stock solution of 1 $\mu g \cdot mL^{-1}$ of PAHs in *n*-hexane. The chromatographic area and the peak ASF of the target compounds were used as responses of the CCD. The precision of the measurements was estimated by the relative standard deviation (RSD %) from the four replicates of the central point (RSD % values were between 1-4% in the case of chromatographic area and between 8-17% in the case of peak ASF for all target compounds).

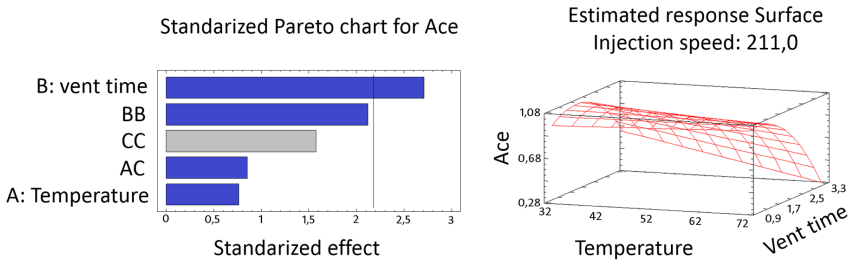


Figure 3.4: *a) Standardized Pareto Charts for the main effects and their interactions and (b) the response surface obtained for Ace varying temperature and vent time (injection speed was fixed at 211 µL·s^{-1}).*

Multiple linear regression was used to build the different response surface models considering only statistically significant variables at 95% of confidence level (p-value < 0.05). In the case of the chromatographic area, no significant effects were observed under studied conditions for any of the target compounds. Besides, regarding the peak ASF, injection speed was also not significant at the studied range for any of the target compounds; thus, it was fixed at 211 µL·s^{-1}. Cryo-focusing temperature was only significant for the late eluting compounds yielding the highest values of ASF (>2) at higher temperatures. Therefore, it was decided to fix T_{cis} at 20 °C since most target compounds gave better responses (ASF values close to 1) at this temperature. The influence of t_{vent} was observed for the earliest and the latest compounds obtaining best results at lower values, whereas there was no effect for the intermediate ones (see Figure 3.4). Indeed, when long vent times were used characteristic pre-peaks (peak splitting) were observed for the earliest PAHs (Acy, Ace and Flu). As a consequence, an optimum vent time of 0.8 min was fixed. The occurrence of this phenomenon is in concordance with other works found in the literature where larger pre-peak areas were obtained using longer venting times [35]. This effect could be probably attributed to a bad focusing of the analytes, first in the injection port and then in the column, during the solvent venting [36]. In addition, the carrier gas also may contribute to the peak splitting because chromatographic efficiency depends on it. Since most of the

published works used helium as carrier gas, the problem of peak splitting is not documented when LVI is used.

3.2.2. Elimination of sulfur interferences

The beachrock samples studied in this work presented high amounts of sulfur. In a first attempt, no clean-up step was used and the obtained chromatograms showed large interferences of sulfur, which impeded the detection of target compounds. One of the methods suggested by the US Environmental Protection Agency to avoid interferences from sulfur is the use of powdered activated copper (US EPA Method 3660B) [37]. Thus, in order to eliminate distortions in the chromatograms, additional steps were included before and after the extraction. Concretely, after the optimization of the activated copper:sample ratio, the solid sample was decided to be mixed with 0.5 g of activated copper prior to the extraction, and once the extraction was done, the liquid fraction was passed through glass cartridges containing 0.75 g of powdered activated copper. This way no interference was observed in the chromatograms.

3.2.3. Optimization of FUSLE

The variables affecting the FUSLE process (extraction solvent, ultrasound amplitude, sonication time and total extraction time) were assessed to improve the efficiency of the extraction step. All the steps involving the optimization were carried out using the Ø<75 µm fraction of homogenized beachrock sample containing PAHs and organic biomarkers but with low content of sulfur. The chromatographic peak areas were corrected with deuterated surrogates that were added after the extraction step and prior to the evaporation step. Moreover, all the areas were normalized to the weighed mass in order to correct differences due to this factor.

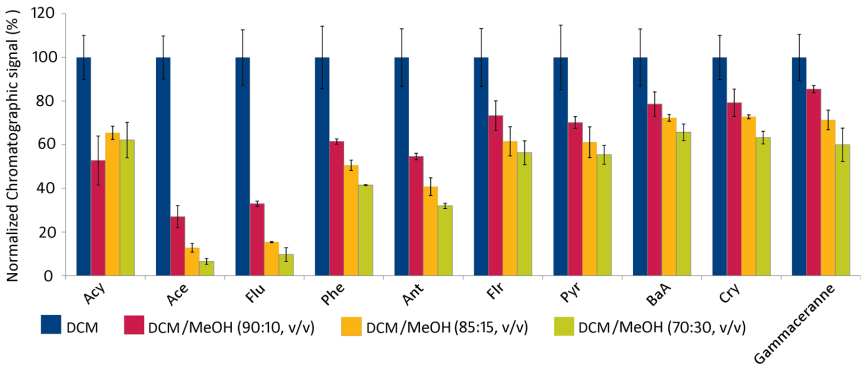


Figure 3.5: *Influence of solvent mixture during FUSLE. Results expressed as chromatographic signals normalized to the highest response (n=3, 95% confidence level).*

In a first approach, the nature of the extraction solvent was evaluated. Mixtures of non-polar and polar solvents have been used in literature for the isolation of PAHs from solid matrices such as biota, sediment and soils [38,39]. Hence, four different mixtures of DCM:MeOH (i.e., pure DCM, DCM:MeOH 90:10 (*v/v*), DCM:MeOH 85:15 (*v/v*) and DCM:MeOH 70:30 (*v/v*)) were tested as extraction solvents. All the experiments were performed in triplicate. Aliquots of 0.25 g of beachrock sample were mixed with 0.50 g of activated copper and they were extracted for 5 min (20% of amplitude and 0.5 $s \cdot s^{-1}$ of duty cycle) with 15 mL of the tested different solvents. Figure 3.5 shows the responses normalized to the highest response obtained for the first nine PAHs and one of the main abundant biomarkers (17α(H), 21β(H) hopane, also known as gammacerane). As can be observed, the extraction efficiencies decreased with the polarity of the solvent mixture, which is consistent with the non-polar nature of the target compounds and also with the literature [39]. Although the reproducibility was slightly higher when 100% of DCM was used (but still acceptable, RSD < 17%), it provided the best extraction yields for all the target compounds compared to the other assessed mixtures. Accordingly, DCM was selected as the extraction solvent for further experiments.

In FUSLE, the success in the extraction yield is mainly attributed to the use of adequate process variables, such as ultrasound amplitude, sonication time and extraction time. In pulsed sonication the extraction time is divided in different cycles, in which each cycle is the sum of the period of time that pulse is on (duty cycle) and off. In this work, cycles of 1 second were used. The influence of these parameters was simultaneously evaluated by means of a CCD and using 0.25 g of the same beachrock sample extracted with 15 mL of DCM. Thus, 18 assays (including four replicates of the central point) were performed varying the amplitude of the delivered ultrasound from 10 to 30%, the duty cycle from 0.2 to 0.8 $s \cdot s^{-1}$ and extraction time from 1 to 15 min. The studied ranges were chosen based on manufacturer's recommendations and previous knowledge [38,39].

The responses obtained for the CCD were analyzed by means of multiple linear regression, and ANOVA results indicated that the studied parameters had a small effect or no significant effect at 95% of confidence level (p-value > 0.05) for any of the target analytes. Similar results were also obtained in the literature for the isolation of PAHs in sediments [38]. Consequently, the amplitude and sonication time were fixed at intermediate values of 20% and 0.5 s, but the extraction time was deeper studied in order to fix the minimum extraction time that gives the largest extraction efficiency.

To this aim, a time profile between 0.5 and 12 min (i.e., 0.5, 0.8, 1.5, 4, 8 and 12 min) was performed in triplicate covering lower times than the ones used during the experimental design approach. As the principal purpose of the optimization was the extraction of all the organic compounds (PAHs and organic biomarkers) present in the sample, data were processed summing up all the PAHs signals detected. According to the time profile shown in Figure 3.6., the highest values were reached after 4 minutes of pulsed sonication and it was selected as optimum extraction time taking into account both method sensitivity and experimental practical aspects. This extraction time is much lower than those used in other methods employed in the field of geochemistry, but it is similar to the

extraction times found in the literature for the isolation of organic compounds in other solid matrices [40]. As a result, optimum extraction conditions were set as follows: extraction time of 4 min, duty cycle of 0.5 $s \cdot s^{-1}$ and amplitude at 20%.

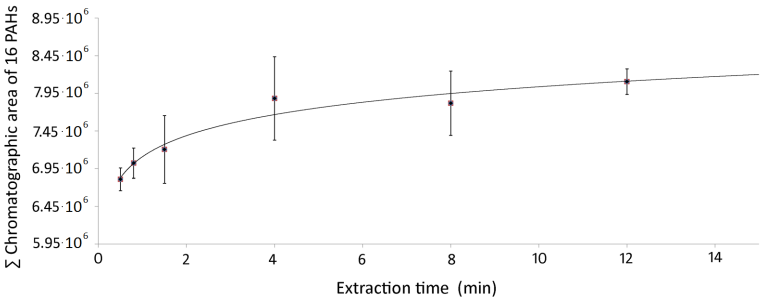


Figure 3.6: *Extraction time profile of organic compounds by means of FUSLE. Signals expressed by summing up all chromatographic areas corresponding to PAHs in one combined response (n=2, 95% confidence level).*

Finally, the number of consecutive extractions required for a quantitative extraction was evaluated by analyzing three replicates of the same real sample containing both PAHs and biomarker compounds. The consecutive extractions were performed under selected conditions and adding fresh solvent. The results showed that above 83% of the extracted amount was achieved in the first fraction for all compounds except for Flu, Phe and Ant, of which extraction efficiencies were of 65%, 66% and 77% respectively. No significant statistical differences were found between second and the third steps of FUSLE at 95% of confidence level (p-values > 0.2 for all the target compounds). Although some of the amounts recovered in the second extraction were not negligible, a second extraction over the same sample would involve the use of more chlorinated organic solvent, which reduces the "green quality" of the method. Moreover, as can be seen in the method's figures of merit section (3.2.4), the results were acceptable in terms of apparent recovery even not

having performed consecutive extractions (i.e., recovery corrected with deuterated analogues), which ensures the suitability of the method for quantitative purposes.

3.2.4. FUSLE-LVI-PTV-GC-MS method validation

The figures of merit of the developed method (FUSLE-LVI-PTV-GC-MS) were only quantitatively calculated for PAHs since the other organic biomarkers, such as hopanes and steranes, are not commercially available (see Table 3.2). Regarding the figures of merit for the LVI-PTV-GC-MS method, external calibration curves showed good linearities over a wide concentration range (i.e., 5-250 $ng \cdot mL^{-1}$) with coefficients of determination (r^2) higher than 0.991 for all the PAHs. Instrumental limits of detection (LODs) were estimated and defined as the average response (n=3) of the lowest concentration level of the calibration curve for each analyte plus three times the standard deviation, as referenced in other works [40,41]. The obtained instrumental LODs were below 41 $ng \cdot mL^{-1}$ for all PAHs (see Table 3.2).

In order to calculate the procedural limits of detection (LOD_{proc}), procedural blanks (i.e., the whole optimized extraction procedure performed without beachrock sample and using 15 mL of DCM) were analyzed (n=3). The LOD_{proc} values were estimated as the average response for each analyte plus three times the standard deviation, and when no signal was detected at the corresponding retention time, LOD_{proc} values were referred to a value of signal-to-noise ratio of 3. The use of other approaches to determine method detection limits that include the presence of matrix (i.e., the definition suggested by the US EPA, [42]) were not possible due to the presence of the target analytes at different concentration levels in real beachrock samples and due to the impossibility to find similar matrices without target compounds. Table 3.2 shows the obtained LOD_{proc} values, which were in the range of 5-32 $ng \cdot g^{-1}$.

The repeatability of the method (expressed in terms of relative standard deviation, RSD %) was assessed using three replicate extractions of a real beachrock sample analyzed

in the same day. As can be seen in Table 3.2, RSD % values were between 10% and 23% for all the target compounds.

Table 3.2: *Main method parameters (limits of detection, repeatability and accuracy) for the developed procedure.*

Analyte	Limits of detection		Repeatability	Accuracy	
	LOD ($ng \cdot mL^{-1}$)	LOD_{proc} ($ng \cdot g^{-1}$)	%RSD (n=3)	Recovery (%)	Apparent recovery (%)[a]
Phe	11	9	10	90	80[b]
Flr	12	10	13	115	89[c]
Pyr	12	9	10	118	89[c]
BaA	41	32	14	127	97[c]
Cry	23	19	14	140	107[c]
B[b]F +B[k]F	14	11	17	91	47[d]
B[a]P	22	17	23	137	77[d]
IcdP+DahA	7	5	18	188	100[d]
BghiP	7	5	17	211	109[d]

(a) Apparent recovery estimated from the certified reference material SRM 1944. (b) Corrected with [$^2H_{10}$] Phenanthrene; (c) corrected with [$^2H_{12}$] Chrysene; (d) corrected with [$^2H_{12}$] Perylene.

In the absence of certified reference material for PAHs in beachrock samples, two approaches were performed to validate the accuracy of the proposed FUSLE-LVI-PTV-GC-MS method. On the one hand, the developed method was applied to SRM 1944 reference material, certified sediment for PAHs. On the other hand, the beachrock sample (particle size of Ø< 0.75 μm) containing both PAHs and a main organic biomarker compound (i.e., gammacerane) was analyzed using the developed method and compared with other classical approaches, such as ultrasound bath extraction (USB) and Soxhlet extraction.

Consequently, 0.25 g of SRM 1944 were treated under the optimal extraction conditions for FUSLE (20 % of amplitude, 0.5 $s \cdot s^{-1}$ of duty cycle and 4 min of extraction time). Adequate recoveries (between 90% and 127%) were obtained for all the PAHs except for the heaviest ones, which showed recoveries higher than 140% (see Table 3.2). These high recoveries may be attributed to the matrix effect, but they were successfully corrected

using isotopically labeled analogues added before the extraction (200 $ng \cdot g^{-1}$). Table 3.2 shows the results obtained with this approach compared to the certified values for selected PAHs in SRM 1944. As it can be seen, the obtained results were acceptable for all target compounds except for B[b]F+B[k]F. Thus, the approach of using deuterated analogue compounds was considered suitable for the quantification of PAHs in real samples.

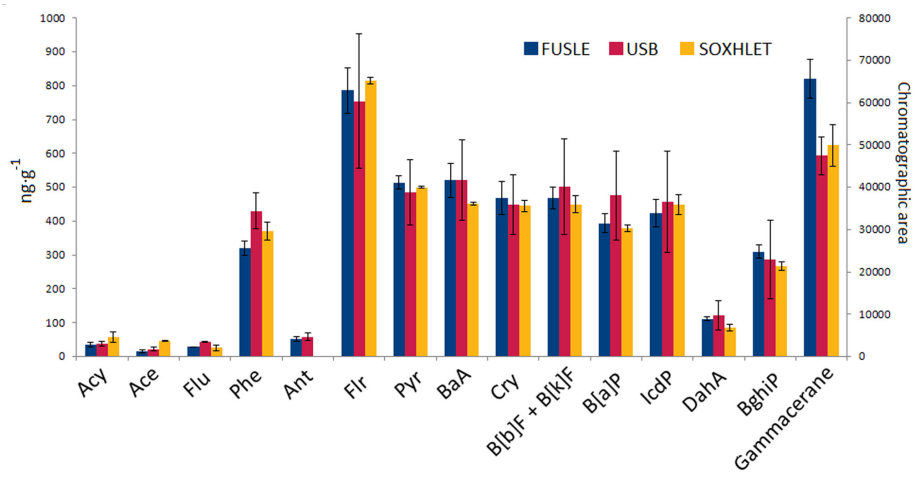


Figure 3.7: *Comparison of the concentrations obtained by means of FUSLE with other traditional techniques used in the literature: ultrasound bath extraction (USB) and Soxhlet extraction. (n=3, 95% confidence level). Ant expressed as the sum of Phe+Ant in the case of Soxhlet extraction. *Gammacerane expressed as chromatographic signal in the second y axis.*

Moreover, in order to compare the trueness and effectiveness of the developed method with those provided by traditional methods, the same amount of the beachrock sample (i.e., 0.25 g) was extracted in triplicate with the different extraction techniques (i.e., Soxhlet, USB and FUSLE). In the case of USB extraction, samples were extracted for 30 min according to the literature [43,44] and for Soxhlet extraction 8 h were needed to complete 8 cycles [24]. Figure 3.7 shows the concentrations ($ng \cdot g^{-1}$) obtained with each extraction technique for all target compounds, as well as the response obtained for gammacerane, which is one of the most representative organic biomarker found in saline environments.

The results showed for gammacerane are expressed as chromatographic signal in the second y axis and, as can be seen, FUSLE provided the highest one among all the assessed extraction techniques. It is worth mentioning that in the case of Soxhlet extraction, the chromatographic resolution was poorer and the retention times changed for all the target compounds, which could be mainly attributed to a high presence of coeluting interferences. This fact implied, for example, the coelution of two main PAHs (i.e., Phe and Ant).

The results got with the three extraction techniques were statistically comparable (t-test p-values between 0.06 and 0.77 for all target compounds). However, in the case of USB, higher RSD % values were achieved in comparison to the other techniques (4-40% for USB, 2-15% for FUSLE and 4-28% for Soxhlet extraction). These results, together with the fact that USB and Soxhlet devices require longer extraction times (24 h) and larger solvent volumes [22,23], make FUSLE a very suitable option to extract organic compounds from beachrock samples.

3.2.5. Application to real samples

We applied the developed analytical procedure to real beachrock samples from Tunelboka cove (Bay of Biscay, Northern Spain). Samples were ground to Ø<75 μm grain size and three replicates were subjected to analysis.

These analyses revealed the presence of the 16 EPA priority PAHs (see Table 3.3). PAH concentrations were estimated with the calibration curve of each analyte and corrected using deuterated analogue compounds. The expanded uncertainty of these results was calculated attending to the guidelines reported by Konieczka et al. [45] (see Table 3.3). PAH ratios were calculated according to De Luca et al. [43] indicating a pyrolitic origin of PAHs (see Tables 3.4 and 3.5). Pyrolitic sources include combustion processes (i.e., fossil fuel combustion) and are largely prevalent in aquatic environments. Pyrolitic PAHs appear to be

more strongly associated with particles and less bioavailable than petroleum-derived PAHs (petrogenic PAHs), and they do not participate in sorption and desorption processes [46]. Consequently, the distributions of pyrolytic PAHs in a variety of sedimentary environments are remarkably constant and they are much more resistant to microbial degradation [47].

Table 3.3: *Retention times, m/z values and concentrations (n=3, 95% confidence level) of the organic compounds measured in a beachrock sample from Tunelboka (Bay of Biscay).*

Compound		t_R (min)	m/z Q , q	Concentration ($ng \cdot g^{-1}$)
PAHs	**Acy**	14.02	152, 153	218 ± 20[a]
	Ace	14.68	153, 154	59 ± 10[a]
	Flu	16.48	165, 166	23 ± 6[b]
	Phe	19.96	178, 179	309 ± 9[b]
	Ant	20.12	178, 179	81 ± 9[b]
	Flr	24.51	202, 203	558 ± 9[c]
	Pyr	25.27	202, 203	397 ± 38[c]
	BaA	30.03	228, 229	586 ± 58[c]
	Cry	30.14	228, 229	564 ± 40[c]
	B[b]F + B[k]F	34.00	252, 253	615 ± 51[d]
	B[a]P	34.92	252, 253	430 ± 55[d]
	IcdP+DahA	38.40	276, 277	480 ± 43[d]
	Bghip	38.99	276, 277	460 ± 54[d]
Organic biomarkers	**17α(H),21β(H)-28,30bisnorhopane**	37.16	191, 384	-
	17α(H),21β(H)-norhopane	37.24	191, 398	-
	Gammacerane	38.07	191, 412	-
	22S-17α(H),21β(H)-homohopane	39.14	191, 426	-
	22R-17α(H),21β(H)-homohopane	39.26	191, 426	-
	22S-17α(H),21β(H)-bishomohopane	39.96	191, 440	-
	22R-17α(H),21β(H)-bishomohopane	40.16	191, 440	-

(a) corrected with [$^2H_{10}$] Acenaphthene; (b) corrected with [$^2H_{10}$] Phenanthrene; (c) corrected with [$^2H_{12}$] Chrysene; (d) corrected with [$^2H_{12}$] Perylene. Q: Quantifier ion, q: qualifier ion.

Table 3.4: *Pyrolytic and petrogenic origins of polycyclic aromatic hydrocarbons. Characteristic values of selected molecular ratios according to de Luca et al. [38].*

	$\frac{\text{LPAHs}^{a}}{\text{HPAHs}}$	$\frac{\text{Phe}}{\text{Ant}}$	$\frac{\text{Chr}}{\text{BaA}}$	$\frac{\text{Flr}}{\text{Pyr}}$	$\frac{\text{Flr}}{\text{Flr}+\text{Pyr}}$	$\frac{\text{Pyr}}{\text{BaP}}$
Pyrolytic origin	Low	< 10	< 1	> 1	> 0.5	> 10*
Petrogenic origin	High	> 15	> 1	< 1	< 0.5	

** From urban origin.*

[a] LPAHs are sum of (Naph, Acy, Ace, Flu, Phe and Ant) concentrations and HPAHs are the sum of (Flr, Pyr, BaA, Chr, B[b]F, B[k]F, B[a]P, IcdP, DahA and BghiP) concentrations.

Table 3.5: *Experimental ratios estimated from concentrations of PAHs measured in a beachrock sample from Tunelboka (n=3, 95% confidence level).*

	$\frac{\sum \text{LMW}}{\sum \text{HMW}}$	$\frac{\text{Phe}}{\text{Ant}}$	$\frac{\text{Chr}}{\text{BaA}}$	$\frac{\text{Flr}}{\text{Pyr}}$	$\frac{\text{Flr}}{\text{Flr}+\text{Pyr}}$	$\frac{\text{Pyr}}{\text{BaP}}$
Beachrock samples from Tunelboka	0.18	3.3	0.96	1.40	0.584	0.99
Standard deviation	0.03	0.8	0.03	0.02	0.003	0.04

In addition to PAHs, we detected several organic biomarkers in the analyzed samples (see Table 3.3 and Figure 3.8). The identification of these compounds was performed based on the data reported in the literature and the interpretation of mass spectra [48-50]. 17α(H), 21β(H) hopane, known as gammacerane (*m/z* 191, 412), a C30 pentacyclic triterpane in which each ring contains six carbon atoms, was the main biomarker found. The presence of this compound may indicate hypersaline/saline conditions during the formation of sedimentary rock [48], an evident fact taking into account the coastal location of samples. However, the presence of gammacerane also indicates anoxic/suboxic conditions during

sediment deposition conditions that can accelerate the process of the beachrock formation [51].

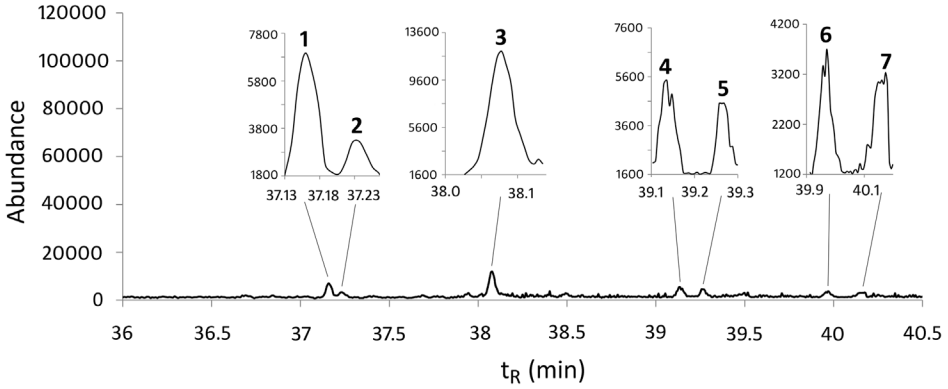


Figure 3.8: *LVI-PTV-GC-MS chromatogram obtained in SCAN mode for Tunelboka samples showing the identified biomarkers: (1) 17α(H),21β(H)-28,30-bisnorhopane; (2) 17α(H), 21β(H)-norhopane; (3) Gammacerane; (4) 22S-17α(H),21β(H)-homohopane; (5) 22R-17α(H),21β(H)-homohopane; (6) 22S-17α(H),21β(H)-bishomohopane; (7) 22R-17α(H),21β(H)-bishomohopane. m/z values of fragment ions are shown in Table 3.3.*

Besides the gammacerane, 17α(H),21β(H),28,30-bisnorhopane (*m/z* 191, 384), which is common in sulfur-rich source rocks, and 17α(H),21β(H)-norhopane (*m/z* 191, 398), were found. Finally, 22S-17α(H),21β(H)-homohopane, 22R-17α(H),21β(H)-homohopane, 22S-17α(H),21β(H)-bishomohopane and 22R-17α(H),21β(H)-bishomohopane pentacyclic triterpanes were also detected. Certainly, it is known that the latter six compounds occur from microbial removal of the methyl group during heavy biodegradation of hopanes [48]. This fact helps to understand that the microbial activity and suboxic depositional environments, together with the high input of organic contaminants, may have promoted the accelerated formation of beachrock at this unusual temperate latitude setting. Indeed, the presence of organic matter is usually linked to microbial proliferation during organic matter degradation. Microbial metabolisms alter the geochemical environment and water

chemistry and ultimately impact on carbonate precipitation reactions by raising locally the level of saturation with respect to carbonate minerals of the ambient water [52]. Various metabolic processes such as C, O, S, N and Fe-based reduction-oxidation reactions under both oxic and anoxic conditions (i.e., photosynthetic carbon fixation by cyanobacteria, hydrolysis of urea or denitrification, sulphate reduction under anoxic conditions, and dissimilatory iron-reduction), that form the basis of microbial metabolisms, greatly affect pH, alkalinity, dissolved organic carbon, and thus, the concentration and nucleation of carbonates [4,53-56].

3.3. Conclusions

The results derived from organic analysis are known to be complementary to those obtained from inorganic analyses of geological rock samples. The analysis of organic biomarkers that are supposed to be at very low $ng \cdot g^{-1}$ levels is a tricky analytical task often performed with conventional extraction techniques, which require high amount of extraction solvent, large amount of sample and/or long extraction times. Ultrasound focused solid-liquid extraction followed by large volume injection – programmable temperature vaporizer – gas chromatography – mass spectrometry (LVI-PTV-GC-MS) turned out to be a very suitable analytical procedure to analyze PAHs and organic biomarkers from beachrock samples. In fact, it resulted to be a fast, eco-friendly and very sensitive procedure that allowed the detection of target compounds at low concentration levels (low $ng \cdot g^{-1}$) with high precision (RSD < 17%) and accuracy (77 – 109%).

The innovative application of the developed method has provided a promising new approach for a fast screening of organic fraction in beachrock samples. Concretely, the detection of the aforementioned organic biomarkers, offers a potentially powerful tool for understanding the past environments and the biophysicochemical conditions in which the nucleation of the cements occurred. Indeed, all of the biomarkers detected, related to a

microbial activity during the depositional period, provided some highlighted evidences that may relate the formation of such unusual beachrock formations with the presence of organic matter and its decay. Therefore, the collected data render some important clues to reinforce the importance of the biological processes involved in the formation of beachrocks located at non-expected temperate latitudes, where the seawaters tend to be subsaturated with respect to carbonates and low rates of evaporation are registered.

Moreover, the ratios of several PAHs detected in the analyzed beachrock samples may indicate an anthropogenic input of such organic pollutants during the sedimentation and the cementation of the beach grains. Thus, the whole characterization of beachrock samples of different origins should be performed in the future in order to get more insights into the beachrock formation phenomena.

Hence, based on this work a new line of research on the identification of microbiological DNA traces within the cement forms and additional elemental, molecular and isotopic compositions of the beachrock deposits could provide new information on the biologically mediated first stages of carbonate precipitation.

3.4. References

[1] E.J. Tarbuck, F.K. Lutgens, D.G. Tasa, Earth: An Introduction to Physical Geology, Prentice Hall, 2016.

[2] N. Arrieta, N. Goienaga, I. Martínez-Arkarazo, X. Murelaga, J.I. Baceta, A. Sarmiento, J.M. Madariaga, Beachrock formation in temperate coastlines: Examples in sand-gravel beaches adjacent to the Nerbioi-Ibaizabal Estuary (Bilbao, bay of Biscay, north of Spain), Spectrochim. Acta Part A Mol. Biomol. Spectrosc. 80 (2011) 55-65.

[3] T. Danjo, S. Kawasaki, Characteristics of Beachrocks: A Review, Geotech. Geol. Eng. 32 (2014) 215-246.

[4] G.E. Webb, J.S. Jell, J.C. Baker, Cryptic intertidal microbialites in beachrock, Heron Island, Great Barrier Reef: Implications for the origin of microcrystalline beachrock cement, Sediment. Geol. 126 (1999) 317-334.

[5] M.M. Vieira, L.F. De Ros, Cementation patterns and genetic implications of Holocene beachrocks from northeastern Brazil, Sediment. Geol. 192 (2006) 207-230.

[6] D. Rey, B. Rubio, A.M. Bernabeu, F. Vilas, Formation, exposure, and evolution of a high-latitude beachrock in the intertidal zone of the Corrubedo complex (Ria de Arousa, Galicia, NW Spain), Sediment. Geol. 169 (2004) 93-105.

[7] E. Gischler, A.J. Lomando, Beachrock and Intertidal Precipitates, in Geochemical Sediments and Landscapes, Blackwell Publishing Ltd, Oxford, 2007.

[8] M.I. Vousdoukas, A.F. Velegrakis, T.V. Karambas, Morphology and sedimentology of a microtidal beach with beachrocks: Vatera, Lesbos, NE Mediterranean, Cont. Shelf Res. 29 (2009) 1937-1947.

[9] N. Arrieta, The study of an unusual temperate latitude beachrock formation. Characterization of the Azkorri beach and Tunelboka cove locations. (2014).

[10] A. Iturregui, N. Arrieta, J. Aramendia, I. Arrizabalaga, X. Murelaga, J.I. Baceta, M.A. Olazabal, I. Martínez-Arkarazo, J.M. Madariaga, In-situ and laboratory Raman spectroscopic analysis on beachrock deposits: Characterisation of the trapped materials, J. Raman Spectrosc. 47 (2016) 329-336.

[11] M.I. Vousdoukas, A.F. Velegrakis, T.A. Plomaritis, Beachrock occurrence, characteristics, formation mechanisms and impacts, Earth Sci. Rev. 85 (2007) 23-46.

[12] F. Calvet, M.C. Cabrera, J.C. Carracedo, J. Mangas, F.J. Pérez-Torrado, C. Recio, A. Travé, Beachrocks from the island of La Palma (Canary Islands, Spain), Mar. Geol. 197 (2003) 75-93.

[13] A. Iturregui, N. Arrieta, X. Murelaga, J.I. Baceta, M.Á Olazabal, I. Martínez-Arkarazo, J.M. Madariaga, The relevance of the analytical methodology in the geochemical study of beachrock outcrops: Arrigunaga Beach inside the Nerbioi-Ibaizabal estuary (Getxo, Basque Country), Anal. Methods. 6 (2014) 8247-8257.

[14] P.M. Medeiros, B.R.T. Simoneit, Gas chromatography coupled to mass spectrometry for analyses of organic compounds and biomarkers as tracers for geological, environmental, and forensic research, J. Sep. Sci. 30 (2007) 1516-1536.

[15] K. Peters, C. Walters, J. Moldovan, The Biomarker Guide Volume 2: Biomarkers and Isotopes in Petroleum Exploration and Earth History, 2nd ed., Cambridge University Press, United Kingdom, 2005.

[16] Z. Wang, J. Chen, P. Yang, X. Qiao, F. Tian, Polycyclic aromatic hydrocarbons in Dalian soils: Distribution and toxicity assessment, J. Environ. Monit. 9 (2007) 199-204.

[17] M.M.R. Mostert, G.A. Ayoko, S. Kokot, Application of chemometrics to analysis of soil pollutants, TrAC Trends Anal. Chem. 29 (2010) 430-445.

[18] M. Tobiszewski, J. Namiesnik, PAH diagnostic ratios for the identification of pollution emission sources, Environ. Pollut. 162 (2012) 110-119.

[19] J.S. Sinninghe Damsté, F. Kenig, M.P. Koopmans, J. Köster, S. Schouten, J.M. Hayes, J.W. de Leeuw, Evidence for gammacerane as an indicator of water column stratification, Geochim. Cosmochim. Acta. 59 (1995) 1895-1900.

[20] A.O. Marshall, F.A. Corsetti, A.L. Sessions, C.P. Marshall, Raman spectroscopy and biomarker analysis reveal multiple carbon inputs to a Precambrian glacial sediment, Org. Geochem. 40 (2009) 1115-1123.

[21] F. Hao, X. Zhou, Y. Zhu, Y. Yang, Lacustrine source rock deposition in response to co-evolution of environments and organisms controlled by tectonic subsidence and climate, Bohai Bay Basin, China, Org. Geochem. 42 (2011) 323-339.

[22] E. Maliński, A. Gąsiewicz, A. Witkowski, J. Szafranek, K. Pihlaja, P. Oksman, K. Wiinamäki, Biomarker features of sabkha-associated microbialites from the Zechstein Platy Dolomite (Upper Permian) of northern Poland, Palaeogeogr. Palaeoclimatol. Palaeoecol. 273 (2009) 92-101.

[23] M. Slowakiewicz, Z. Mikolajewski, Upper Permian Main Dolomite microbial carbonates as potential source rocks for hydrocarbons (W Poland), Mar. Pet. Geol. 28 (2011) 1572-1591.

[24] V. Suchý, I. Sýkorová, M. Havelcová, V. Machovic, A. Zeman, H. Trejtnarová, Cementation and blackening of Holocene sands by peat-derived humates: A case study from the Great Dune of Pilat, Landes des Gascogne, Southwestern France, Int. J. Coal Geol. 114 (2013) 19-32.

[25] H. Budzinski, M. Letellier, P. Garrigues, K. Le Menach, Optimisation of the microwave-assisted extraction in open cell of polycyclic aromatic hydrocarbons from soils and sediments. Study of moisture effect, J. Chromatogr. A. 837 (1999) 187-200.

[26] H.W. Mielke, G. Wang, C.R. Gonzales, B. Le, V.N. Quach, P.W. Mielke, PAH and metal mixtures in New Orleans soils and sediments, Sci. Total Environ. 281 (2001) 217-227.

[27] C. Bendicho, I. de la Calle, F. Pena, M. Costas, N. Cabaleiro, I. Lavilla, Ultrasound-assisted pretreatment of solid samples in the context of green analytical chemistry, Trends Anal. Chem. 31 (2012) 50-60.

[28] M. Fabianska, K. Miotlinski, A. Kowalczyk, Geochemical features of re-deposited organic matter occurring in fluvioglacial sediments in the Racibórz region (Poland): A case study, Chem. Geol. 253 (2008) 151-161.

[29] W. Gbenga Akande, Assessment of Thermal Maturity of the Mesozoic Organic-Rich Rocks of Southern England, Pac. j. sci. technol. 13 (2012) 407-416.

[30] M. Olivares, A. Vallejo, M. Irazola, X. Murelaga, J.I. Baceta, A. Tarriño, N. Etxebarria, Optimisation of focused ultrasound extraction (FUSE) and microwave-assisted extraction (MAE) of hydrocarbons in geological chert samples, Talanta. 83 (2010) 605-612.

[31] N. Prieto-Taboada, O. Gómez-Laserna, I. Martínez-Arkarazo, M.A. Olazabal, J.M. Madariaga, Optimization of two methods based on ultrasound energy as alternative to European standards for soluble salts extraction from building materials, Ultrason. Sonochem. 19 (2012) 1260-1265.

[32] S.F. Ortiz de Vallejuelo, A. Barrena, G. Arana, A. de Diego, J.M. Madariaga, Ultrasound energy focused in a glass probe: An approach to the simultaneous and fast extraction of trace elements from sediments, Talanta. 80 (2009) 434-439.

[33] J.P.M. Syvitski, Principles, methods, and application of particle size analysis, Principles, methods, and application of particle size analysis. Cambridge University Press, Cambridge, 1991.

[34] A. Vallejo, L.A. Fernández, M. Olivares, A. Prieto, N. Etxebarria, A. Usobiaga, O. Zuloaga, Optimization of large volume injection-programmable temperature vaporization-gas chromatography-mass spectrometry analysis for the determination of estrogenic compounds in environmental samples, J. Chromatogr. A. 1217 (2010) 8327-8333.

[35] A.J. Buczynska, B. Geypens, R. Van Grieken, K. De Wael, Large-volume injection combined with gas chromatography/isotope ratio mass spectrometry for the analysis of polycyclic aromatic hydrocarbons, Rapid Commun. Mass Spectrom. 28 (2014) 200-208.

[36] J.A. Gómez-Ruiz, F. Cordeiro, P. López, T. Wenzl, Optimisation and validation of programmed temperature vaporization (PTV) injection in solvent vent mode for the analysis of the 15+1 EU-priority PAHs by GC–MS, Talanta. 80 (2009) 643-650.

[37] U.S. Environmental Protection Agency (EPA), Method 3660B, Sulfur Cleanup, Revision 2, In Online Test Methods for Evaluating Solid Waste Physical/Chemical Methods (SW-846), Office of Solid Waste, (1996).

[38] A. Errekatxo, A. Prieto, O. Zuloaga, A. Usobiaga, N. Etxebarria, L.A. Fernández, Simultaneous extraction of several persistent organic pollutants in sediment using focused ultrasonic solid-liquid extraction, Anal. Bioanal. Chem. 392 (2008) 1471-1478.

[39] J.L. Capelo, M.M. Galesio, G.M. Felisberto, C. Vaz, J.C. Pessoa, Micro-focused ultrasonic solid-liquid extraction (μFUSLE) combined with HPLC and fluorescence detection for PAHs determination in sediments: Optimization and linking with the analytical minimalism concept, Talanta. 66 (2005) 1272-1280.

[40] I. Zabaleta, E. Bizkarguenaga, A. Iparragirre, P. Navarro, A. Prieto, L.T. Fernández, O. Zuloaga, Focused ultrasound solid-liquid extraction for the determination of perfluorinated compounds in fish, vegetables and amended soil, J. Chromatogr. A. 1331 (2014) 27-37.

[41] M. Moeder, S. Schrader, U. Winkler, R. Rodil, At-line microextraction by packed sorbent-gas chromatography-mass spectrometry for the determination of UV filter and polycyclic musk compounds in water samples, J. Chromatogr. A. 1217 (2010) 2925-2932.

[42] U.S. Environmental Protection Agency (EPA), Definition and Procedure for the Determination of the Method Detection Limit, Revision 2, (2016).

[43] G. De Luca, A. Furesi, R. Leardi, G. Micera, A. Panzanelli, P.C. Piu, G. Sanna, Polycyclic aromatic hydrocarbons assessment in the sediments of the Porto Torres Harbor (Northern Sardinia, Italy), Mar. Chem. 86 (2004) 15-32.

[44] E. Martinez, M. Gros, S. Lacorte, D. Barceló, Simplified procedures for the analysis of polycyclic aromatic hydrocarbons in water, sediments and mussels, J. Chromatogr. A. 1047 (2004) 181-188.

[45] P. Konieczka, J. Namiesnik, Estimating uncertainty in analytical procedures based on chromatographic techniques, J. Chromatogr. A. 1217 (2010) 882-891.

[46] S.E. Mcgroddy, J.W. Farrington, Sediment Porewater Partitioning of Polycyclic Aromatic Hydrocarbons in Three Cores from Boston Haihor, Massachusetts, Environ. Sci. Technol. 29 (1995) 1542-1550.

[47] S.D. Killops, M.S. Massoud, Polycyclic aromatic hydrocarbons of pyrolytic origin in ancient sediments: evidence for Jurassic vegetation fires, Org. Geochem. 18 (1992) 1-7.

[48] K. Peters, C. Walters, J. Moldovan, The Biomarker Guide Volume 1: Biomarkers and Isotopes in the Environment and Human History, Cambridge University Press, United Kingdom, 2005.

[49] S. Fleck, R. Michels, S. Ferry, F. Malartre, P. Elion, P. Landais, Organic geochemistry in a sequence stratigraphic framework. The siliciclastic shelf environment of Cretaceous series, SE France, Org. Geochem. 33 (2002) 1533-1557.

[50] A. Šajnovic, K. Stojanovic, B. Jovancicevic, A. Golovko, Geochemical investigation and characterisation of Neogene sediments from Valjevo-Mionica Basin (Serbia), Environ. Geol. 56 (2009) 1629-1641.

[51] A.M. Alonso-Zarza, A. Martín-Pérez, Dolomite in caves: Recent dolomite formation in oxic, non-sulfate environments. Castañar Cave, Spain, Sediment. Geol. 205 (2008) 160-164.

[52] H. Hillgärtner, C. Dupraz, W. Hug, Microbially induced cementation of carbonate sands: Are micritic meniscus cements good indicators of vadose diagenesis? Sedimentology. 48 (2001) 117-131.

[53] C. Dupraz, R.P. Reid, O. Braissant, A.W. Decho, R.S. Norman, P.T. Visscher, Processes of carbonate precipitation in modern microbial mats, Earth Sci. Rev. 96 (2009) 141-162.

[54] U. Neumeier, Experimental modelling of beachrock cementation under microbial influence, Sediment. Geol. 126 (1999) 35-46.

[55] Y. Van Lith, R. Warthmann, C. Vasconcelos, J.A. McKenzie, Microbial fossilization in carbonate sediments: A result of the bacterial surface involvement in dolomite precipitation, Sedimentology. 50 (2003) 237-245.

[56] R. Riding, Microbial carbonates: The geological record of calcified bacterial-algal mats and biofilms, Sedimentology. 47 (2000) 179-214.

Chapter 4

Water soluble organic compounds in aerosol and historical mortars affected by marine atmosphere: the case of Punta Begoña Galleries (Getxo, Northern Spain)

Under review in: Talanta

"A rock pile ceases to be a rock pile the moment a single man contemplates it, bearing within him the image of a cathedral."

Antoine de Saint-Exupery - The Little Prince

Built heritage is one of the most important cultural assets in the world and it supports the clues to enlarge the knowledge of the History. The scientific community has made significant efforts to conserve or to slow down the decaying processes of these materials as long as possible [1]. Several environmental stressors (i.e., atmospheric acid pollutants, sunlight, humidity, infiltration waters containing dissolved ions, etc.) can affect the materials promoting weathering and decay and, consequently, several pathologies on their surfaces can appear. These decaying sources have led to a significant increase in the number of research works focused on the assessment of the integrity of the building materials [2-4].

In coastal areas, the atmosphere is enriched with particles that are naturally generated by the action of wind on the seawater surface. These particles compose the sea spray or the so-called marine aerosol that mainly contains inorganic salts and water soluble organic matter [5]. The major component of marine aerosol is sodium chloride, but other salts such as sulfates and nitrates may also appear at significant quantities. Although in a lesser extent, other dissolved ions (i.e., Ca^{2+}, K^{+}, Mg^{2+}, Fe^{3+}, Al^{3+}, Sr^{2+}, NH_4^{+}, HCO_3^{-}, Br^{-}, etc.) and suspended materials can be also part of marine aerosol [6]. Once these salts reach the material's surface, they can react with other components producing new salts formations (efflorescence and white crusts) or migrate inside the material through the pores precipitating new salts (sub-efflorescence) with the subsequent material degradation [7,8]. The appearance of these pathologies, mainly attributed to the high presence of ions in marine aerosols, has driven the assessment of the conservation state of different buildings sited in coastal areas [9-12].

In addition, the 10% of marine aerosol is composed of organic matter and occasionally, metals such as As, Ni, Cd, Pb, Ti, V, Cr, Mn, Cu, Mo and Hg. $PM_{2.5}$ and PM_{10} airborne particulate material can be also transported by the marine aerosol. Most pollutants outdoor generated are stable enough to accumulate on building surfaces through wet and dry

depositions, and after some physico-chemical reactions they would end up exerting structural degradations [13].

In general, the concentration of the organic contaminants found in marine aerosol are correlated with traffic-related pollutants, combustion processes and other industrial and harbor activities [14]. In addition to persistent organic pollutants, the so-called water soluble organic compounds (WSOCs) require attention in this field. Dicarboxylic acids and related compounds are an important class of WSOCs, accounting for up to 3% and 16% of the total organic carbon in urban areas and marine aerosols, respectively [15,16]. Small organic anions, such as formate, acetate and oxalate, were consistently found in black crusts in the work published by Sabionni [17]. In a recent study, the reactivity of some WSOCs in aerosol, namely, dicarboxylic acids, with carbonaceous building materials was also evaluated showing damages in the porous matrix [18].

The presence of dicarboxylic acids in aerosols may result from a wide range of sources. Primary sources include vehicular exhausts, biomass combustions and natural marine sources, but they are mainly originated from the atmospheric photooxidation of volatile and semi-volatile organic compounds (secondary sources) [19]. Among low molecular weight dicarboxylic acids, oxalic acid (C2) is the most abundant species and it is considered an end product from the degradation of many organic compounds [20]. Besides, oxalic (C2), malonic (C3), succinic (C4) and phthalic (C8, Ph) acids are the dicarboxylic acids mostly found in urban and marine areas [21]. Although higher dicarboxylic acids are also found in a lesser extent, they are often monitored in research works focused on atmospheric pollution since they can be used as tracers to identify different sources of contamination. In this regard, glutaric acid (C5) and adipic acid (C6) are considered to be originated from the oxidation of cyclic and aliphatic olefins, while pimelic acid (C7), suberic acid (C8) and azelaic acid (C9) are thought to be formed via photooxidation of unsaturated carboxylic acids like oleic and linoleic acids [22,23].

The pollutants present in air, water and marine aerosol can act as catalysts of the chemical weathering damage and, therefore, they are considered more harmful in urban-industrial contaminated areas [10]. Within this scenario, while SO_2 deposition processes affecting building materials have been extensively studied, few works in the literature aim to analyze the organic contaminants trapped on the surface of building materials [24]. The matrix complexity and low concentration levels of WSOCs require a preconcentration step that typically accounts for more than half of the total analysis time, and it can affect the quality of the final results. Moreover, the physicochemical characteristics of WSOCs, such as their low molecular weight, relative high polarity and limited stability, add more challenges to the analysis [25]. Some researchers decided on using a rotary evaporator to evaporate large volumes of water [26,27]. However, this approach shows many disadvantages, such as loss of volatile organic compounds and adsorption of polar compounds on the glass walls of the flask. Liquid-liquid extraction can solve some of the previous problems when using large volumes of organic solvents and performing consecutive extractions [28,29]. However, these strategies do not allow the elimination of interferences arising from the presence of compounds with similar physicochemical properties as those of the target compounds.

Solid phase extraction (SPE) is the preferred technique to isolate a wide variety of organic contaminants from water samples avoiding the previously mentioned drawbacks. Activated charcoal and adsorption on ion exchange like Amberlite XAD-type resins have been used for the preconcentration of small organic acids [30]. However, the use of activated charcoal is effective for the retention of medium to large carbon chain carboxylic compounds but not for the smallest ones [31]. New hydrophilic polymeric sorbents with enhanced retention mechanisms have been used for the preconcentration of dicarboxylic acids from aqueous extracts [32] but the breakthrough volumes for very small, highly polar molecules (namely, oxalic, malonic or succinic acids) in water are too small for their appropriate retention and quantitative isolation. The so-called mixed-mode sorbents have

recently appeared in order to isolate target compounds of a wide variety of polarities allowing selective extractions through a dual mechanism: ion-exchange and reversed-phase mode. The mixed-mode SPE procedure renders cleaner extracts and has lower matrix effects and improved method detection limits as it has been proven in many scientific works [33,34].

In this context, the principal aim of this work was the development of an analytical method based on solid phase extraction coupled to gas chromatography-mass spectrometry (SPE-GC-MS) for the determination of short chain dicarboxylic acids (C2-C10) in marine aerosol water samples as well as in mortars, in which a previous focused ultrasound solid-liquid extraction (FUSLE) was necessary. After the optimization and validation of the whole procedure, the method was applied to marine aerosol water samples affecting the facade of a 20th century historic building (Punta Begoña Galleries, Getxo), located in a coastal area with an important industrial harbor (Biscay Gulf, Basque Country, Northern Spain), and to the mortars from the affected columns located inside the building.

4.1. Experimental procedure

4.1.1. Cleaning procedure

As dicarboxylic acids are ubiquitous, special care was taken to avoid any cross-contamination. Dicarboxylic acids are prone to absorbing in glass surfaces, thus, plastic material or silanized glass material was used throughout the experimentation. All the laboratory material was washed with abundant Ellix quality water (< 0.2 µS·cm^{-1}, Millipore, Bedford, MA, USA) and then sonicated under clean acetone (Q.P., Panreac Química, Barcelona, Spain) for at least an hour or maintained in a clean acetone bath overnight. After that, the material was rinsed with abundant Milli-Q water (< 0.057 µS·cm^{-1}, Milli-Q Model 185, Millipore, Bedford, MA, USA).

4.1.2. Reagents and materials

The thirteen dicarboxylic acids: oxalic acid (C2), malonic acid (C3), maleic acid (C4, M), succinic acid (C4), fumaric acid (C4, F), glutaric acid (C5), adipic acid (C6), pimelic acid (C7), phthalic acid (C8, Ph), suberic acid (C8), azelaic acid (C9) and sebacic acid (C10) were obtained from Fluka (Sigma Aldrich, Steinheim, Germany). The deuterated dodecanoic acid-d23 used as surrogate was purchased from Sigma Aldrich (Steinheim, Germany).

Solid standards were dissolved to prepare individual stock solutions of approximately 3000 $\mu g \cdot g^{-1}$ in acetonitrile (AcN, HPLC-grade, 99.9%, Sigma Aldrich, Germany) with the exception of fumaric acid, phthalic acid, suberic acid, azelaic acid and sebacic acid, which were dissolved in acetone (HPLC-grade, 99.8%, LabScan, Ireland). All stock solutions were stored in silanized amber glass vials at -20 °C.

A mixed fresh stock solution containing 40 $\mu g \cdot mL^{-1}$ of all target compounds was prepared monthly in acetonitrile, whereas intermediate dilutions were prepared daily according to the experimentation.

The derivatization reagents N,O-bis(trimethylsilyl) trifluoroacetamide with 1% trimethylchlorosilane (BSTFA + 1%TMCS) and N-(t-butyldimethylsilyl)-N-methyltrifluoroacetamide (MTBSTFA) were supplied by Supelco (Walton-on-Thames, UK). Pyridine and ethyl acetate used as derivatization solvents were obtained by Honeywell Riedel-de Haën™ (Germany) and by Sigma Aldrich (EtOAc, HPLC-grade, 99.9%, Germany), respectively. Empty SPE tubes and ENVI-Carb graphitized carbon (200-mg, 6 mL, 100 $m^2 \cdot g^{-1}$ specific surface area) used during the preconcentration step were purchased from Supelco (Bellefonte, PA, USA). Plexa (200-mg, 6 mL, 550 $m^2 \cdot g^{-1}$ specific surface area, 320 mesh) and OASIS-MAX (poly(divinylbenzene-co-*N*-vinylpirrilidone) + quaternary amine polymer, 150-mg) SPE cartridges were purchased from Agilent Technologies (Avondale, PA, USA) and Waters (Milford, USA), respectively.

4.1.3. Sampling procedure and samples

Punta Begoña Galleries, a 20th century historical building, are located in front of the Ereaga beach and the industrial harbor of Bilbao, in the Basque Country (Northern Spain) (43°20´26.8´´N 3°00´51.5´´W) (see Figure 4.1). Northwest direction winds are predominant in the area where Punta Begoña Galleries are located. However, in summer, there are many days with north wind and also southwest in autumn.

Figure 4.1: *Location of the galleries of Punta Begoña (Getxo, Basque Country) and sampling points depending on the wind directions. (SW: southwest oriented sampling point; NW: northwest oriented sampling point).*

The galleries are composed of two levels: the upper gallery (oriented to the southwest) and the lower gallery (oriented to the northwest) (see Figure 4.1). Two different sampling locations were selected according to the predominant wind directions of the area: (i) part of the building in front of the sea (northwest orientation, samples named as AN) and (ii) part of the building oriented to the harbor (southwest orientation, samples named as AW). Aerosol water samples (A) were collected for 24 hours in June 2016 using a dehumidifier (Qlima D510) and a 20 L plastic bottle collector.

Mortars exposed to the marine environment were sampled in the same sampling points of aerosol water samples. Exposed mortar samples (M) were collected in the upper gallery (named as MW) and in the lower gallery (named as MN). Non-exposed mortars were also collected in the inner part of the walls of the same gallery and were used for method development purposes.

4.1.4. Extraction and preconcentration of WSOCs

4.1.4.1. Extraction of WSOCs from mortars

WSOCs were extracted from the mortars by means of FUSLE. The extractions were carried out using a Bandelin Sonopuls HD 3100 sonifier ultrasonic cell disruptor/homogenizer (100 W, 20 kHz; Bandelin Electronic, Berlin, Germany) equipped with a 3 mm titanium micro-tip, and the extraction process variables (i.e., duty cycle, sonication amplitude and extraction time) were fixed according to previous works [35,36].

Briefly, 0.5 g of exposed mortar samples were spiked with dodecanoic acid-d23 used as surrogate and extracted by means of FUSLE (20% of amplitude, 0.5 $s \cdot s^{-1}$ of duty cycle and 4 min of extraction time) using 7 mL of an AcN:H_2O mixture (2:1, *v/v*). The extracts were filtered through 0.45 µm PTFE filters and the AcN was evaporated under a gentle stream of nitrogen at 40 ^{o}C (Turbovap® LV, Caliper, Life sciences, USA) until approximately 2.5 mL of

water remained. The extracts were diluted to a final volume of 5 mL of water and submitted to a further preconcentration of analytes by means of SPE.

4.1.4.2. Preconcentration of WSOCs from water samples and mortars

The extraction and preconcentration of WSOCs from environmental aerosol water samples was performed by means of SPE. The optimization assays were performed with aliquots of 100 mL of Milli-Q water fortified at 8.5 $ng \cdot mL^{-1}$ of the target compounds and using dodecanoic acid-d23 as surrogate. In order to achieve the maximum extraction efficiencies and taking into account the chemical properties of the target compounds, reversed-phase and mixed-mode SPE were tested.

For the reverse-phase SPE approach (RP-SPE), 100 mL of spiked Milli-Q water (pH adjusted to 1 with HCl) were loaded onto 200-mg Envicarb or 200-mg Plexa cartridges, which were previously conditioned with 5 mL of AcN and 5 mL of acidified Milli-Q water (pH=1). Afterwards, 5 mL of acidified Milli-Q water (pH=1) were added as washing step and the cartridge was completely dried for 1 h under vacuum. Finally, the analytes were recovered using 5 mL of AcN.

In the case of mixed-mode SPE, aliquots of 100 mL of spiked Milli-Q water were loaded onto 150-mg Oasis MAX cartridges (mixed-mode strong anion exchanger). Before sample loading, the cartridges were conditioned with 5 mL of AcN and 5 mL of Milli-Q water. In this case, the adjustment of pH of the sample was not required since all the target compounds were in the ionized form at pH∼7. After the sample loading, 5 mL of Milli-Q water and 5 mL of AcN were added. The cartridges were completely dried for 1 h under vacuum before recovering the analytes with 5 mL of 0.8% HCl in AcN.

Regardless of the preconcentration strategy used, the extracts were concentrated to dryness under a gentle stream of nitrogen at 40 °C, reconstituted in 150 µL of 75:25 EtOAc:AcN (*v/v*) and derivatized with 20 µL of BSTFA + 1%TMCS prior to the GC-MS analysis.

In the case of the mortar samples, once the mixed-mode SPE was selected as the optimum approach, the extracts obtained by means of FUSLE (see section 4.1.4.1) were charged into the cartridges following the same procedure described above.

4.1.5. Optimization of the derivatization step

Several variables affecting the derivatization step such as the derivatization reagent nature and volume, the derivatization solvent, temperature and time were optimized. During the optimization, 20 µL of standard solution of a mixture containing all the target analytes at 40 $\mu g \cdot mL^{-1}$ each were evaporated to dryness under a gentle stream of nitrogen at 40 °C. Aliquots of 130 µL of different solvents (EtOAc, AcN and pyridine) and different volumes of the BSTFA + 1% TMCS used as derivatization reagent (20 µL, 40 µL and 60 µL) were added and submitted to the derivatization step (30 min at 60 °C). Under the optimum conditions, sample extracts were evaporated to dryness in chromatographic amber vials, reconstituted in 150 µL of 75:25 EtOAc:AcN (*v/v*) and derivatized with 20 µL of BSTFA + 1% TMCS at 60°C for 30 min.

4.1.6. Instrumentation

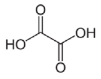
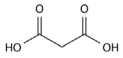
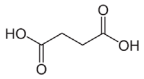
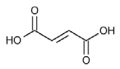
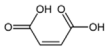
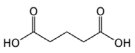
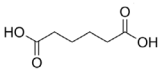
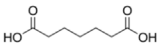
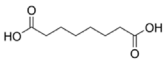
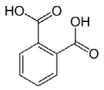
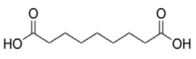
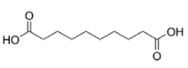
The separation and detection of the extracts was performed in a 6890N gas chromatograph (Agilent Technologies, Avondale, PA, USA) equipped with an Agilent 5973N electron impact ionization mass spectrometer using an Agilent 7683 autosampler. 2µL of the derivatized extracts were injected in splitless mode at 250°C in a capillary column HP-5MS (30 m x 0.25 mm, 0.25 µm, Agilent Technologies). Hydrogen (AD-1020 Hydrogen Generator, Cinel Strumenti Scientifi, Padova, Italy) was used as a carrier gas at a constant flow of 1.3 $mL \cdot min^{-1}$. The following oven temperature program was used for the separation

of the target analytes: 60 °C (hold 4 min), temperature increase at 10°C·min^{-1} to 150°C and hold 3 min, a second temperature increase at 10°C·min^{-1} to 220°C to continue rising temperature at 25°C·min^{-1} to 300°C that was maintained for 5 min.

The mass spectrometer worked in the electron impact mode with a potential difference of 70 eV. The MS transfer line temperature was kept at 310 °C and the ion source and quadrupole temperatures were maintained at 230 °C and 150 °C, respectively. Detection was carried out both in the scan (*m/z* 50-450) and in the selected ion monitoring (SIM) modes. The *m/z* values of the fragment ions monitored in the SIM mode are listed in Table 4.1. The first ion was used as quantifier, whereas the second and third ions were considered as qualifiers.

The quantification of soluble salts (only anions) was carried out by a Dionex ICS 2500 suppressed ion chromatograph coupled to a conductivity detector ED50. An IonPac AS23 (4 x 250 mm) column and IonPac AG23 (4 x 50 mm) precolumn were used for the separation of anions. 4.5 mM Na_2CO_3 /0.8 mM $NaHCO_3$, 25 mA and 1 mL·min^{-1} were used as mobile phase, suppression current and flow, respectively.

Table 4.1: *Analyzed compounds, pKa, log K_{ow} and characteristic ions of C2-C10 dicarboxylic acids. The first ion (bold) was used as quantifier and the rest of ions were used as qualifiers.*

Compound	Chemical structure	pKa	log K_{OW}	Characteristic ions (m/z)
Oxalic acid, C2		1.36; 4.11	-0.26	**147**, 73
Malonic acid, C3		2.43;5.92	-0.33	**147**, 73, 233
Succinic acid, C4		3.55 ;5.69	-0.40	**147**, 73, 247
Fumaric acid, C4		3.55; 4.41	-0.04	**245**, 73, 147
Maleic acid, C4		3.05; 5.91	-0.04	**147**, 73, 245
Glutaric acid, C5		3.76; 4.56	0.05	**73**, 147, 261
Adipic acid, C6		3.92; 4.70	0.49	**73**, 147, 275
Pimelic acid, C7		4.05; 4.81	0.94	**73**, 147, 289
Suberic acid, C8		4.15; 4.90	1.38	**73**, 147, 303
Phthalic acid, C8		2.94; 5.42	1.29	**73**, 147,295
Azelaic acid, C9		4.15; 4.98	1.82	**73**, 147, 303
Sebacic acid, C10		4.72; 5.32	2.27	**73**, 147, 331

4.2. Results and discussion

4.2.1. Optimization of the derivatization step

The influence of some conditions (nature and volume of the derivatization reagent, nature of the derivatization solvent and derivatization time and temperature) on the efficiency of the derivatization process was assessed.

Although BSTFA, with addition of TMCS as a catalyst, is the most frequently used reagent to derivatize carboxylic compounds, some authors suggest that MTBSTFA renders better results in terms of detection limits as well as detection capabilities (i.e., more reliable fragment ions of MTBSTFA-derivatives) [37]. Hence, the use of both alternatives was evaluated using a 5 $\mu g \cdot mL^{-1}$ WSOCs standard solution prepared in 150 µL EtOAc, which was mixed with 20 µL of the corresponding derivatization reagent at 60°C for 30 minutes. As proposed by Schummer et al. [37], the main characteristic fragmentation ions of the MTBSTFA-derivatives ($[M]^+$, $[M-57]^+$ and $[M-131]^+$) were observed, but dirty chromatograms with raised baseline were obtained in comparison to those obtained with BSTFA + 1%TMCS. This fact would turn into higher detection limits and thus, the use of BSTFA + 1%TMCS was considered in further experiments.

Regarding the solvent, pyridine is often used since it acts as catalyst increasing the reactivity of BSTFA [38], but other aprotic solvents such as AcN and EtOAc are also suitable. The influence of pyridine, AcN and EtOAc in the derivatization process was assessed using a 5 $\mu g \cdot mL^{-1}$ WSOCs standard solution prepared in 150 µL of the corresponding solvent where 20 µL of silylation reagent were added and the derivatization was run at 60°C for 30 minutes.

On the one hand, the use of pyridine produced peak tailing and dirty chromatograms, thus, its use was discarded. On the other hand, both AcN and EtOAc allowed the reaction forward efficiently obtaining adequate chromatographic baselines and peak shapes. Nevertheless, the derivatization yield can also depend on the reaction temperature and

time. The effect of both parameters was evaluated for each compound in different solvent media (EtOAc and AcN). Derivatization was performed using stock solutions of WSOCs (5 $\mu g \cdot mL^{-1}$) prepared in the corresponding solvents and 20 µL of BSTFA + 1%TMCS. The influence of the reaction time was tested at 60°C and 80°C using AcN and at 60°C and 70°C using EtOAc for 30, 60, 90 and 120 minutes. Higher temperatures were not examined for EtOAc due to the risk of solvent evaporation throughout derivatization reaction (EtOAc boiling point 77°C). These assays were performed in duplicate. Figure 4.2 shows the results (expressed as chromatographic peak areas) obtained for oxalic acid, fumaric acid, suberic acid and succinic acid as examples of the behavior of the other studied compounds.

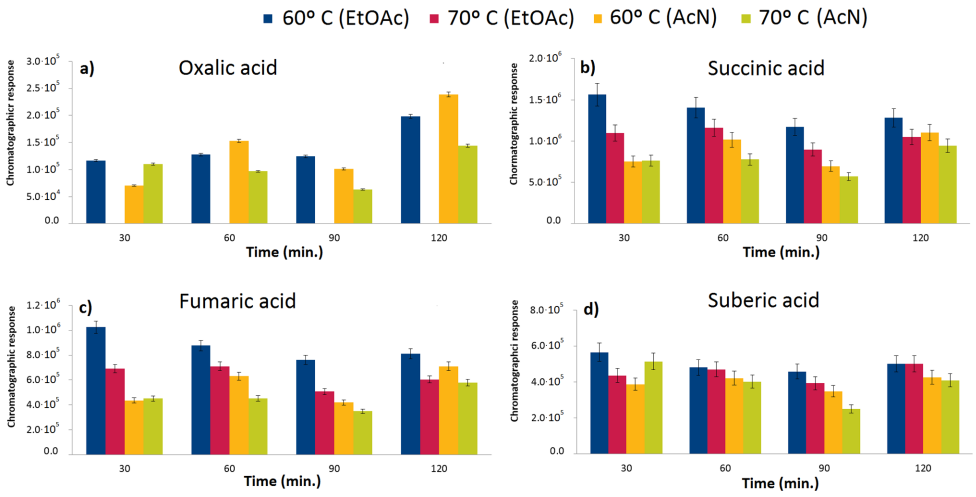


Figure 4.2: *Chromatographic signals of the silyl-derivatives of four target compounds at different derivatization conditions (derivatization time, solvent and temperature).*

Overall, it was found that derivatization time had only limited effects on the analytical responses, hence, the minimum time (i.e., 30 min) was established as optimum. Besides, the temperature showed a weak effect on the derivatization reaction. Whilst statistically comparable results (p-level > 0.05) were obtained at tested temperatures in EtOAc media,

the increase of temperature showed a negative effect on the derivatization yield using AcN as solvent. Thus, the derivatization temperature was fixed at 60°C.

The use of AcN or EtOAc showed an unequal effect on the reaction progress depending on the number of carbons of the WSOCs. The use of AcN rendered higher chromatographic responses for oxalic acid (i.e., the compounds with the shortest carbon chain), whereas EtOAc seemed to be more adequate for the rest of compounds. Due to these two trends, the effect of the solvent nature was further studied in detail by performing assays using different mixtures of EtOAc:AcN. Figure 4.3 shows the responses obtained for silyl-derivatives of oxalic acid, fumaric acid, suberic acid and succinic acid after their derivatization with 20 µL of BSTFA + 1%TMCS and the corresponding derivatization solvent at 60°C for 30 min. Similar results to those mentioned above were obtained. The results prove that the presence of AcN favors the derivatization of the most polar WSOCs, especially for oxalic acid. Thus, the use of a 75:25 EtOAc:AcN (*v/v*) mixture was considered as the best option from then on.

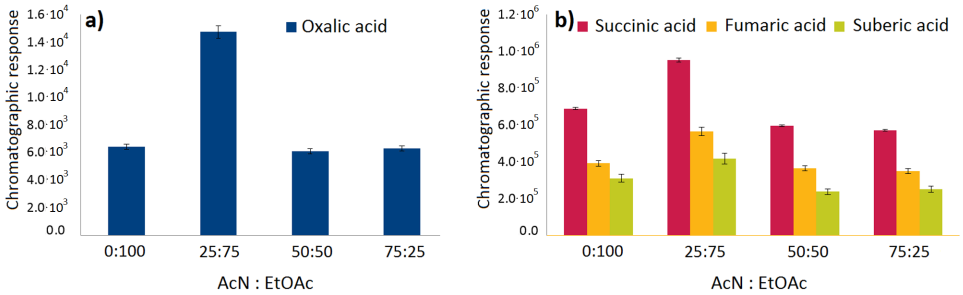


Figure 4.3: *Chromatographic signals of the silyl-derivatives of four target compounds obtained with 20 µL BSTFA + 1%TMCS in 150 µL of 0:100, 25:75, 50:50 and 100:0 AcN:EtOAc (v/v) mixtures: a) oxalic acid, b) succinic, fumaric acid and suberic acids.*

Finally, the volume of derivatization reagent was optimized. Three different volumes of BSTFA + 1 %TMCS were evaluated (i.e., 20 µL, 40 µL and 60 µL) using a stock solution of WSOCs in 75:25 EtOAc:AcN (*v/v*) and using the derivatization conditions fixed previously. In general, the use of large quantities of silylation reagent did not render higher yields, but favored the presence of additional derivatives or artifacts that lead to more complex and dirtier chromatograms (data not shown). Hence, to minimize these drawbacks, the minimum amount of sylilation reagent was fixed as optimum value (20 µL of BSTFA + 1%TMCS). Under optimum conditions, the extracts were reconstituted in 150 µL of 75:25 EtOAc:AcN (*v/v*) and 20 µL of BSTFA + 1%TMCS and derivatized at 60°C for 30 min.

4.2.2. Preconcentration of WSOCs from water samples

4.2.2.1. Selection of sorbent material

In the first step, the use of several combinations of RP-SPE (i.e., 200-mg ENVI-Carb, 200-mg Plexa and their mixture ENVI-Carb:Plexa (1:1) 100-mg each) and mixed-mode (200-mg Oasis-MAX) sorbents were assayed. These experiments were carried out by triplicate using 100 mL of Milli-Q water aliquots fortified at 8 ng·mL^{-1} of WSOCs.

In the case of reversed-phase cartridges, the initial assays were performed according to methods published in the literature with some modifications [30,32]. In these experiments, the water aliquots were adjusted to pH 1 with HCl 1 mol·L^{-1} (to ensure that all the compounds are non-charged) and submitted to the SPE conditions described in experimental section (see section 4.1.4.2.). Regarding the use of Oasis MAX cartridges, the preliminary assays performed with them were based on a method found in the literature for other analytes with similar properties [39] (see section 4.1.4.2.). In this case, the adjustment of the pH was not required before sample loading.

The results of these assays are expressed as chromatographic signal normalized to the highest chromatographic response in Figure 4.4. Two different trends can be distinguished.

On the one hand, using pure reversed-phase sorbent Envicarb, the compounds with the largest carbon chain were mainly retained (i.e., C6 – C10). Similar results were also reported in the literature, where the difficulties found to retain small dicarboxylic acids in reversed-phases are pointed out [32]. Pure hydrophilic reversed-phase sorbent Plexa did not improve the abovementioned results, except for phthalic acid, which is more non-polar in comparison to the other screened small molecules. These results were also in agreement with those obtained by Jurado-Sánchez et al. [32]. Similar conclusions were derived from the experiments conducted with the combination of both phases (i.e., Envicarb:Plexa (1:1)).

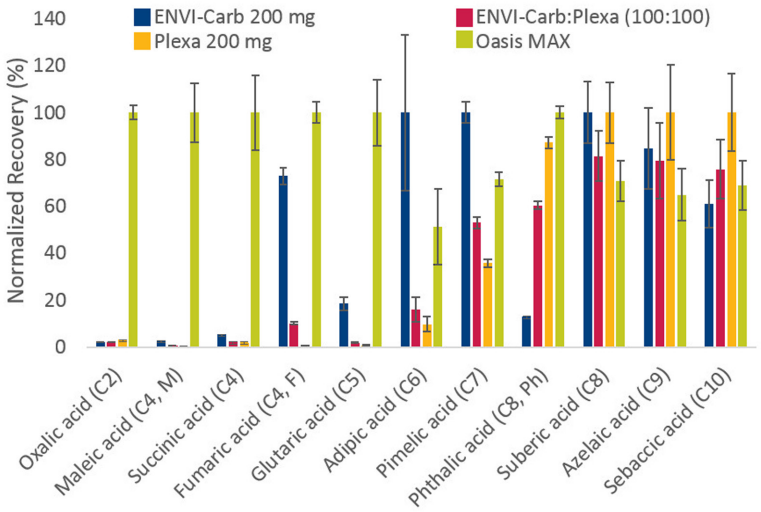


Figure 4.4: *Recoveries (%, normalized to the highest ones) obtained for Milli-Q water samples (n=3) with ENVI-Carb (200-mg), Plexa (200-mg , a mixture of them (ENVI-Carb:Plexa) (100-mg:100-mg) and mixed-mode (Oasis MAX) cartridges.*

According to literature, the use of a higher amount of sorptive material could improve the retention of these compounds [30], thus, some extra analyses were performed using 400-mg of Envicarb instead of 200-mg. However, doubling the sorbent bed mass did not increase the retention capacity of the smallest WSOCs, whereas the results obtained for the

highest ones (i.e., C6–C10) were statistically comparable (p-value > 0.05) to those obtained using 200-mg.

On the other hand, the mixed-mode Oasis MAX cartridges allowed a better retention of acidic compounds. As can be seen in Figure 4.4, whereas the highest WSOCs were retained by reversed-phase interaction, the smallest ones (i.e., C2–C5) were mostly retained by an ion-exchange mechanism, as they were always eluted in the acidic acetonitrile fraction. Thus, the use of mixed-mode cartridges was required for the quantitative retention of all the target compounds in the same run.

4.2.2.2. Elution profile

The use of an organic solvent in acidic media is often required to elute quantitatively short chain dicarboxylic acids from mixed-mode SPE cartridges. According to the literature, both MeOH and AcN can be used, but owing to the instability of the target compounds in MeOH (especially in acidic media) [40], elution profiles were studied using pure AcN (0.8% HCl). For this purpose, aliquots of 100 mL of Milli-Q water fortified at 8 $ng \cdot mL^{-1}$ were loaded onto Oasis WAX cartridges. Three consecutive 2 mL fractions of the solvent were collected in separate vials to get the elution profile corresponding to a total volume of 6 mL. All the fractions were evaporated to dryness, reconstituted in 150 µL of 75:25 EtOAc:AcN (*v/v*) and derivatized with 20 µL of BSTFA + 1%TMCS for their subsequent individual analysis by means of GC-MS. As can be seen in Figure 4.5, analytes were almost not recovered with 2 mL, and the use of 6 mL of elution solvent was required to recover quantitatively all the analytes.

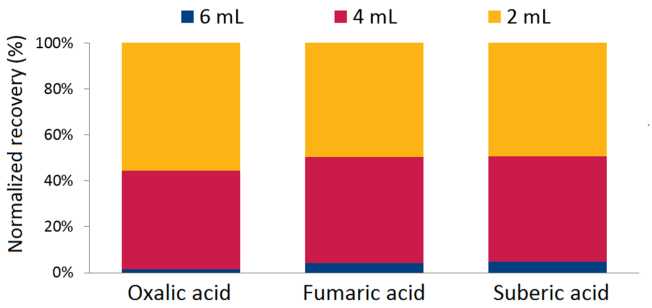


***Figure 4.5**: Percentage of representative WSOCs recovered in the consecutive 2 mL aliquots of AcN (0.8% HCl) eluted from Oasis-MAX cartridges.*

4.2.3. SPE-GC-MS method validation

The main figures of merit of the whole analytical method are summarized in Table 4.2. Experimental calibration curves were built with standard solutions derivatized in 150 µL 75:25 EtOAc:AcN (*v/v*) and 20 µL BSTFA + 1%TMCS and were analyzed by means of GC-MS at ng·mL^{-1} concentration levels in the range of 200 to 7500 ng·mL^{-1} for all the compounds. Adequate linearity was observed in that range for all the dicarboxylic acids under study obtaining determination coefficients (r^2) between 0.9988 and 0.9999. Instrumental limits of detection (LODs), estimated and defined as the average response (n=3) of the lowest concentration level plus three times the standard deviation, were below 20 ng·mL^{-1} levels for all the target analytes.

Table 4.2: *Main figures of merit of the SPE followed by derivatization and GC-MS analysis for Milli-Q water and aerosol water samples spiked at 5000 ng·L^{-1} level and for mortar samples spiked at 0.85 µg·g^{-1} level.*

Analyte	Milli-Q water (5000 ng·L^{-1})				Aerosol water (5000 ng·L^{-1})			Mortar samples					
									Low concentration level (0.34 µg·g^{-1})		High concentration level (1.7 µg·g^{-1})		
	%RSD (n=3)	Extraction efficiency (%)	Apparent Recovery (%)	LOD $_{proc}$ (ng·mL^{-1})	%RSD (n=3)	Extraction efficiency (%)	Apparent Recovery (%)	%RSD (n=3)	Extraction efficiency	Apparent recovery	Extraction efficiency	Apparent recovery	LOD $_{proc}$ (ng·g^{-1})
Oxalic acid (C2)	3	103	163	1503	20	94	148	12	45	62	18	27	220
Malonic acid (C3)	15	45	69	165	25	30	47	23	5	7	5	8	33
Maleic acid (C4, M)	3	69	109	40	11	71	111	6	4	45	37	59	58
Succinic acid (C4)	1	80	126	130	9	83	130	7	64	88	52	86	60
Fumaric acid (C4, F)	4	72	113	26	13	69	109	11	30	42	38	54	47
Glutaric acid (C5)	3	70	110	86	10	76	119	1	52	73	58	90	54
Adipic acid (C6)	4	68	107	110	11	62	97	7	64	90	62	89	57
Pimelic acid (C7)	7	67	105	75	4	57	90	7	69	96	65	92	55
Phthalic acid (C8,Ph)	4	73	112	125	11	60	94	23	47	65	34	44	104
Suberic acid (C8)	7	70	109	191	4	68	107	18	74	103	66	95	97
Azelaic acid (C9)	5	71	115	304	4	74	116	18	87	121	66	98	116
Sebacic acid (C10)	5	75	122	39	6	71	111	7	61	85	62	93	142

LOD: limit of detection; %RSD: relative standard deviation

4.2.3.1. Milli-Q and aerosol water samples

The trueness of the method for aqueous matrices was determined in terms of recovery using 100 mL of Milli-Q water and aerosol water samples (collected in the less exposed side of Punta Begoña Gallery, that is, the northwest), both fortified with WSOCs and the deuterated surrogate (dodecanoic acid d-23) at 5 ng·mL^{-1}. In both cases, non-spiked samples were also measured and their concentration was considered in recovery calculations.

Recoveries were in the range of 45 and 103% for spiked Milli-Q samples and slightly lower for spiked aerosol water samples (30 and 94%). Apparent recoveries, calculated with the deuterated surrogate, ranged between 105% and 126% in the case of Milli-Q water samples and between 90% and 130% in the case of aerosol water samples for the target compounds except for oxalic and malonic acids. These results can be considered as satisfactory as those reported in the literature [32].

The method repeatability, calculated in terms of relative standard deviation (%RSD), was obtained from the same abovementioned assays performed within a day. The %RSD values were lower than 7% for all the target analytes, except for malonic acid, in Milli-Q and aerosol water samples, which was about 20%.

It is worth mentioning that matrix effects in the proposed methodology are mainly attributed to the presence of inorganic salts in the aqueous samples that compete with dicarboxylic acids in the anion exchange process. In the marine aerosol sample used for the validation 3.3 ± 0.2 µg·mL^{-1} of chloride, 0.17 ± 0.01 µg·mL^{-1} of nitrate and 0.85 ± 0.04 µg·mL^{-1} of sulfate were determined by means of ion chromatography. Since statistically comparable results were obtained for Milli-Q water samples and marine aerosol samples (p-level > 0.05), the absence of matrix effect was assured for this type of samples. Nevertheless, the deuterated surrogate corrected the extraction efficiencies of those

analytes with values lower than 80%. Thus, its use was not discarded for the subsequent analysis of real marine aerosol samples.

One of the main problems reported in the literature related to the analysis of short chain dicarboxylic acids at trace level is the background contamination in the analytical blanks. All the target compounds were detected in procedural blanks performed with purified Milli-Q water samples, which raised the procedural limits of detection (LOD_{proc}) to the range of 26 $ng \cdot mL^{-1}$ – 1503 $ng \cdot mL^{-1}$ in the case of aqueous matrices. In fact, the concentrations found in non-spiked marine aerosol samples used for validation purposes were lower (between 102 $ng \cdot L^{-1}$ to 344 $ng \cdot L^{-1}$) than those detected in Milli-Q water samples. Oxalic acid deserves more attention since this compound was detected at high concentration values in non-spiked Milli-Q water samples, which impeded the method validation for this compound at the low $ng \cdot mL^{-1}$ levels. However, the LOD values obtained for short chain dicarboxylic acids are still adequate to determine these compounds in environmental water samples [30].

4.2.3.2. Mortar samples

In the case of solid matrices, the trueness of the method was stablished similarly using a non-exposed mortar sample (collected from the inner walls of the galleries) containing 25.6 ± 0.3 $\mu g \cdot mL^{-1}$ of chloride, 7.68 ± 0.02 $\mu g \cdot mL^{-1}$ of nitrate and 2.34 ± 0.03 $\mu g \cdot mL^{-1}$ of sulfate, according to the ion chromatography measurements. 0.5 g of non-exposed mortar was fortified at two levels of concentration by adding 0.17 µg and 0.85 µg of WSOCs and was extracted by means of FUSLE after the addition of dodecanoic acid d-23 as surrogate at the same concentration levels. In parallel, the same non-exposed and non-fortified mortar sample was also analyzed in order to subtract the concentration of WSOCs present in such real sample (n=3).

Extraction efficiencies were generally low for all the analytes, but especially for oxalic, malonic, maleic, fumaric and phthalic acids in which values below 50% were obtained. The low extraction efficiency found for these compounds can be attributed to their higher interaction with the matrix via conjugated systems (π bonds). Besides this fact, the lower extraction efficiencies obtained for oxalic and malonic acids can be also related to their lower stability when heating (during the evaporation step) or their higher reactivity when treated with acids (0.8% of HCl during the evaporation step, after elution), especially for oxalic acid [41-43].

After the correction of the extraction efficiencies with the deuterated surrogate (dodecanoic acid d-23) adequate apparent recoveries were obtained: between 73 and 121%, and between 86% and 98% for low and high concentration levels, respectively, except for the previously mentioned analytes (i.e., oxalic, malonic, maleic, fumaric and phthalic acids) that cannot be corrected with dodecanoic acid d-23 and their recovery should be considered to provide accurate results.

The method repeatability for solid samples in terms of % RSD, was below 23% for all the analyzed WSOCs in mortar samples except for oxalic and malonic acids (see Table 4.2). The higher values (%RSD > 30) obtained for these two compounds can be attributed to instability and reactivity problems as well as to the background contamination issues previously explained (see section 4.2.3.1).

In order to calculate the procedural limits of detection, procedural blanks (i.e., the whole optimized extraction procedure performed without mortar sample) were analyzed in triplicate. The LOD_{proc} were estimated as the average response for each analyte plus three times the standard deviation. Each WSOC was found in procedural blanks raising the values of the LOD_{proc} to the range 33-116 $ng \cdot g^{-1}$ (values estimated considering 0.5 g of mortar sample), except for oxalic acid which had a higher limit of detection (220 $ng \cdot g^{-1}$) owing to its ambient ubiquity.

4.2.4. Application to real samples: the case of Punta Begoña Galleries

The developed SPE-GC-MS method was applied to real aerosol water samples and mortars collected both in two different exposed sides (northwest and southwest) of the building of Punta Begoña. Table 4.3 summarizes the concentration of dicarboxylic acids detected in aerosol water samples and mortars analyzed in triplicate.

4.2.4.1. Marine aerosol samples

All the analytes were detected at ng·mL^{-1} level in the samples collected in the southwest side. Regarding the samples collected in the northwest side only succinic, fumaric, adipic, pimelic, phthalic and sebacic acids were detected, being the rest of the target compounds under LODs.

Oxalic acid (C2) was the second most abundant compound found in the exposed zone (southwest oriented). In the literature, oxalic acid is described as the most commonly detected dicarboxylic acid in aerosol water samples [14,30,44], being the last transformation product in the degradation pathway of a variety of gas and particle-phase constituents. Nevertheless, in this case, the dicarboxylic acids were all found at similar concentrations in the range 100-700 ng·mL^{-1}, being adipic acid (C6) the compound found at higher concentration. However, more monitoring campaigns should be performed to infer relevant information about the possible trends among the different compounds.

Overall, lower concentrations were found for all the analytes in the aerosol water that impacts the northwest side of the facade of Punta Begoña in comparison to the southwest side. Considering the orientation of the facade to the sea and harbor (see section 4.1.3.), it might be possible that the source of these compounds could be the photochemical degradation of organic contaminants of the atmosphere due to the harbor activities. Although this is the first time that these compounds have been monitored nearby the facade of Punta Begoña Galleries, other organic contaminants such as polycyclic aromatic

hydrocarbons and polychlorinated byphenyls were detected in previous works performed on the walls of the same building [45].

Table 4.3: *Concentrations (n=3, 95% confidence level, 2s) measured in aerosol water and mortar samples of two different sides of Punta Begoña (southwest and northwest) Galleries.*

Analyte	Punta Begoña Galleries			
	Southwest galleries		Northwest galleries	
	Aerosol water ($ng \cdot mL^{-1}$)	Mortar ($ng \cdot g^{-1}$)	Aerosol water ($ng \cdot mL^{-1}$)	Mortar ($ng \cdot g^{-1}$)
Oxalic acid (C2)	723 ± 289	4120 ± 590	< LOD	< LOD
Malonic acid (C3)	< LOD	92 ± 4	< LOD	61 ± 2
Maleic acid (C4, M)	239 ± 52	173 ± 59	< LOD	127 ± 27
Succinic acid (C4)	450 ± 81	517 ± 49	139 ± 25	518 ± 132
Fumaric acid (C4, F)	457 ± 119	170 ± 21	306 ± 80	121 ± 15
Glutaric acid (C5)	132 ± 26	158 ± 27	< LOD	117 ± 7
Adipic acid (C6)	920 ± 202	420 ± 15	344 ± 76	319 ± 98
Pimelic acid (C7)	631 ± 50	163 ± 32	114 ± 9	127 ± 14
Phthalic acid (C8,Ph)	287 ± 63	358 ± 79	177 ± 39	265 ± 78
Suberic acid (C8)	298 ± 24	258 ± 22	< LOD	202 ± 18
Azelaic acid (C9)	652 ± 52	331 ± 57	< LOD	554 ± 46
Sebacic acid (C10)	293 ± 35	578 ± 30	102 ± 12	597 ± 75

LOD: limit of detection

4.2.4.2. Mortar samples

In the case of mortar samples, higher differences were observed when comparing samples taken from the northwest oriented galleries with the southwest oriented ones, which are supposed to be more exposed to the industrial harbor. The most interesting case involves oxalic acid, which was not identified in mortars from the gallery oriented to the northwest, whereas noticeable high values were quantified in the southwest oriented gallery (4121 ± 590 $ng \cdot g^{-1}$). A similar situation was found for maleic acid, which is a more reactive species among the organic acids and may be seen as an intermediate by-product of different substances, including aromatic hydrocarbons [46]. Maleic acid was not found among northwest oriented samples, whereas concentrations over the LOD were detected in samples oriented to the southwest.

For the rest of the WSOCs, similar concentrations were found both in the northwest and the southwest oriented mortar samples, being generally slightly higher for those from the southwest gallery. Exceptionally, azelaic and sebacic acids were found at significantly higher levels in mortars from the northwest gallery. However, their presence can be attributed to many different sources other than harbor contamination, including fatty acids and their by-products from vegetation [47-49].

4.3. Conclusions

To assess the risks arising from the presence of short chain dicarboxylic acids in any building affected by marine environment, new sensitive and robust analytical methodologies are required for routine analysis.

In the present work a cost effective and environmental friendly analytical method for the determination of 12 short chain dicarboxylic acids in aerosol water and mortar samples was developed. Mixed-mode SPE-GC-MS was successfully applied for the determination of

these compounds after optimizing several variables affecting both the extraction and analysis steps. Moreover, the presented method is interesting for routine analysis in monitoring programs in the low $ng \cdot mL^{-1}$ and $ng \cdot g^{-1}$ ranges.

The developed method was applied to aerosol water and mortar samples and, according to the results, harbor activities might have a key role in the concentrations of dicarboxylic acids of aerosol water since the side exposed to the industrial harbor is more contaminated. This theory was confirmed with the analysis of mortar samples, in which significant high values were obtained for oxalic acid, which is supposed to dominate the composition of the aerosol particles [46]. Those high values were only determined in mortars from the galleries oriented to the southwest, that is, the facade exposed to the industrial harbor, whereas no oxalic was found in mortars from the northwest galleries.

The developed method extracts low chain organic acids from the mortars. If we consider that these mortars are partially composed by calcium carbonate, a neutralization reaction is expected between oxalic acid and calcium carbonate to form the highly insoluble salt calcium oxalate, either in the monohydrate (whewellite) or dehydrate (weddellite) form. This is a reaction that takes time to be completed but not more than a couple of days. As we have extracted an important amount of oxalic acid from samples, we must conclude that this low chain organic compound arrives daily in important quantities to the building.

In view of these results, it can be considered that one of the main sources of WSOCs in the facade of the historical building under study is related to harbor activities. This problem makes necessary a long-term monitoring of the zone to study the long-term impact of those activities on the galleries of Punta Begoña.

To conclude, the developed method is suitable for the determination of WSOCs in aerosol and mortar samples in any historical building located near industrial complexes in order to diagnose their influence on the facades of built heritage.

4.4. References

[1] M. Maguregui, I. Martínez-Arkarazo, K. Castro, J.M. Madariaga, The impact of environmental pollution on immovable cultural heritage: Decay mechanisms, products and analytical techniques to evaluate the impact, in: Environmental Pollution and its Relation to Climate Change, 2012.

[2] A.E. Charola, J. Pühringer, M. Steiger, Gypsum: A review of its role in the deterioration of building materials, Environ. Geol. 52 (2007) 207-220.

[3] A. Deneckere, W. Schudel, M. Van Bos, H. Wouters, A. Bergmans, P. Vandenabeele, L. Moens, In situ investigations of vault paintings in the Antwerp cathedral, Spectrochim. Acta Part A Mol. Biomol. Spectrosc. 75 (2010) 511-519.

[4] I. Martínez-Arkarazo, M. Angulo, L. Bartolomé, N. Etxebarria, M.A. Olazabal, J.M. Madariaga, An integrated analytical approach to diagnose the conservation state of building materials of a palace house in the metropolitan Bilbao (Basque Country, North of Spain), Anal. Chim. Acta. 584 (2007) 350-359.

[5] E.A.L. Costa, V.P. Campos, L.C.P. da Silva Filho, H.A. Greven, Evaluation of the aggressive potential of marine chloride and sulfate salts on mortars applied as renders in the Metropolitan Region of Salvador - Bahia, Brazil, J. Environ. Manage. 90 (2009) 1060-1068.

[6] B. Gantt, N. Meskhidze, The physical and chemical characteristics of marine primary organic aerosol: A review, Atmos. Chem. Phys. 13 (2013) 3979-3996.

[7] H. Morillas, M. Maguregui, C. Paris, L. Bellot-Gurlet, P. Colomban, J.M. Madariaga, The role of marine aerosol in the formation of (double) sulfate/nitrate salts in plasters, Microchem. J. 123 (2015) 148-157.

[8] H. Morillas, J. García-Galan, M. Maguregui, I. Marcaida, C. García-Florentino, J.A. Carrero, J.M. Madariaga, Assessment of marine and urban-industrial environments influence on built heritage sandstone using X-ray fluorescence spectroscopy and complementary techniques, Spectrochimica Acta Part B: Atom. Spectrosc. 123 (2016) 76-88.

[9] C. García-Florentino, M. Maguregui, H. Morillas, U. Balziskueta, A. Azcarate, G. Arana, J.M. Madariaga, Portable and Raman imaging usefulness to detect decaying on mortars from Punta Begoña Galleries (Getxo, North of Spain), J. Raman Spectrosc. 47 (2016) 1458-1466.

[10] A.C. Antunes, J. Coroado, D. Boaventura, F. Rocha, The environmental factors impact on the conservation of an historic marine quay—a baseline study, Environ. Sci. Pollut. Res. 22 (2015) 9563-9569.

[11] O. Gómez-Laserna, N. Prieto-Taboada, H. Morillas, I. Arrizabalaga, M.A. Olazabal, G. Arana, J.M. Madariaga, Analytical study to evaluate the origin and severity of damage caused by salt weathering in a historical Palace House: the attack of infiltration water, Anal. Methods. 7 (2015) 4608-4615.

[12] H. Morillas, M. Maguregui, O. Gómez-Laserna, J. Trebolazabala, J.M. Madariaga, Could marine aerosol contribute to deteriorate building materials from interior areas of lighthouses? An answer from the analytical chemistry point of view, J. Raman Spectrosc. 44 (2013) 1700-1710.

[13] H. Morillas, M. Maguregui, C. García-Florentino, I. Marcaida, J.M. Madariaga, Study of particulate matter from Primary/Secondary Marine Aerosol and anthropogenic sources collected by a self-made passive sampler for the evaluation of the dry deposition impact on built heritage, Sci. Total Environ. 550 (2016) 285-296.

[14] K. Kawamura, S. Bikkina, A review of dicarboxylic acids and related compounds in atmospheric aerosols: Molecular distributions, sources and transformation, Atmos. Res. 170 (2016) 140-160.

[15] K. Kawamura, O. Yasui, Diurnal changes in the distribution of dicarboxylic acids, ketocarboxylic acids and dicarbonyls in the urban Tokyo atmosphere, Atmos. Environ. 39 (2005) 1945-1960.

[16] H. Wang, K. Kawamura, K. Yamazaki, Water-soluble dicarboxylic acids, ketoacids and dicarbonyls in the atmospheric aerosols over the Southern Ocean and Western Pacific Ocean, J. Atmos. Chem. 53 (2006) 43-61.

[17] C. Sabbioni, N. Ghedini, A. Bonazza, Organic anions in damage layers on monuments and buildings, Atmos. Environ. 37 (2003) 1261-1269.

[18] M.Z.H. Rozaini, The atmospheric deposition of dicarboxylic acids aerosols to the formation of the damage layers, J. Sustainability Sci. Manage. 7 (2012) 137-144.

[19] C.L. Heald, J.H. Kroll, J.L. Jiménez, K.S. Docherty, P.F. Decarlo, A.C. Aiken, Q. Chen, S.T. Martin, D.K. Farmer, P. Artaxo, A simplified description of the evolution of organic aerosol composition in the atmosphere, Geophys. Res. Lett. 37 (2010).

[20] M. Hyder, J. Genberg, M. Sandahl, E. Swietlicki, J.T. Jönsson, Yearly trend of dicarboxylic acids in organic aerosols from south of Sweden and source attribution, Atmos. Environ. 57 (2012) 197-204.

[21] A. Limbeck, H. Puxbaum, Organic acids in continental background aerosols, Atmos. Environ. 33 (1999) 1847-1852.

[22] S. Koch, R. Winterhalter, E. Uherek, A. Kolloff, P. Neeb, G.K. Moortgat, Formation of new particles in the gas-phase ozonolysis of monoterpenes, Atmos. Environ. 34 (2000) 4031-4042.

[23] E.G. Stephanou, N. Stratigakis, Oxocarboxylic and α,ω-Dicarboxylic Acids: Photooxidation Products of Biogenic Unsaturated Fatty Acids Present in Urban Aerosols, Environ. Sci. Technol. 27 (1993) 1403-1407.

[24] H. Morillas, J. García-Galan, M. Maguregui, I. Marcaida, C. García-Florentino, J.A. Carrero, J.M. Madariaga, Assessment of marine and urban-industrial environments influence on built heritage sandstone using X-ray fluorescence spectroscopy and complementary techniques, Spectrochim. Acta Part B At. Spectrosc. 123 (2016) 76-88.

[25] D. Kloos, H. Lingeman, O.A. Mayboroda, A.M. Deelder, W.M.A. Niessen, M. Giera, Analysis of biologically-active, endogenous carboxylic acids based on chromatography-mass spectrometry, Trends Anal. Chem. 61 (2014) 17-28.

[26] M. Piñeiro-Iglesias, M. Miñones-Vázquez, E. Vázquez-Blanco, S. Muniategui-Lorenzo, P. López-Mahía, D. Prada-Rodríguez, Concentration and clean-up of trace hydrocarbons from atmospheric particulate matter, Chromatographia. 56 (2002) 483-488.

[27] P. Hegde, K. Kawamura, I.A. Girach, P.R. Nair, Characterisation of water-soluble organic aerosols at a site on the southwest coast of India, J. Atmos. Chem. 73 (2016) 181-205.

[28] M. Rezaee, Y. Assadi, M.R. Milani Hosseini, E. Aghaee, F. Ahmadi, S. Berijani, Determination of organic compounds in water using dispersive liquid-liquid microextraction, J. Chromatogr. A. 1116 (2006) 1-9.

[29] J.G. Huddleston, H.D. Willauer, R.P. Swatloski, A.E. Visser, R.D. Rogers, Room temperature ionic liquids as novel media for 'clean' liquid-liquid extraction, Chem. Commun. 16 (1998) 1765-1766.

[30] M. Tedetti, K. Kawamura, B. Charrièra, N. Chevalier, R. Sempéré, Determination of low molecular weight dicarboxylic and ketocarboxylic acids in seawater samples, Anal. Chem. 78 (2006) 6012-6018.

[31] A. Limbeck, H. Puxbaum, L. Otter, M.C. Scholes, Semivolatile behavior of dicarboxylic acids and other polar organic species at a rural background site (Nylsvley, RSA), Atmos. Environ. 35 (2001) 1853-1862.

[32] B. Jurado-Sánchez, E. Ballesteros, M. Gallego, Determination of carboxylic acids in water by gas chromatography–mass spectrometry after continuous extraction and derivatisation, Talanta. 93 (2012) 224-232.

[33] N. Fontanals, R.M. Marcé, F. Borrull, P.A.G. Cormack, Mixed-mode ion-exchange polymeric sorbents: dual-phase materials that improve selectivity and capacity, Trends Anal. Chem. 29 (2010) 765-779.

[34] N. Gilart, F. Borrull, N. Fontanals, R.M. Marcé, Selective materials for solid-phase extraction in environmental analysis, Trends Anal. Chem. 1 (2014) 8-18.

[35] L. Blanco-Zubiaguirre, M. Olivares, K. Castro, J.G. Iñañez, J.M. Madariaga, An alternative analytical method based on ultrasound micro bath hydrolysis and GC-MS analysis for the characterization of organic biomarkers in archaeological ceramics, Anal. Bioanal. Chem. 408 (2016) 8001-8012.

[36] L. Blanco-Zubiaguirre, N. Arrieta, A. Iturregui, I. Martinez-Arkarazo, M. Olivares, K. Castro, M.A. Olazabal, J.M. Madariaga, Focused ultrasound solid-liquid extraction for the determination of organic biomarkers in beachrocks, Ultrason. Sonochem. 27 (2015) 430-439.

[37] C. Schummer, O. Delhomme, B.M.R. Appenzeller, R. Wennig, M. Millet, Comparison of MTBSTFA and BSTFA in derivatization reactions of polar compounds prior to GC/MS analysis, Talanta. 77 (2009) 1473-1482.

[38] M.C. Pietrogrande, D. Bacco, GC–MS analysis of water-soluble organics in atmospheric aerosol: Response surface methodology for optimizing silyl-derivatization for simultaneous analysis of carboxylic acids and sugars, Anal. Chim. Acta. 689 (2011) 257-264.

[39] N. Fontanals, R.M. Marcé, F. Borrull, New materials in sorptive extraction techniques for polar compounds, J. Chromatogr. A. 1152 (2007) 14-31.

[40] I.W. Ashworth, E. Bush, L.C. Chan, J. Cherryman, B.G. Cox, J. Muir, S.R. Korupoju, J. Keshwan, Where has my acid gone? Understanding the self-catalyzed esterification of maleic acid in methanol during salt formation, Org. Process Res. Dev. 16 (2012) 1646-1651.

[41] K.H. Weber, F.J. Morales, F. Tao, Theoretical study on the structure and stabilities of molecular clusters of oxalic acid with water, J. Phys. Chem. A. 116 (2012) 11601-11617.

[42] H. Strittmatter, S. Hildbrand, P. Pollak, Malonic acid and derivatives in: Ullmann's Encyclopedia of industrial chemistry., Wiley-VCH, 2007.

[43] OSHA Occupational Chemical Database. Occupational Safety and Health Administration, Oxalic acid. https://www.osha.gov/dts/chemicalsampling/data/CH_259000.html (Last accessed: December 2017)

[44] C. Cheng, G. Wang, B. Zhou, J. Meng, J. Li, J. Cao, S. Xiao, Comparison of dicarboxylic acids and related compounds in aerosol samples collected in Xi'an, China during haze and clean periods, Atmos. Environ. 81 (2013) 443-449.

[45] N. Prieto-Taboada, I. Ibarrondo, O. Gómez-Laserna, I. Martinez-Arkarazo, M.A. Olazabal, J.M. Madariaga, Buildings as repositories of hazardous pollutants of anthropogenic origin, J. Hazard. Mater. 248-249 (2013) 451-460.

[46] R. Koppmann, Volatile Organic Compounds in the Atmosphere, in Volatile Organic Compounds in the Atmosphere, Oxford, 2007.

[47] E. Ribechini, F. Modugno, C. Baraldi, P. Baraldi, M.P. Colombini, An integrated analytical approach for characterizing an organic residue from an archaeological glass bottle recovered in Pompeii (Naples, Italy), Talanta. 74 (2008) 555-561.

[48] M.P. Colombini, G. Giachi, F. Modugno, E. Ribechini, Characterisation of organic residues in pottery vessels of the Roman age from Antinoe (Egypt), Microchem. J. 79 (2005) 83-90.

[49] E. Fedina, A. Yarin, F. Mukhitova, A. Blufard, I. Chechetkin, Brassinosteroid-induced changes of lipid composition in leaves of Pisum sativum L. during senescence, Steroids. 117 (2017) 25-28.

Chapter 5

Determination of wine related biomarkers in archaeological potteries at trace level

Prepared to be sent

"In vino veritas"

Gaius Plinius Secundus

Analytical research in the field of organic archaeological residues found in ancient vessels has grown into a recognized field in its own right and has developed rapidly in recent decades. The characterization of such residues has led to the clarification of several aspects of daily life related to diet, trade, rituals, etc. [1-3]. As explained in Chapter 1, those residues can survive as actual contents preserved in situ as vessel fills (rarely observed), surface residues appearing as visible residues on the interior or exterior of vessels, or absorbed residues preserved within the vessel wall, invisible to the naked eye, being the latter the most common category [4].

For centuries, wine has been one of the most produced, consumed and traded liquid worldwide. Wine has been widely consumed, especially in different regions of the Mediterranean area [5-7], and besides, it has been used for medicinal purposes in China and Middle East [8] or as offering in funerary rituals in ancient Egypt [9]. In any case, *dolia* and *amphorae* were the ceramic containers that were used in past periods of time for storage and transport of foodstuff such as wine, oil and fish sauces [6]. The analysis of organic residues preserved in such materials using hyphenated analytical techniques allows uncovering hidden information about ancient activities and about the stored or transported foodstuff [10]. Lipids and their by-products are the most detected organic residues in archaeological amphorae since they remain adsorbed in the pores of the ceramics that contained fatty foodstuff (e.g., animal fats, vegetal fats, etc.) [11,12]. Some small organic acids are widely considered as wine-related organic markers (e.g., tartaric, malic and syringic acid) [6,7,13-26] and wine fermentation-related markers (e.g., fumaric and citric acid). In fact, the presence of tartaric acid, hardly found in nature, is a chemical evidence of the presence of wine in ancient ceramics [9,13,27,28]. However, all these compounds linked to grapes and wine are often labile and do not easily remain in the ceramic surface throughout the ages. Consequently, their detection at trace levels is still an analytical challenge [16,19,20].

Malvidin, an extremely stable and persistent anthocyanin that can be hydrolized to syringic acid [13], is another important wine biomarker that gives information about the color of ancient wine, since it is the responsible of the red color of grapes and red wines [13]. Concretely, the anthocyanin malvidin-3-glucoside gives the characteristic red-purple color to young red wines, which changes to red-brown tonality when they are aged due to the formation of polymeric pigments [9]. In addition to the already mentioned wine biomarkers, resins and resin-derived compounds can also be found in this type of archaeological ceramics [7]. The presence of these water-insoluble tree and plant exudate stable compounds could be related to their use as waterproofing agents or to give flavor to the stored liquid [5].

Many efforts have been done in the literature to develop new analytical methods to detect wine-related organic biomarkers. Folin-Denis and Folin-Ciocalteu methods to determine tannins and polyphenols, respectively, by UV-VIS in amphorae [29] and Fiegl spot test to identify tartaric acid in organic residues when irradiated with UV light [8,21], are some routine analytical methods to detect wine-related organic compounds. However, detection at trace levels is not feasible using these methods and they could also bring false positives as they do not allow a specific identification [16]. Owing to these limitations, hyphenated chromatographic techniques such as liquid chromatography coupled to a wide range of detectors (e.g., diode array detector (HPLC-DAD) [16], mass spectrometry (HPLC-MS) [7,30], tandem mass spectrometry (HPLC-MS/MS) [9,13,21]), and specially, gas chromatography coupled to mass spectrometry (GC-MS), have been widely used in research works dealing with organic acids and other wine-related compounds [6,7,15,16,19-21,24,30].

Regardless of the detection technique used, the effectiveness of the analysis relies on the extraction and preconcentration protocols used for the isolation of wine biomarkers. Moreover, the high polarity and solubility of some wine-related organic compounds (e.g.,

tartaric acid, malic acid, etc.) difficulties their isolation, being critical both the extraction technique and solvent used. Ultrasound bath is by far the most used technique for the extraction of wine-related compounds from ceramic materials [19] but, recently, other techniques such as focused ultrasound solid-liquid extraction (FUSLE) [31], ultrasound micro-bath (UMB) [32] and microwave assisted extraction (MAE) [33] have also shown their efficiency to determine organic compounds at trace levels in archaeological remains. Owing to the wide range of polarities of wine-related organic compounds, mixtures of solvents of different polarities (water, methanol or dichloromethane) have been used in the literature [20,21]. Some authors suggest the use of pure alkaline solutions (KOH) to favor the cleavage of compound-matrix bonds as well as to determine malvidin [19] (see Figure 5.1). Malvidin is a specific marker of dark grape that can be hydrolyzed to syringic acid, which is a wine-related marker but not as specific as malvidin because syringic acid is also present in barley, wheat and even in soils due to microbiological activity [13]. Alternatively to the use of KOH to differentiate free syringic acid from that released by malvidin, recently, a two-step protocol based on a first extraction with traditional solvents (i.e., dichloromethane, methanol) and a second extraction using boron trifluoride has been proposed in the literature [16].

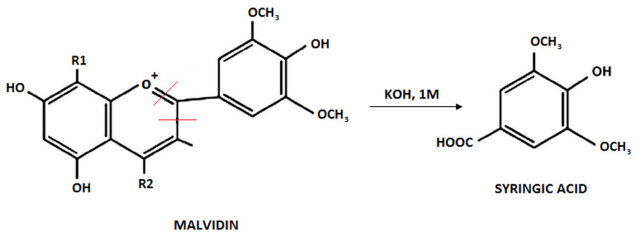


Figure 5.1: *Alkaline treatment of malvidin (specific marker of red wine) with KOH 1 M and release of syringic acid (non specific wine related compound).*

No matter the extraction solvent and technique used, a preconcentration step is almost always required in order to get lower limits of detection. Liquid-liquid extraction

(LLE) is the most classical technique used in the literature [11,34], but the use of more specific extraction techniques such as ion-pair LLE, solid-phase extraction (SPE) [35,36] and mixed-mode SPE [37] can render higher extraction efficiencies for compounds with a wide range of polarities.

The efficiency of all these methods is widely accepted for the identification of wine-related compounds in archaeological residues. However, the improvement of those methods in terms of sensitivity and extraction efficiency is still required in order to be able to detect those compounds, especially in archaeological remains with no visible organic residues. In this context, the present work describes a thorough optimization and validation of an analytical method to determine wine-related organic compounds (including tartaric acid, malic acid, fumaric acid, succinic acid, citric acid and syringic acid) in archaeological ceramics at trace levels. The method was applied to real archaeological ceramic fragments together with organic residues found inside two amphorae from Zaragoza (Spain) suspected to have been used to store wine.

5.1. Experimental part

5.1.1. Archaeological samples

The ceramic models used during the optimization and validation of the methodology were prepared and supplied by the Department of Sculpture of the Fine Arts Faculty from the University of the Basque Country (UPV/EHU). The models were prepared according to a previous work [32].

In this work, fragments of two different amphorae (ca. II-I BCE) suspected to be related to wine production and collected in an archaeological site found in Tarazona (Zaragoza, Spain) in 2015 were analyzed using the developed method. These samples were named as DC4 and DC7. Another two samples of visible residues (M47 and M48) recovered from the

inner walls of two different non-restored and not cleaned amphorae, stored in the museum of Zaragoza and catalogued as possible wine containers, were also analyzed. The samples of Roman origin containing these residues were found in 2014 in the archaeological site located in Tenerías (Zaragoza, Spain).

5.1.2. Cleaning procedure

Since the target compounds are prone to be adsorbed in glass material, plastic or silanized glass material was used throughout the experimentation. All the laboratory material was washed with abundant Ellix quality water (<0.2 $\mu S \cdot cm^{-1}$, Millipore, Bedford, MA, USA) and then sonicated under clean acetone (Q.P., Panreac Química, Barcelona, Spain) for at least an hour. After that, the material was rinsed with abundant Milli-Q water (< 0.057 $\mu S \cdot cm^{-1}$, Milli-Q Model 185, Millipore, Bedford, MA, USA).

5.1.3. Reagents and materials

The six wine markers: succinic acid, fumaric acid, malic acid, tartaric acid, citric acid and syringic acid were obtained from Sigma-Aldrich (Steinheim, Germany). Individual stock solutions from each solid standard were dissolved to prepare approximately 1000 $\mu g \cdot g^{-1}$ in acetonitrile (AcN, HPLC-grade, 99.9%, Sigma Aldrich, Germany) with the exception of fumaric and syringic acids which were dissolved in acetone (Ace, HPLC-grade, 99.8%, LabScan, Ireland). All stock solutions were stored in silanized amber glass vials at -20 °C. A mixed fresh stock solution containing 20 $\mu g \cdot mL^{-1}$ of all target compounds was prepared monthly in acetonitrile, whereas intermediate dilutions were prepared daily according to the experimentation.

The solvents ethyl acetate (EtOAc), acetone, propan-2-ol (IPA), dichloromethane (DCM), methanol (MeOH) and acetonitrile (AcN) (all HPLC grade, 99.8%) were purchased from LabScan (Dublin, Ireland). Potassium hydroxide pellets (KOH) were supplied by Merck (Darmstadt, Germany). The derivatization reagent N,O-bis(trimethyl)silyltrifluoro-

acetamide containing 1% trimethylchlorosilane (BSTFA + 1% TMCS) and the tetrabutylammonium bromide (TBA) were acquired from Sigma-Aldrich (Milan, Italy). OASIS-MAX (poly(divinylbenzene-co-*N*-vinylpirrilidone) + quaternary amine polymer, 150-mg) and OASIS-HLB (*N*-vinylpyrrolidone and divinylbenzene, 200-mg) SPE cartridges were purchased from Waters (Milford, USA).

5.1.4. Sample preparation

Special care was taken in all analytical steps including sample crushing, fractionation and extraction to avoid any cross-contamination. In the case of ceramic samples DC4 and DC7, sub-samples were taken both from the inner wall surface and from the cross section of the ceramic fragments using a scalpel. For samples M47 and M48, the residues were crushed in the mortar in order to obtain a fine powder. All samples were weighed and stored in glass vessels for their preservation until their analysis and subsamples of 1 g and 0.5 g were taken from the glass vessels for ceramic samples (DC4 and DC7) and residues (M47 and M48), respectively. The analyses were performed in triplicate.

5.1.5. Extraction of wine biomarkers from solid matrix using focused ultrasound bath and ultrasound probe

According to the literature, KOH (1M, aq.) [19,24] or a mixture of a non-polar solvent (dichloromethane, chloroform, etc.) and MeOH using a ratio of 2:1 *v/v* [7,20,21] are the most frequently used extraction solvents for this type of compounds. However, due to the wide range of polarities of the target compounds, the efficiency of several extraction solvents was evaluated: H_2O, KOH (1M, aq.), 0.1% formic acid in H_2O:MeOH (4:1, *v/v*), DCM: MeOH (2:1, *v/v*), AcN:H_2O (1:1, *v/v*) and AcN:H_2O (2:1, *v/v*).

Briefly, 1 g of the synthetic ceramic was spiked with ~0.85 µg of the target compounds and subjected to extraction by means of ultrasound micro bath (UMB). The assays were

carried out using a Bandelin Sonopuls HD 3100 sonifier ultrasonic cell disruptor/homogenizer (100 W, 20 kHz; Bandelin Electronic, Berlin, Germany) with a cup booster BR made of stainless steel. The extraction process variables (i.e., duty cycle, sonication amplitude and extraction time) were fixed according to a previous work [31]. Wine related compounds were extracted from ceramics using 3 mL of the solvents under study, immersed in an ice-water bath for 4 min using a duty cycle of 0.5 $s \cdot s^{-1}$ and a sonication amplitude of 20%. Two consecutive extractions were performed using fresh extraction solvents to ensure a quantitative extraction of the target compounds. After the extraction procedure, the extracts were centrifuged, filtered through 0.45 µm PTFE disks and subjected to a LLE according to Pecci et al. [19]. Briefly, all the extracts (except those in KOH, which were acidified before LLE) were directly extracted with 2 mL of EtOAc and vortexed for 2 min, and the organic layer containing the target compounds was recovered. The process was carried out in triplicate. The extracts were combined, dried under a gentle stream of nitrogen (Turbovap® LV, Caliper, Life sciences, USA) and derivatized with 20 µL of BSTFA and 150 µL of EtOAc at 70 ^{o}C for 30 min before being analyzed by means of GC-MS.

The efficiency of the UMB and FUSLE was assessed according to the results obtained in the previous assays. To this aim, 1 g of the synthetic ceramic was spiked with ~0.85 µg of the target compounds and extracted with 7 mL of the optimum extraction solvent using FUSLE and extraction conditions fixed according to a previous work (4 min, duty cycle of 0.5 $s \cdot s^{-1}$ and 20% amplitude) [31].

5.1.6. Preconcentration step

5.1.6.1. Liquid-liquid extraction and ion-pair formation

Owing to the wide range of polarities of the target compounds (i.e., from tartaric acid (log P= -1.32) to syringic acid (log P=1.01)) the efficiency of LLE using four extraction solvents combining ethyl acetate (EtOAc) and propan-2-ol (IPA) was studied: EtOAc (100%), EtOAc:IPA (95:5, *v/v*), EtOAc:IPA (90:10, *v/v*) and EtOAc:IPA (80:20, *v/v*). For this purpose,

an aqueous solution containing ~0.85 µg of the target compounds was acidified with HCl (to pH=1) and extracted with 2 mL of the solvents under study by vortexing for 2 min (3 x 2 mL). The organic phases were combined, dried under a gentle stream of nitrogen, derivatized (20 µL of BSTFA, 150 µL of EtOAc, 30 min, 70 ^{o}C) and analyzed by GC-MS.

The use of ion-pair reagents can improve the extraction efficiency of the target compounds in the LLE step, and hence, the use of tetrabutylammonium bromide (TBA) as ion-pairing agent was studied. TBA was added at different concentration levels (50 mM, 75 mM and 100 mM) onto 3 mL of Milli-Q water (fixed at pH>6) containing ~0.85 µg of the target compounds. LLE was performed in triplicate using 2 mL of EtOAc and vortexing for 2 min. Phases were separated under centrifugation (5 min, 3500 rpm) and the supernatant was recovered. The organic phases were combined and acidified with HCl to pH 1 in order to recover the target compounds in their free acidic forms. The combined extracts were dried under a gentle stream of nitrogen, derivatized (20 µL of BSTFA, 150 µL of EtOAc, 30 min, 70 ^{o}C) and injected in the GC-MS.

5.1.6.2. Solid phase extraction (SPE)

Reversed-phase SPE (RP-SPE) and mixed-mode SPE were also tested to preconcentrate the target compounds. For this purpose, aliquots of 5 mL of Milli-Q water were fortified with ~0.85 µg of the target analytes and subjected to the SPE procedure in triplicate.

For RP-SPE, 5 mL of spiked Milli-Q water were loaded onto 200-mg OASIS HLB cartridges, which were previously conditioned with 5 mL of EtOAc and 5 mL of acidified Milli-Q water (pH=1). Afterwards, 5 mL of acidified Milli-Q water was added as washing step and the cartridge was completely dried for 2 h under vacuum. Finally, the analytes were recovered using 6 mL of EtOAc.

For mixed-mode SPE, aliquots of 5 mL of spiked Milli-Q water were loaded onto 150-mg Oasis MAX cartridges (mixed-mode strong anion exchanger). Before sample loading, the

cartridges were conditioned with 5 mL of AcN and 5 mL of Milli-Q water. The adjustment of pH of the sample was not required since all the target compounds were in the ionized form at pH∼7. After the sample loading, 5 mL of Milli-Q water and 5 mL of AcN were added in order to eliminate non-charged polar interferences and non-polar interferences, respectively. The cartridges were completely dried for 1 h under vacuum before recovering the analytes with 5 mL of 0.8% HCl in AcN.

The recovered extracts in both preconcentration strategies were concentrated to dryness under a gentle stream of nitrogen at 40 ^{o}C. Afterwards, they were reconstituted in 150 µL of EtOAc and derivatized with 20 µL of BSTFA (70 ^{o}C, 30 min) prior to the GC-MS.

5.1.7. Derivatization

Target compounds need to be derivatized prior to their analysis by means of GC-MS. The variables affecting the derivatization step such as derivatization solvent (100% EtOAc and 25:75 EtOAc:AcN), temperature (60 °C, 70 °C and 80 °C) and time (30, 60 and 90 min) were optimized, whereas derivatization reagent (BSTFA + 1%TMCS) and volume (20 µL) were fixed according to previous works [32]. Under optimum conditions, the extracts were evaporated to dryness in chromatographic amber vials and derivatized with 20 µL of BSTFA + 1% TMCS and 150 µL of EtOAc at 70 °C for 30 min.

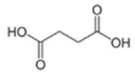
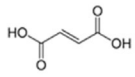
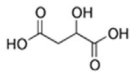
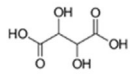
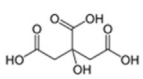
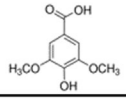
5.1.8. GC-MS analysis

Separation and detection of the derivatized extracts was performed in a 6890N gas chromatograph (Agilent Technologies, Avondale, PA, USA) equipped with an Agilent 5973N electron impact ionization mass spectrometer using an Agilent 7683 autosampler. 2 µL were injected in splitless mode at 250 °C in a capillary column HP-5MS (30 m x 0.25 mm, 0.25 µm, Agilent Technologies). Hydrogen (AD-1020 Hydrogen Generator, Cinel Strumenti Scientifi, Padova, Italy) was used as a carrier gas at a constant flow of 1.5 mL·min^{-1}. The

following oven temperature program was used for the separation of the target analytes: 60 °C (hold 4 min), temperature increase at 10 °C·min^{-1} to 150 °C and hold 3 min, a second temperature increase at 10 °C·min^{-1} to 220 °C to continue rising temperature at 25 °C·min^{-1} to 300 °C that was maintained for 5 min (total analysis run time lasts 31.20 min).

The mass spectrometer worked in the electron impact mode with a potential difference of 70 eV. The MS transfer line temperature was kept at 310 °C and the ion source and quadrupole temperatures were maintained at 230 °C and 150 °C, respectively. Detection was carried out both in the scan (50-450 *m/z*) and in the selected ion monitoring (SIM) modes. The *m/z* values of the fragment ions monitored in the SIM mode are listed in Table 5.1. The first ion was used as quantifier, whereas the second and third ions were considered as qualifiers.

Table 5.1: *Chemical structure, pKa, log K_{ow} and characteristic ions of wine biomarkers. The first ion (bold) was used as quantifier and the second (and third) ion was used as qualifier.*

Compound	Chemical structure	pKa	log K_{OW}	Characteristic ions (*m/z*)
Succinic acid		3.55	-0.40	**147**, 73, 247
Fumaric acid		3.35	-0.04	**245**, 73, 147
Malic acid		3.20	-1.11	**73**, 147, 233
Tartaric acid		2.72	-1.83	**73**, 147, 292
Citric acid		3.05	-1.32	**73**, 147, 273
Syringic acid		3.93	1.01	**327**, 312, 73

The quantification of soluble salts (only anions) was performed by a Dionex ICS 2500 suppressed ion chromatograph coupled to a conductivity detector ED50. An IonPac AS23 (4 x 250 mm) column and IonPac AG23 (4 x 50 mm) precolumn were used for the separation of anions. 4.5 mM Na_2CO_3 / 0.8 mM $NaHCO_3$, 25 mA and 1 $mL \cdot min^{-1}$ were used as mobile phase, suppression current and flow, respectively.

5.2. Results and discussion

5.2.1. Optimization of the derivatization step

Several variables, such as the nature and volume of derivatization reagent, the nature of derivatization solvent and derivatization time and temperature can affect the efficiency of the derivatization reaction. In the present work it was decided to use directly BSTFA due to its frequent use in literature related to wine markers [5,16,19-21,24,30].

EtOAc has been reported in literature as an adequate derivatization solvent [32]. Nevertheless, the addition of a small percentage of acetonitrile has shown to be effective in order to favor the derivatization process of polar compounds [31]. Thus, the use of 150 µL of 100% of EtOAc and a mixture of 75:25 *v/v* EtOAc:AcN was tested under fixed derivatization conditions (i.e., 20 µL of BSTFA + 1% TMCS, 30 min, 60 ^oC). The use of 100% EtOAc rendered better chromatographic responses for all the target compounds, hence, it was fixed for further experiments (see Figure 5.2a).

Regarding derivatization temperature, assays were performed at 60 ^oC, 70 ^oC and 80 ^oC for 30 min. As shown in Figure 5.2b, the lowest (60 ^oC) and the highest temperatures tested (80 ^oC) meant lower chromatographic signals and poorer repeatability in comparison to the ones obtained at 70 ^oC for all the target compounds. Consequently, 70 ^oC was fixed as optimum derivatization temperature for subsequent analysis.

Finally, in order to evaluate the influence of time on the derivatization reaction, the extracts were derivatized using 20 µL of BSTFA + 1% TMCS and 150 µL of EtOAc at 70 ^oC for

30, 60 and 90 min. As it can be observed in Figure 5.2c, no statistical differences were found for the studied time intervals, so that 30 minutes were chosen as optimum derivatization time in order to shorten the analytical procedure. To sum up, the silylation was performed using 20 µL of BSTFA + 1 %TMCS and 150 µL of EtOAc at 70 °C for 30 min.

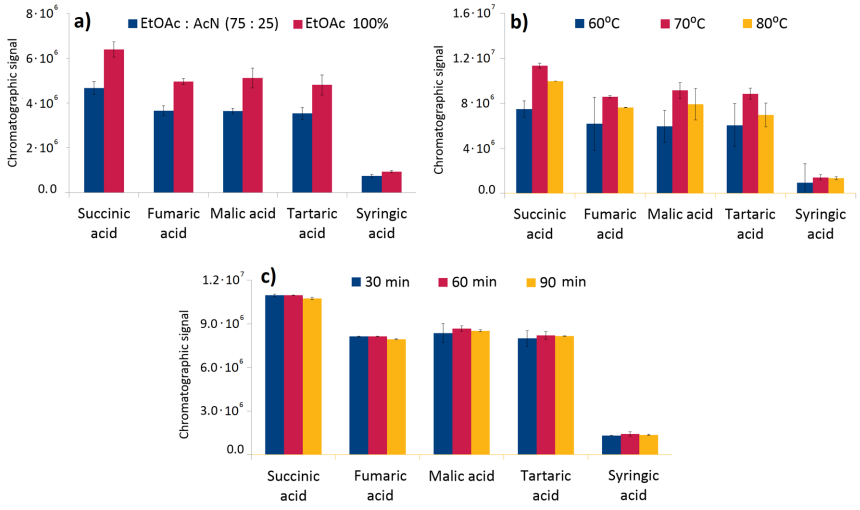


Figure 5.2: *Obtained chromatographic signals of the silyl-derivatives of the target compounds when optimizing: a) the derivatization solvent (20 µL of BSTFA + 1 % TMCS, 30 min, 60 ºC), b) the derivatization temperature (20 µL of BSTFA + 1 % TMCS, 100% EtOAc, 30 min) and c) the derivatization time (20 µL of BSTFA + 1 % TMCS, 100 % EtOAc, 70 ºC).*

5.2.2. Optimization of the extraction of wine biomarkers from solid matrix

5.2.2.1. Selection of extraction solvent

Several extraction solvents were evaluated to extract wine related compounds based on the physicochemical properties of the target compounds. On the one hand, polar compounds such as tartaric, citric or malic acids require the use of polar solvents (H_2O, 0.1% formic acid in H_2O:MeOH (4:1, *v/v*)). Aqueous KOH (1 M) is necessary to ensure the hydrolysis of malvidin to syringic acid, but it may not be adequate for the rest of the target

compounds. Finally, less polar solvents such as DCM:MeOH (2:1, *v/v*), AcN:H_2O (1:1, *v/v*) and AcN:H_2O (2:1, *v/v*) can be more adequate to extract those slightly less polar compounds (fumaric and succinic acids), together with the less polar syringic acid. Hence, the efficiency of all these solvents to extract target compounds from the synthetic ceramic sample was tested.

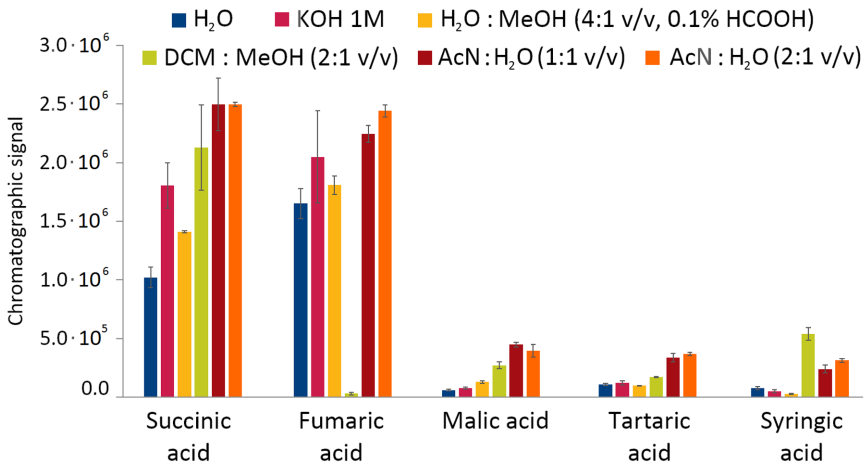


Figure 5.3: *Chromatographic signals obtained during the optimization of the solvent used for the solid-liquid extraction by means of UMB (4 min of extraction, duty cycle of 0.5 s·s^{-1} and sonication amplitude of 20%) (n=3).*

Figure 5.3 shows the chromatographic responses obtained for the target compounds extracted with the different solvents. Overall, the use of mixtures of polar/non-polar solvents (i.e., DCM:MeOH (2:1, *v/v*) or AcN:H_2O (1:1 and 2:1, *v/v*)) rendered better responses for all target compounds in comparison to the use of polar solvents (i.e., H_2O, KOH (1M, aq.) or H_2O:MeOH (4:1 *v/v*, 0.1% formic acid)). Moreover, polar solvents are not adequate to extract syringic acid. Poor extraction recoveries were obtained for fumaric acid using DCM:MeOH, so that AcN:H_2O (2:1, *v/v*) was selected as optimum extraction solvent in order to: (i) get the highest extraction efficiencies for all the target compounds, and (ii)

reduce water content that can promote the extraction of other ionic interfering compounds.

It is noteworthy that, according to the literature, the use of KOH (1 M, aq.) is required to hydrolyze malvidin to free syringic acid [7]. Hence, in order to determine all wine specific biomarkers in the same run, a two-step strategy was proposed. Once the extraction of tartaric, malic, fumaric, succinic and citric acid was over using 7 mL of AcN:H_2O (2:1, *v/v*), the remaining residue was extracted with fresh 7 mL of KOH (1 M, aq.) by means of FUSLE (4 min, duty cycle of 0.5 $s \cdot s^{-1}$ and 20% of amplitude) in order to determine the putative content of malvidin as free syringic acid.

5.2.2.2. Ultrasound micro bath (UMB) vs. titanium probe (FUSLE)

The duality of the ultrasound equipment allowed to make a comparison between the micro bath and the titanium probe. The extraction by means of UMB and FUSLE was performed using 3 mL and 7 mL of AcN:H_2O (2:1, *v/v*), respectively, and using the same extraction parameters (i.e., 4 min, duty cycle of 0.5 $s \cdot s^{-1}$ and 20% amplitude). The samples were extracted once using FUSLE, whereas two consecutive extractions using 3 mL of fresh solvent each time were performed using UMB. Results were statistically comparable (p-level > 0.05) regardless of the extraction technique used and the number of extractions performed. Thus, in order to shorten the analytical procedure, FUSLE was used thereafter.

5.2.3. Optimization of the preconcentration step

5.2.3.1. Liquid-liquid extraction and ion-pair formation

The compounds in the AcN:H_2O (2:1, *v/v*) phase after FUSLE procedure can be re-extracted to a small volume of organic phase (LLE), and the organic extract can be easily evaporated and derivatized afterwards.

The efficiency of LLE was evaluated using several solvents: 100% EtOAc, EtOAc:IPA (95:5, *v/v*), EtOAc:IPA (90:10, *v/v*) and EtOAc:IPA (80:20, *v/v*). The recoveries obtained for the target compounds are summarized in Figure 5.4. Overall, good repeatability and extraction efficiencies were obtained for the most hydrophobic compounds using EtOAc:IPA (80:20, *v/v*) by LLE, but poor recovery was obtained for the most important wine biomarker, tartaric acid (< 15%), and for malic acid (< 20%). The high polarity of these two analytes makes difficult their extraction from the aqueous media. Moreover, the addition of a 20% of 2-propanol enlarges significantly the subsequent evaporation step prior to the derivatization (almost 90 minutes were necessary to evaporate the extracts to dryness) and, consequently, the analysis throughput.

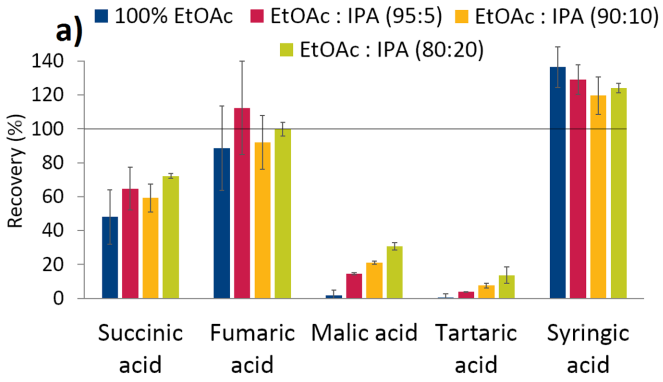


Figure 5.4: *Recoveries (%) obtained during LLE with EtOAc and EtOAc : IPA mixtures at different ratios.*

In order to promote the transference of malic and tartaric acids to the organic phase, 10% of NaCl was added to the AcN:H_2O (2:1, *v/v*) solution spiked with the target compounds. The addition of salt increases the ionic strength of aqueous samples, reducing the solubility of the organic compounds and consequently, improving the mass transference to the organic phase. The recovery for tartaric and malic acids was only increased up to 30% using this strategy, thus, we evaluated another approach based on ion-pair formation to improve the mass transference to the organic phase. The use of TBA as ion-pair reagent

rendered dirty chromatograms and did not provide adequate results in terms of extraction efficiency. Consquently, LLE approach was discarded to preconcentrate the biomarkers of wine and other alternatives based on SPE approaches had to be evaluated.

5.2.3.2. Solid phase extraction

5.2.3.2.1. Selection of sorbent material

The use of RP-SPE (200-mg, Oasis HLB) and mixed-mode (150-mg, Oasis-MAX) was studied to preconcentrate wine biomarkers. For this purpose, assays were performed in triplicate spiking 5 mL of Milli-Q water with ~0.85 µg of the target analytes and following the procedures described in section 5.1.6.2. In these assays, the target compounds were eluted with 6 mL of EtOAc when Oasis-HLB cartridges were used and with 6 mL of AcN (0.8% HCl) when Oasis-MAX cartridges were used.

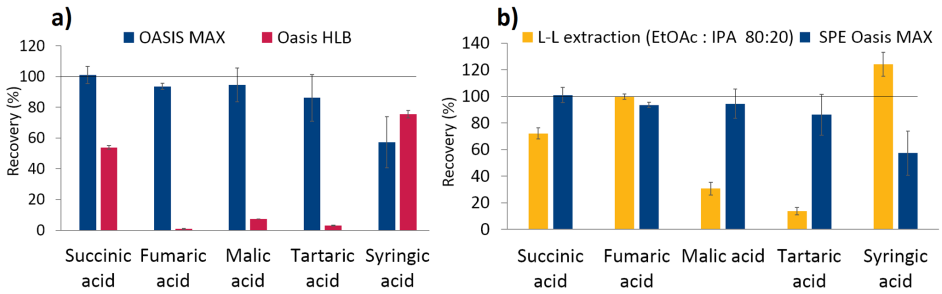


Figure 5.5: *a) % Recoveries of the target compounds after mixed-mode SPE (Oasis MAX) and RP-SPE (Oasis HLB). b) Comparison of liquid-liquid extraction with optimum solvent mixture with SPE using Oasis-Max cartridges.*

The best SPE cartridge for the preconcentration of wine related compounds was selected according to the recoveries (%) that are shown in Figure 5.5a. The dual retention mechanism (i.e., partition and ion-exchange) in Oasis-MAX cartridges in comparison to the partition mechanism in Oasis-HLB, enhanced the retention of the target compounds and it

was quantitative (> 80%) for all the target compounds except for syringic acid. On the other hand, due to its more non-polar nature in comparison to the rest screened small molecules, syringic acid is prone to be retained by the reversed-phase mode, and hence, slightly higher recovery was obtained for this compound using Oasis-HLB cartridges (75%) in comparison to the one obtained using Oasis-MAX (46%).

Moreover, the results obtained with Oasis-MAX cartridges were also compared to the ones obtained with the classical extraction mechanism LLE. As it can be seen in Figure 5.5b, although acceptable results can be obtained for the more hydrophobic compounds (i.e., syringic acid, fumaric acid and succinic acid), LLE did not render quantitative recoveries for the most hydrophilic compounds including the most important wine biomarker (i.e. tartaric acid).

According to these results, the compounds recovered in AcN:H_2O FUSLE fraction were preconcentrated using Oasis-MAX cartridges. Moreover, in order to improve the efficiency of the procedure to determine syringic acid formed after the alkaline hydrolysis of malvidin, the syringic acid recovered in the KOH FUSLE fraction was preconcentrated using Oasis-HLB cartridges.

5.2.3.2.2. Elution profile

In order to improve the elution of analytes from the mixed-mode anion exchange sorbent, the influence of elution solvent was evaluated. The use of an organic solvent in acidic media is required to quantitatively elute short chain dicarboxylic acids from mixed-mode SPE cartridges. Although both MeOH and AcN can be used, owing to the more non-polar nature of syringic acid, target compounds were eluted with AcN (0.8% HCl) and the need of a second elution using pure AcN was also evaluated.

For this purpose, aliquots of 5 mL of Milli-Q water fortified with ~0.85 µg of target analytes were loaded onto Oasis WAX cartridges. Three consecutive 3 mL fractions of the solvent (pure AcN, 0.8% HCl) were collected in separate vials to get the elution profile corresponding to a total volume of 9 mL. Afterwards, 5 mL of pure AcN were also loaded and collected separately. All the fractions were evaporated to dryness, reconstituted in 150 µL of EtOAc and derivatized with 20 µL of BSTFA + 1% TMCS for their subsequent individual analysis by means of GC-MS.

The results of these assays were expressed as the chromatographic signal normalized to the sum of chromatographic responses of each fraction. According to the results summarized in Figure 5.6a, the use of 6 mL of elution solvent was required to recover all the compounds. Moreover, in order to quantitatively elute succinic and fumaric acids (see Figure 5.6b), the addition of 5 mL of AcN was necessary.

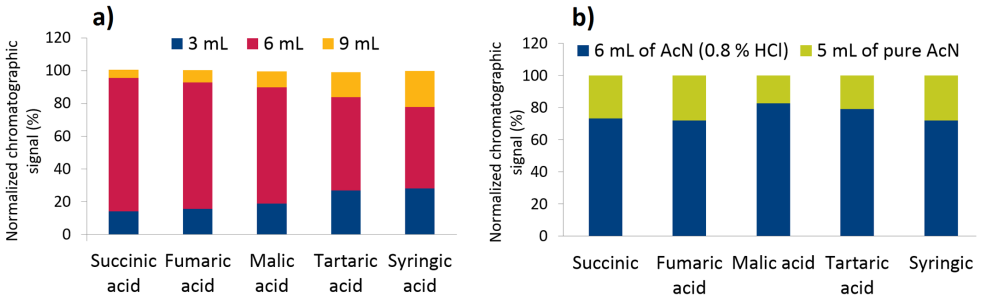


Figure 5.6: *Obtained normalized chromatographic signal of wine biomarkers during the elution profile from Oasis-MAX cartridges using: a) consecutive 3 mL aliquots of AcN (0.8% HCl) and b) a subsequent 5 mL aliquot of pure AcN after elution with optimum amount of AcN (0.8% HCl).*

Similarly, we designed an elution profile experiment for the quantitative elution of syringic acid from reversed-phase Oasis HLB cartridges. Thus, aliquots of 5 mL of Milli-Q water fortified with ~0.85 µg of syringic acid were loaded onto Oasis HLB cartridges. Four consecutive 3 mL fractions of the EtOAc were collected in separate vials to get the elution

profile corresponding to a total volume of 12 mL. According to the results displayed in Figure 5.7, 6 mL of EtOAc were required to quantitatively elute the syringic acid.

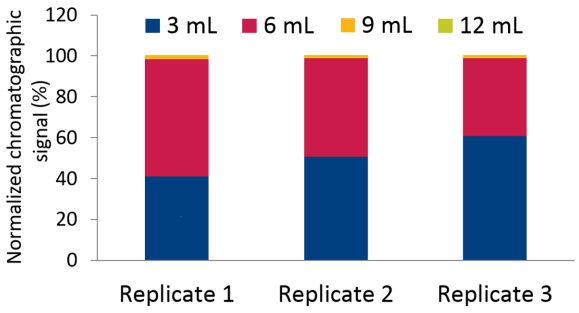


Figure 5.7: *Obtained normalized chromatographic signal of syringic acid during the elution profile from Oasis-HLB cartridges using consecutive 3 mL aliquots of EtOAc.*

5.2.3.2.3. Matrix effect

The extraction efficiency can be affected by the composition of the sample since high levels of anions co-extracted with the target compounds from the ceramic sample may compete in the anion-exchange process occurring in the mixed-mode sorptive material. Therefore, the efficiency of the mixed-mode SPE was tested using the synthetic ceramic samples spiked with the target compounds and extracted with the solvent fixed as optimum (i.e., 7 mL AcN:H_2O (2:1, *v/v*)). Under these conditions, it was determined by ion chromatography that, in addition to the target compounds, 4.3 ± 0.3 µg·mL^{-1} of chlorine, 0.40 ± 0.02 µg·mL^{-1} of nitrate and 4.91 ± 0.03 µg·mL^{-1} of sulfate were also extracted from the synthetic ceramic sample.

The matrix effect during the preconcentration step was evaluated by the comparison of a FUSLE synthetic ceramic sample extract (n=3) spiked with ~0.85 µg of the target compounds before the mixed-mode SPE step with the FUSLE clean solvent extract (n=3) spiked with the same mass of the target compounds.

Figure 5.8 shows the ratio between the recovery of the analytes achieved in presence and in absence of matrix interferences. The most affected compounds by the co-extracted interfering anions were the most polar target compounds (i.e., tartaric and citric acid), whereas no effect was observed for the rest of the compounds. Anyhow, since the ratio for all the target analytes was between 75% and 114% for all the target analytes (see Figure 5.8), matrix effect during the preconcentration using mixed-mode Oasis MAX cartridges can be considered negligible.

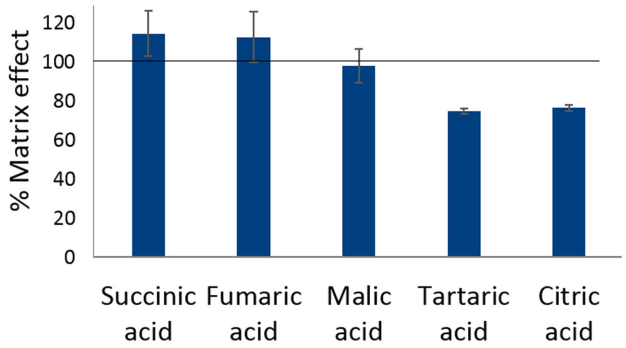


Figure 5.8: *Evaluation of matrix effects (%) for wine biomarkers in the presence and in the absence of interfering anions extracted from the synthetic ceramics.*

5.2.4. SPE-GC-MS method validation

The main figures of merit of the analytical procedure can be found in Table 5.2. External calibration curves were built from standard solutions at ng·mL^{-1} concentration levels in the range of 500 to 10000 ng·mL^{-1} for all the target compounds and derivatized in 150 µL of EtOAc and 20 µL BSTFA + 1% TMSC and analyzed by means of GC-MS. Linearities were found to be good with determination coefficients (r^2) in the range 0.9950-0.9997 for all the trimethylsilylated wine markers under study.

Table 5.2: *Main method parameters for the developed procedure.*

	Sylilation-GC-MS			FUSLE-SPE (MAX)-GC-MS Ceramic spiked at 1.7 µg·g^{-1}			
Analyte	**% RSD (n=3)**	**Linearity (r^2)**	**LOD_{instr} (µg·mL^{-1})**	**Absolute Recovery (%)**	**Apparent Recovery[c] (%)**	**%RSD (n=3)**	**LOD_{proc} (ng·g^{-1})**
Succinic acid	7	0.999	0.01	100	-	3	54
Fumaric acid	9	0.998	0.01	86	-	4	40
Malic acid	6	0.999	0.01	34	123	6	55
Tartaric acid	4	0.998	0.02	22	80	2	68
Citric acid	7	0.995	0.02	28	99	1	76
Syringic acid[a]	8	0.998	0.02	18	-	6	71
Syringic acid[b]	8	0.998	0.02	47	-	10	66

[a] FUSLE performed with 7 mL of AcN:H_2O (2:1 v/v) and preconcentration by mixed-mode SPE (MAX).

[b] FUSLE performed with 7 mL of KOH 1M and preconcentration by RP-SPE (Oasis-HLB).

[c] Extraction recovery corrected with 3-hydroxy-3-methyl pentadienoic acid used as surrogate.

The workflow of the procedure to analyze wine-related organic compounds is shown in Figure 5.9. Briefly, it consisted in a first extraction of the ceramic sample with 7 mL of AcN:H_2O (2:1, *v/v*) and the analytes recovered in this fraction were preconcentrated by means of mixed-mode SPE. In a second step, the remaining solid residue was treated with 7 mL of KOH (1M, aq.) to hydrolyze malvidin to syringic acid, which was preconcentrated by means of reversed-phase SPE.

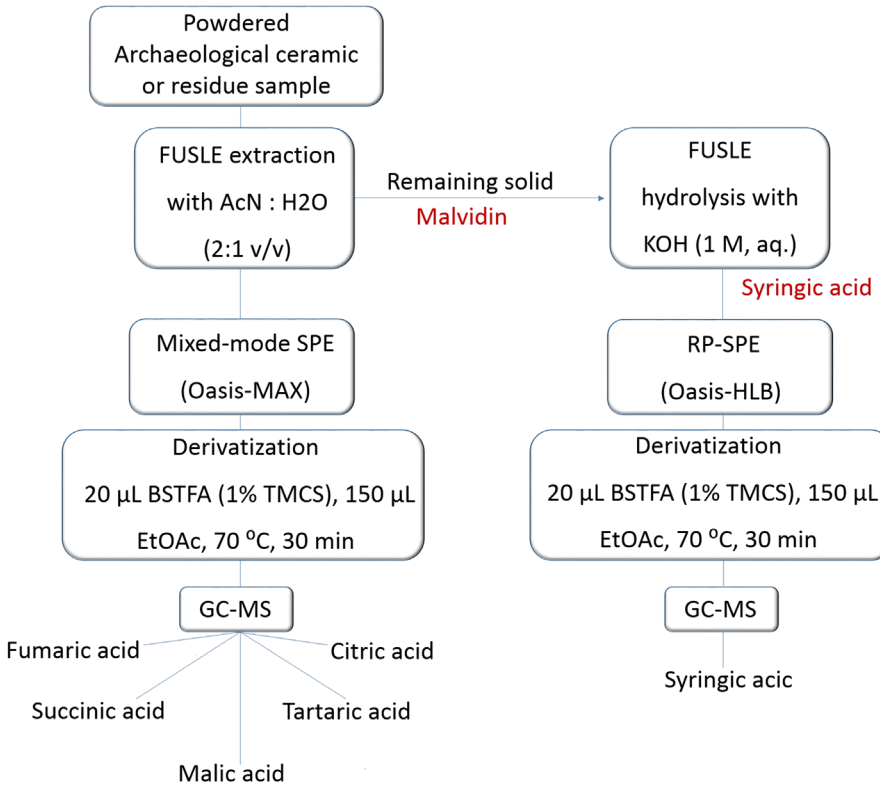


Figure 5.9: *Schematic flow-chart of the developed analytical procedure for the determination of wine biomarkers in archaeological ceramics.*

The accuracy of the whole analysis procedure for the determination of all wine related compounds (except malvidin) was determined in terms of recovery using 1 g of ceramic model fortified with 0.85 µg of the aforementioned wine markers.

The fortified ceramics were first treated with 7 mL of AcN:H_2O (2:1, *v/v*), the extracts were submitted to mixed-mode SPE preconcentration and finally, wine biomarkers were analyzed by GC-MS after derivatization. As it can be observed in Table 5.2, acceptable recoveries were obtained for succinic and fumaric acids, 100% and 86% respectively,

whereas low recoveries were obtained for malic, tartaric and citric acids (in the range of 22% and 34%). Tartaric, malic and citric acids (all with hydroxyl substituents) are prone to be more deeply impregnated and strongly bonded to the clay structure (i.e., via hydrogen bonds). Therefore, the low absolute recoveries obtained for these compounds may be related to the weak efficiency of AcN:H_2O mixture and ultrasound energy to cleavage the bonds between the compounds and the matrix. In order to correct the extraction efficiency, the use of 3-hydroxy-3-methyl-pentadienoic acid, a substituted dicarboxylic acid of similar nature, as surrogate was evaluated. This compound was added at the beginning of the whole procedure together with the target compounds. As can be seen in Table 5.2, adequate apparent recoveries (> 80%) were obtained after correcting the concentration of the target compounds with the surrogate.

The remaining fortified ceramic samples were re-extracted with 7 mL of KOH, and although malvidin was not present in the matrix, the absolute recovery for syringic acid was evaluated. We detected that in presence of matrix, the recovery decreased up to 47%, but the use of 3-hydroxy-3-methyl-pentadienoic acid was not adequate for this compound and we did not find any compound that could correct this lack of accuracy. Even so, the obtained absolute recovery was still much better than those obtained after performing liquid extraction (12%) as preconcentration technique.

Method repeatability was calculated in terms of relative standard deviation (% RSD) (see Table 5.2) and adequate % RSD values, between 1-10%, were obtained for all the target analytes.

The whole method was performed without using solid matrices but all the organic solvents and steps involved in order to calculate procedural limits of detection (LOD_{proc}). They were calculated as the average signal of blank (n=3) plus three times its standard deviation. In the case that no signal was detected at the corresponding retention time,

LOD_{proc} were referred to a ratio signal-to-noise of 3. The calculated limits of detection were estimated considering 1 g of archaeological ceramic sample (recalculations should be performed for 0.5 g of archaeological organic residues). The values of the LOD_{proc} were raised to the range 40-76 $ng \cdot g^{-1}$ but even adequate to detect them in ancient ceramic residues.

5.2.5. Application to real samples

The developed method was applied to real archaeological samples in order to detect wine biomarkers. Figure 5.10 shows the obtained chromatograms in SIM mode for the residue M48 (Figure 5.10a) and the archaeological ceramic fragment DC7 (Figure 5.10b).

Tartaric acid was found in both residue samples (M47 and M48), together with succinic, fumaric, malic and citric acids, which, according to the literature, confirms the archaeological hypothesis of being a wine residue and the use of the original amphorae to store wine [19]. Moreover, we detected two tricyclic diterpenoid acids in these samples: dehydroabietic acid and 7-oxo-dehydroabietic acid. Dehydroabietic acid and its oxidation by-product, 7-oxo-dehydroabietic acid, are characteristic compounds of a resin from *Pinaceae* family, and they have been widely detected in archaeological ceramics or glass containers [20,38,39]. The use of pine resin is documented not only as sealant or waterproofing material, but also as medicine, antiseptic or balms used in rituals [1]. Nevertheless, when pine resin is detected together with wine biomarkers, it is thought to be used in order to coat the interior surface of the ceramic artifacts, making it less porous [7,19,24], or to act as antioxidant promoting wine preservation or flavor enhancement [11,13,20,40]. Apart from the already mentioned wine related compounds, the alkaline treatment of the ceramic residues allowed the identification of syringic acid derived from malvidin, which reveals the red grape origin of the wine.

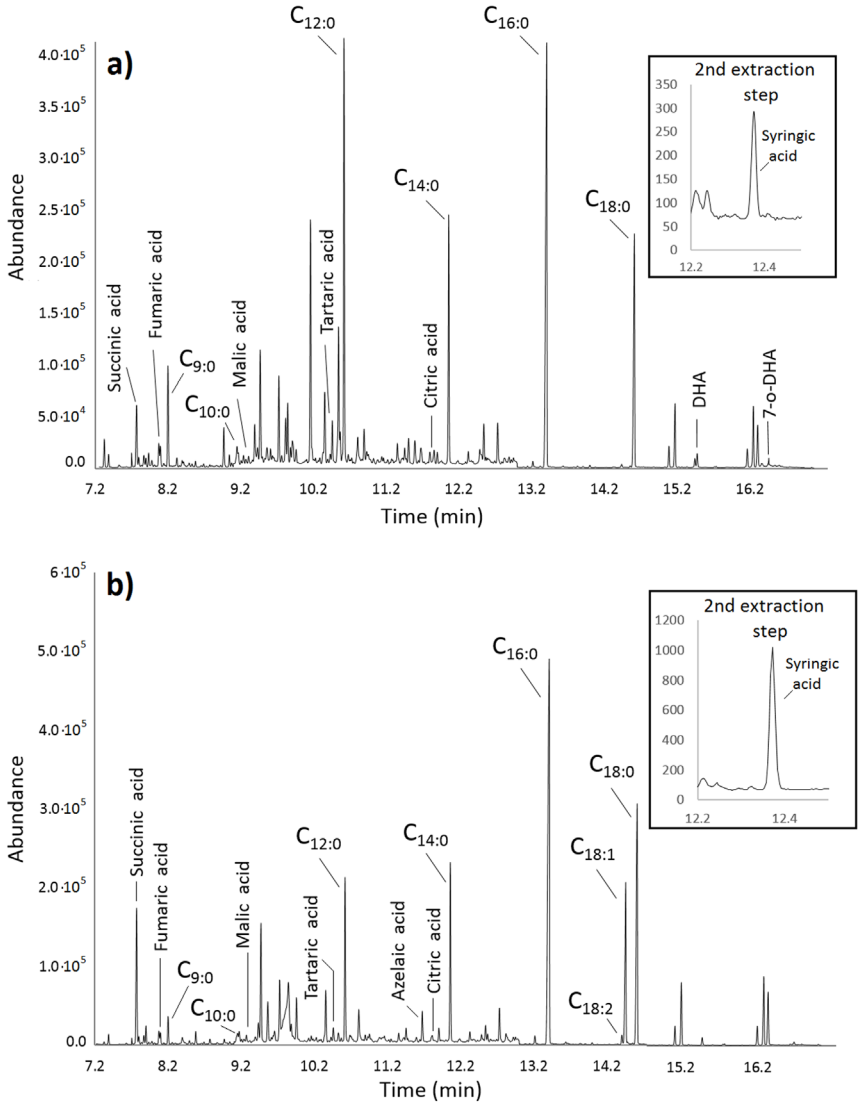


Figure 5.10: *Obtained chromatograms in SIM mode after analysis by GC-MS for a) the organic residue M48 found in the interior walls of an amphora and b) the potsherd sample named DC7. Shortened chromatograms in a frame correspond to the obtained result after performing the second extraction step to identify syringic acid. Cx:y are fatty acids of chain length x and degree of unsaturation y.*

In the case of potsherds DC4 and DC7, all the organic acids (i.e. tartaric, fumaric, succinic, malic and citric acids) were detected confirming their use as wine storing devices. Syringic acid was also found in both archaeological ceramics after the alkaline treatment at higher concentration than the concentration found in the residues (see Figure 5.10.b). Pine resin biomarkers were not found in the potsherds. In the case of sample DC7, in addition to wine related markers, high concentrations of oleic acid ($C_{18:1}$), together with palmitic ($C_{16:0}$), stearic ($C_{18:0}$) and azelaic (C9 diacid) acids, and low levels of linoleic acid ($C_{18:2}$) were also found (see Figure 5.10b), which are characteristic compounds of vegetable oil [5,41]. Thus, these data suggest that the archaeological ceramic was used for multiple purposes: to store wine but to store vegetable oil as well. Nevertheless, a more exhaustive analysis using a specific methodology to obtain the lipid profile of the organic residues in the ceramic should be performed in order to get more specific information about the vegetable oil.

5.2.6. Conclusions

Many archaeologists and analytical chemists have been concerned about the wine use in antiquity and consequently, the search for wine related compounds in archaeological samples has increased during the last decades. Despite several different analytical methodologies have been developed to detect these biomarkers, few are the works that aim developing new analytical methods that render high extraction efficiencies in order to detect organic biomarkers at trace levels. Herein, an entire analytical procedure was fully developed, optimized and validated in order to identify wine biomarkers in visible or absorbed organic residues in archaeological ceramic samples. FUSLE extraction rendered the best results in a single and short extraction time (4 min) using first 7 mL of a AcN:H_2O mixture (2:1, *v/v*) and, then, 7 mL of KOH. For the preconcentration step, mixed mode SPE using a strong anion exchanger cartridge rendered clean extracts, with negligible matrix effect and satisfactory recoveries for fumaric and succinic acids, and also for the rest of the target compounds after the correction with 3-hydroxy-3-methyl-pentadienoic acid as

surrogate. Reversed phase Oasis HLB was used to preconcentrate syringic acid formed after the alkaline hydrolysis of malvidin, and even if it was not quantitatively recovered, the recoveries were good enough to detect it in the analyzed archaeological samples.

The developed procedure was successfully applied to two archaeological ceramic samples (ca. II-I BCE) and two organic residues found inside the walls of two amphorae (Roman origin). Despite the antiquity of the archaeological samples, all the studied wine markers were detected above the LODs confirming the use of DC4 and DC7 archaeological ceramics as wine containers and the nature of M47 and M48 samples as wine residues. Additionally, the developed procedure allowed to detect simultaneously resin biomarkers from *Pinaceae* family, which, according to the literature, has been largely used for the preservation of wine.

On the other hand, several fatty acids were also identified in the analyzed samples, which helped searching for other uses of the materials under study. In this sense, one of the analyzed samples (DC7) was suggested to have also been used to store vegetable oil apart from wine. However, this analytical method was specifically developed for the identification of wine-markers; thus, it should only be used as a screening tool for the detection of fatty acids and more specific analysis that can be found in literature should be performed in order to go into detail.

5.4. References

[1] M.P. Colombini, G. Giachi, F. Modugno, E. Ribechini, Characterisation of organic residues in pottery vessels of the Roman age from Antinoe (Egypt), Microchem. J. 79 (2005) 83-90.

[2] M.P. Colombini, F. Modugno, E. Ribechini, Organic mass spectrometry in archaeology: Evidence for Brassicaceae seed oil in Egyptian ceramic lamps, J. Mass Spectrom. 40 (2005) 890-898.

[3] E. Ribechini, F. Modugno, C. Baraldi, P. Baraldi, M.P. Colombini, An integrated analytical approach for characterizing an organic residue from an archaeological glass bottle recovered in Pompeii (Naples, Italy), Talanta. 74 (2008) 555-561.

[4] R.P. Evershed, Organic residue analysis in archaeology: The archaeological biomarker revolution, Archaeometry. 50 (2008) 895-924.

[5] A. Pecci, M.T. Cau Ontiveros, N. Garnier, Identifying wine and oil production: Analysis of residues from Roman and Late Antique plastered vats, J. Archaeol. Sci. 40 (2013) 4491-4498.

[6] I. Jerkovic, Z. Marijanovic, M. Gugic, M. Roje, Chemical profile of the organic residue from ancient amphora found in the adriatic sea determined by direct GC and GC-MS analysis, Molecules. 16 (2011) 7936-7948.

[7] K. Romanus, J. Baeten, J. Poblome, S. Accardo, P. Degryse, P. Jacobs, D. De Vos, M. Waelkens, Wine and olive oil permeation in pitched and non-pitched ceramics: relation with results from archaeological amphorae from Sagalassos, Turkey, J. Archaeol. Sci. 36 (2009) 900-909.

[8] P.E. McGovern, J. Zhang, J. Tang, Z. Zhang, G.R. Hall, R.A. Moreau, A. Nuñez, E.D. Butrym, M.P. Richards, C.-. Wang, G. Cheng, Z. Zhao, C. Wang, Fermented beverages of pre- and proto-historic China, Proc. Natl. Acad. Sci. U. S. A. 101 (2004) 17593-17598.

[9] M.R. Guasch-Jané, M. Ibern-Gómez, C. Andrés-Lacueva, O. Jáuregui, R.M. Lamuela-Raventós, Liquid Chromatography with Mass Spectrometry in Tandem Mode Applied for the Identification of Wine Markers in Residues from Ancient Egyptian Vessels, Anal. Chem. 76 (2004) 1672-1677.

[10] R.P. Evershed, Experimental approaches to the interpretation of absorbed organic residues in archaeological ceramics, World Archaeol. 40 (2008) 26-47.

[11] M.P. Colombini, F. Modugno, Organic Mass Spectrometry in Art and Archaeology, in: Anonymous John Wiley & Sons, Ltd, Pisa, 2009, 1-493.

[12] R.P. Evershed, S.N. Dudd, S. Charters, H. Mottram, A.W. Stott, A. Raven, P.F. Van Bergen, H.A. Bland, Lipids as carriers of anthropogenic signals from prehistory, Philos. Trans. R. Soc. B Biol. Sci. 354 (1999) 19-31.

[13] H. Barnard, A.N. Dooley, G. Areshian, B. Gasparyan, K.F. Faull, Chemical evidence for wine production around 4000 BCE in the Late Chalcolithic Near Eastern highlands, J. Archaeol. Sci. 38 (2011) 977-984.

[14] D. Arobba, F. Bulgarelli, F. Camin, R. Caramiello, R. Larcher, L. Martinelli, Palaeobotanical, chemical and physical investigation of the content of an ancient wine amphora from the northern Tyrrhenian sea in Italy, J. Archaeol. Sci. 45 (2014) 226-233.

[15] N. Garnier, P. Richardin, V. Cheynier, M. Regert, Characterization of thermally assisted hydrolysis and methylation products of polyphenols from modern and archaeological vine derivatives using gas chromatography-mass spectrometry, Anal. Chim. Acta. 493 (2003) 137-157.

[16] N. Garnier, S.M. Valamoti, Prehistoric wine-making at Dikili Tash (Northern Greece): Integrating residue analysis and archaeobotany, J. Archaeol. Sci. 74 (2016) 195-206.

[17] Y. Hamilakis, Wine, oil and the dialectics of power in Bronze Age Crete: A review of the evidence, Oxf. J. Archaeol. 15 (1996) 1-32.

[18] J. Hopkins, R.A. Armitage, Characterizing organic residues on ceramics by direct analysis in real time time-of-flight mass spectrometry, ACS Symp. Ser. 1103 (2012) 131-142.

[19] A. Pecci, G. Giorgi, L. Salvini, M.Á Cau Ontiveros, Identifying wine markers in ceramics and plasters using gas chromatography-mass spectrometry. Experimental and archaeological materials, J. Archaeol. Sci. 40 (2013) 109-115.

[20] E. Manzano, S. Cantarero, A. García, A. Adroher, J.L. Vílchez, A multi-analytical approach applied to the archaeological residues in Iberian glasses. Earliest evidences on the consumption of fermented beverages in votive rituals, Microchem. J. 129 (2016) 286-292.

[21] B. Stern, C. Heron, T. Tellefsen, M. Serpico, New investigations into the Uluburun resin cargo, J. Archaeol. Sci. 35 (2008) 2188-2203.

[22] C. Milanesi, I. Bigliazzi, C. Faleri, B. Caterina, M. Cresti, Microscope observations and DNA analysis of wine residues from Roman amphorae found in Ukraine and from bottles of recent Tuscan wines, J. Archaeol. Sci. 38 (2011) 3675-3680.

[23] P.E. McGovern, G.R. Hall, Charting a Future Course for Organic Residue Analysis in Archaeology, J. Archaeol. Method and Theory. 23 (2015) 592-622.

[24] E. Allevato, M. Buonincontri, M. Vairo, A. Pecci, M.A. Cau, M. Yoneda, G.F. De Simone, M. Aoyagi, C. Angelelli, S. Matsuyama, K. Takeuchi, G. Di Pasquale, Persistence of the cultural landscape in Campania (Southern Italy) before the AD 472 Vesuvius eruption: archaeoenvironmental data, J. Archaeol. Sci. 39 (2012) 399-406.

[25] M.R. Guasch-Jané, The meaning of wine in Egyptian tombs: The three amphorae from Tutankhamun's burial chamber, Antiquity. 85 (2011) 851-858.

[26] E.D. Teodor, G.I. Badea, A. Alecu, L. Calu, G.L. Radu, Interdisciplinary study on pottery experimentally impregnated with wine, Chem. Pap. 68 (2014) 1022-1029.

[27] R.H. Michel, P.E. McGovern, V.R. Badler, The First Wine & Beer: Chemical Detection of Ancient Fermented Beverages, Anal. Chem. 65 (1993) 408A-413A.

[28] F. Inserra, A. Pecci, M.ÁC. Ontiveros, J.R. Buxó, Organic residues analysis of Late Antique pottery from Plaça Major-Horts de Can Torras (Castellar del Vallés, Catalonia, Spain), Period. Mineral. 84 (2015) 123-138.

[29] M.D. Petit-Domínguez, R. García-Giménez, M.I. Rucandio, Chemical characterization of Iberian amphorae and tannin determination as indicative of amphora contents, Mikrochim. Acta. 141 (2003) 63-68.

[30] K.L. Rasmussen, J. Gunneweg, J. Van Der Plicht, I. Kralj Cigic, A.D. Bond, B. Svensmark, M. Balla, M. Strlic, G. Doudna, On the age and content of Jar-35-A sealed and intact storage jar found on the southern plateau of Qumran, Archaeometry. 53 (2011) 791-808.

[31] L. Blanco-Zubiaguirre, N. Arrieta, A. Iturregui, I. Martinez-Arkarazo, M. Olivares, K. Castro, M.A. Olazabal, J.M. Madariaga, Focused ultrasound solid-liquid extraction for the determination of organic biomarkers in beachrocks, Ultrason. Sonochem. 27 (2015) 430-439.

[32] L. Blanco-Zubiaguirre, M. Olivares, K. Castro, J.G. Iñañez, J.M. Madariaga, An alternative analytical method based on ultrasound micro bath hydrolysis and GC-MS analysis for the characterization of organic biomarkers in archaeological ceramics, Anal. Bioanal. Chem. 408 (2016) 8001-8012.

[33] A. Lluveras, I. Bonaduce, A. Andreotti, M.P. Colombini, GC/MS analytical procedure for the characterization of glycerolipids, natural waxes, terpenoid resins, proteinaceous and polysaccharide materials in the same paint microsample avoiding interferences from inorganic media, Anal. Chem. 82 (2010) 376-386.

[34] J.G. Huddleston, H.D. Willauer, R.P. Swatloski, A.E. Visser, R.D. Rogers, Room temperature ionic liquids as novel media for 'clean' liquid-liquid extraction, Chem. Commun. (1998) 1765-1766.

[35] N. Fontanals, R.M. Marcé, F. Borrull, New hydrophilic materials for solid-phase extraction, Trends Anal. Chem. 24 (2005) 394-406.

[36] N. Gilart, F. Borrull, N. Fontanals, R.M. Marcé, Selective materials for solid-phase extraction in environmental analysis, Trends Anal. Chem. 1 (2014) e8-e18.

[37] N. Fontanals, R.M. Marcé, F. Borrull, P.A.G. Cormack, Mixed-mode ion-exchange polymeric sorbents: dual-phase materials that improve selectivity and capacity, Trends Anal. Chem. 29 (2010) 765-779.

[38] E. Ribechini, F. Modugno, M.P. Colombini, R.P. Evershed, Gas chromatographic and mass spectrometric investigations of organic residues from Roman glass unguentaria, J. Chromatogr. A. 1183 (2008) 158-169.

[39] M.P. Colombini, F. Modugno, E. Ribechini, Direct exposure electron ionization mass spectrometry and gas chromatography/mass spectrometry techniques to study organic coatings on archaeological amphorae, J. Mass Spectrom. 40 (2005) 675-687.

[40] P. McGovern, M. Jalabadze, S. Batiuk, M.P. Callahan, K.E. Smith, G.R. Hall, E. Kvavadze, D. Maghradze, N. Rusishvili, L. Bouby, O. Failla, G. Cola, L. Mariani, E. Boaretto, R. Bacilieri, P. This, N. Wales, D. Lordkipanidze, Early Neolithic wine of Georgia in the South Caucasus, Proc. Natl. Acad. Sci. U. S. A. 114 (2017) E10309-E10318.

[41] A. Pecci, M.ÁC. Ontiveros, C. Valdambrini, F. Inserra, Understanding residues of oil production: Chemical analyses of floors in traditional mills, J. Archaeol. Sci. 40 (2013) 883-893.

Chapter 6

An alternative analytical method based on ultrasound micro bath hydrolysis and GC-MS analysis for the characterization of organic biomarkers in archaeological ceramics

Analytical and Bioanalytical Chemistry 408 (2016) 8001–8012

"I try all things, I achieve what I can."
Herman Melville—Moby-Dick

Organic residues can remain for a long time in ceramic pottery mainly absorbed in the porous matrix. The analysis of organic remains in these potsherds offers an insight into vessel use, site and regional economies and technologies. Among the wide range of investigated compounds, lipids and their transformation products have been the most widely studied ones due to the wide use of animal fats and oils of various nature and origin in the past and their relative stability [1,2]. Marine animal fats are easily distinguished from modern terrestrial ones through their lipid profiles being the formers richer in monounsaturated ($C_{16:1}$, $C_{18:1}$, $C_{20:1}$ and $C_{22:1}$) and polyunsaturated ($C_{20:5}$ and $C_{22:6}$) fatty acids. Unfortunately, unsaturated compounds rarely survive to the oxidation processes that take place during vessel use and burial [3]. In this sense, many investigations have focused their attention on the determination of oxidation products such as dihydroxy derivatives of monounsaturated fatty acids and ω-(o-alkylphenyl)-alkanoic acids containing 16–20 carbon atoms [4]. Isoprenoid fatty acids [4,8,12-trimethyltridecanoic acid (4,8,12-TMTD) and 3,7,11,15-tetramethylhexadecanoic (phytanic acid)] have also been demonstrated to be important biomarkers for the processing of marine commodities in archaeological pottery vessels [5-7].

From an analytical point of view, the isolation and characterization of unknown constituents within complex organic mixtures is a challenging analytical task that requires the use of many analytical techniques involving mainly chromatographic techniques coupled to mass spectrometry (MS) [2,8,9]. Gas chromatography – mass spectrometry (GC-MS) provides valuable structural information on each of the separated components and allows recognizing diagnostic lipid distributions and identifying the previously mentioned stable molecules (biomarkers) present in the ancient material or formed over the centuries due to ageing [8]. Previous published works have generally focused their attention on the identification of the target molecules without giving too much importance to the analytical procedure itself, which often requires tedious and long pre-treatment steps.

The isolation of suspect organic compounds from archaeological vessels often requires several consecutive steps that normally consist of an extraction, hydrolysis and saponification step, followed by a liquid extraction and a final derivatization step prior to the GC-MS analysis [10,11]. The combined hydrolysis and saponification procedure is the most critical one and is often assisted by external energies such as temperature and/or ultrasound energy [10,11]. Nowadays, however, the use of ultrasound probes has gained interest in many research fields since, as explained in previous chapters, they provide 100 times higher sonication power, as well as higher reproducibility and efficiency. However, when the quantity of solution is limited, the ultrasound instrument can be used as a small water bath (ultrasound micro bath or UMB) in which the sample is immersed, and this way, the ultrasound energy's action is restricted only to one sample, allowing fast, reproducible and quantitative extraction assays [12,13]. Nevertheless, the use of ultrasound micro bath for the extraction or hydrolysis of organic biomarkers from archaeological ceramic samples has not yet been reported in the literature.

In this framework, we present an improved analytical approach based on an alkaline hydrolysis by means of an ultrasound micro bath (UMB) followed by liquid extraction in combination with GC-MS, which was fully optimized and validated. For this purpose, fatty acid methyl esters were used in order to simulate the processes that biomarkers from real samples would undergo to be successfully identified. This main goal has been achieved after the optimization of the main parameters affecting the hydrolysis step (% MeOH/H_2O and volume and ultrasound micro bath parameters such as ultrasound amplitude, duty cycle and hydrolysis time), the extraction procedure (extractant nature and volume) and the derivatization step (% BSTFA and derivatization solvent). Finally, the method was applied to analyze organic remains preserved in ceramic vessels, concretely, samples collected from a deposit in Lekeitio (Basque Country, Northern Spain). These vessels are suspected to have been used by Basque Whalers in the period from 16th to 17th Century to store whale oil.

6.1. Materials and methods

6.1.1. Archaeological samples

The ceramic samples used in the application of the optimized analytical procedure were collected in an archaeological site in Lekeitio (Basque Country, Northern Spain). No entire ceramic amphora was found but several fragments corresponding to different vessels were recovered from the deposit. In this work, we analyzed five ceramic samples labeled as follows: Lekeitio 002 (L002), Lekeitio 003 (L003), Lekeitio 006 (L006), Lekeitio 016 (L016) and Lekeitio 020 (L020). No visible residues were detected among the different pieces of ceramic, which made largely difficult the characterization and identification of potential organic residues.

6.1.2. Reagents and materials

Laboratory material was carefully cleaned with abundant pure water (<0.2 $\mu S \cdot cm^{-1}$, Millipore, USA) and without using detergent to avoid possible contamination produced by detergent residues. The material was sonicated under clean acetone (Q.P., Panreac Química, Spain) for an hour and then rinsed with ultrapure water (<0.057 $\mu S \cdot cm^{-1}$, Milli-Q model, Millipore, USA). Finally, the glass material was dried in an oven at 400 °C for 4 h.

Fatty acid methyl esters (FAME) mix containing methyl octanoate ($C_9H_{18}O_2$), methyl decanoate ($C_{11}H_{22}O_2$), methyl laurate ($C_{13}H_{26}O_2$), methyl tetradecanoate ($C_{15}H_{30}O_2$), methyl palmitate ($C_{17}H_{34}O_2$), methyl palmitoleate ($C_{17}H_{32}O_2$), methyl stearate ($C_{19}H_{38}O_2$), cis-9-oleic methyl ester, methyl linoleate, methyl linolenate ($C_{19}H_{34}O_2$), methyl arachidate ($C_{21}H_{42}O_2$), methyl docosanoate ($C_{23}H_{46}O_2$), methyl erucate ($C_{23}H_{44}O_2$) and methyl lignocerate ($C_{25}H_{50}O_2$) at 2000 $\mu g \cdot mL^{-1}$ was purchased from Supelco (Sigma-Aldrich, Germany). Standard individual solutions of octanoic acid ($C_{8:0}$), decanoic acid ($C_{10:0}$), dodecanoic acid (lauric acid or $C_{12:0}$), tetradecanoic acid (myristic acid or $C_{14:0}$), hexadecanoic acid (palmitic acid or $C_{16:0}$), octadecanoic acid (stearic acid or $C_{18:0}$), oleic acid ($C_{18:1}$) and linoleic acid ($C_{18:2}$) were

acquired from Supelco (Sigma-Aldrich, Germany). Mixed fresh solutions with ≈50 $\mu g \cdot g^{-1}$ of each target compound were monthly prepared and stored in amber vials at -20 ^{o}C. Dilutions of these stock solutions were daily prepared in *n*-hexane according to the experimentation.

The used solvents, *n*-hexane (Hex), ethyl acetate (EtOAc), dichloromethane (DCM), methanol (MeOH) and isooctane (all HPLC grade, 99.8 %) were purchased from LabScan (Dublin, Ireland). Diethyl ether, potassium hydroxide pellets (KOH) and N,O-bis(trimethyl)silyltrifluoro-acetamide containing % trimethylchlorosilane (BSTFA + 1% TMCS) were purchased from Sigma-Aldrich (Milan, Italy).

The ceramic models used during the validation of the methodology were prepared and supplied by the department of sculpture of the Fine Arts faculty from the University of the Basque Country (UPV/EHU). The models were prepared according to chemical and mineralogical information of the archaeological vessels, which is described elsewhere [14].

6.1.3. Sample preparation

Special care was taken in all analytical steps (including sample crushing, fractionation and extraction) in order to minimize cross-contamination sources and other side effects. Ceramic powder samples were collected with a scalpel, both from the external and internal part of the archaeological pieces. The powdered samples were crushed in a mortar in order to obtain a more homogeneous sample since a heterogeneous particle size was obtained using the scalpel. Finally, the samples were weighed and stored in glass vessels in order to preserve them until their analysis. For the analysis of the ceramics, sub-samples of 0.5 g were taken from the glass vessels in order to have representative aliquots of the archaeological pieces and the assays were performed using three sub-samples of each real sample.

6.1.4. Hydrolysis and extraction of fatty acids

6.1.4.1. Optimization of the hydrolysis step

According to the literature, KOH dissolved in a hydroalcoholic mixture of MeOH and H_2O is the most used hydrolyzing agent [15]. Thus, the composition of this mixture as well as the volume required for a quantitative hydrolysis was evaluated in the first assays. The volume (i.e., 1, 3 and 5 mL) of the hydrolyzing mixture fixed as KOH_{MeOH} (10 % weight)/KOH_{H2O} (10% weight), 2:3 (*v/v*) was studied using a ceramic model in order to simulate the volume occupied by 1 g of the archaeological samples. Next, in order to maximize the corresponding recovery of this step, a mixture of 3 mL of MeOH with different percentages of KOH (2.5%, 5% and 10%) and ratios of MeOH:H_2O (0:5, 1:4, 2:3, 3:2, 4:1 and 5:0) were tested. All these previous assays were performed according to the traditional method of hydrolysis, which consists of heating and stirring the samples in a water bath at 60 ^{o}C for 3 hours [16].

Afterwards, the applicability of the ultrasound micro bath in order to accelerate the hydrolysis step was assessed. The assays were carried out using a Bandelin Sonopuls HD 3100 sonifier ultrasonic cell disruptor/homogenizer (100 W, 20 kHz; Bandelin Electronic, Berlin, Germany) with a cup booster BR made of stainless steel. A mixture of ~0.85 µg of FAMEs were hydrolyzed using 3 mL of 5% KOH in MeOH:H_20 (2:3, *v/v*). The extraction process variables (i.e., duty cycle and sonication amplitude) were thoroughly optimized by means of an experimental design approach and the hydrolysis time by means of a time profile (up to 10 min). Under optimum conditions, FAMEs were hydrolyzed from ceramic vessels using a mixture of 3 mL of 5% hydroalcoholic KOH (MeOH:H_2O, 2:3 *v/v*), immersed in an ice-water bath for 2.5 minutes using a duty cycle of 0.5 s and a sonication amplitude of 20%. The process was repeated three times using fresh hydrolyzing agent.

After the hydrolysis step, the neutral fraction was extracted with *n*-hexane (3 x 500 µL) and quantitatively recovered. The remaining aqueous fraction was acidified (HCl 12 mol·L^{-1}, to pH=2) and the acid fraction containing fatty acids was subsequently extracted with diethyl ether (3 x 500 µL). The extracts were evaporated to dryness and derivatized with 20 µL of BSTFA + 1% TMCS and 150 µL of isooctane and analyzed by means of GC-MS. The neutral fractions were also analyzed in order to ensure that no fatty acid remained as methyl ester after the analytical pretreatment.

6.1.4.2. Optimization of the extraction step

The extraction of both neutral and acid fractions was optimized. In a first attempt, the neutral fraction was extracted using different extraction volumes (3 x 250, 500 or 1000 µL) of *n*-hexane. The remaining aqueous phase was acidified with hydrochloric acid (12 mol·L^{-1}; to pH 2) and the free fatty acids were extracted from the hydrolysate testing different extraction solvents (diethyl ether, dichloromethane and *n*-hexane) and volumes (3 × 250, 500 or 1000 µL). The extracts were concentrated to dryness under a gentle stream of nitrogen at 25 ^{o}C, reconstituted in the derivatization solvent, derivatized with BSTFA + 1% TMCS (60 ^{o}C, 30 min) and analyzed by means of GC-MS system. Under optimum conditions, 1000 µL of *n*-hexane were used to isolate both neutral compounds and fatty acids from the neutral and acid fractions, respectively.

6.1.5. Optimization of the derivatization step

The analysis of fatty acids by means of GC-MS requires a previous derivatization step, which was optimized. During optimization, 42.6 µL of standard solution of a mixture containing all the analytes at 20 µg·mL^{-1} each were evaporated to dryness under a gentle stream of nitrogen (Turbovap® LV, Caliper, Life sciences, USA) at 25 °C. Aliquots of 150 µL of different solvents (ethyl acetate, *n*-hexane and isooctane) and different volumes of the

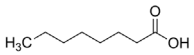
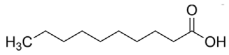
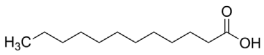
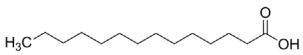
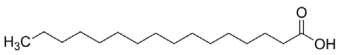
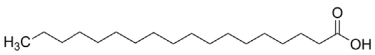
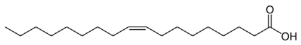
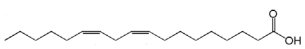
BSTFA + 1% TMCS used as derivatization reagent (8.5 µL, 20 µL and 34 µL) were added and submitted to the derivatization step (30 min at 60 ^{o}C). In the case of sample extracts, they were evaporated to dryness in chromatographic amber vials and re-dissolved in the optimum conditions. Hence, under optimum conditions, the extracts were reconstituted in 150 µL of EtOAc and 20 µL of BSTFA + 1% TMCS and derivatized at 60°C for 30 min.

6.1.6. Analysis by GC-MS

2 µL of the derivatized extract were injected into a 6890N gas chromatographer (Agilent Technologies, Avondale, PA, USA) coupled to a 5973N electron impact ionization mass spectrometer and a 7683 Agilent autosampler. The analysis was performed in the splitless mode for 1.5 min at 300 ^{o}C using HP5-MS capillary column (30m x 0.25mm, 0.25 µm, Agilent). The oven program temperature used was established as follows: 55 ^{o}C (5min), a temperature increase of 45 ^{o}C·min^{-1} to 90 ^{o}C to continue rising at 6 ^{o}C·min^{-1} to 300 ^{o}C, maintaining it for 15 min.

Hydrogen (Hydrogen generator AD-1020, Cinel Strumenti Scientifici, Padova, Italy) was used as carrier gas at constant flow of 1.5 mL·min^{-1}. The MS transfer line temperature was maintained at 310 ^{o}C, the ion source and quadrupole at 230 ^{o}C and 150 ^{o}C, respectively. Measurements were performed in both SCAN (*m/z*: 50–450) and SIM modes using the most abundant *m/z* fragment of trimethylsilylated *n*-alkanoic acids (*m/z*: 73) as quantifier and *m/z*: 117 and each fatty acid trimethylsilyl ester's molecular ion minus 15 (corresponding to the loss of a methyl group) as qualifiers (see Table 6.1).

Table 6.1: *Name, abbreviature, structure and monitored m/z ion fragments (quantitative in bold) for each fatty acid under study.*

Compound	Abbreviation	Structure	m/z
Octanoic acid *(Caprilic acid)*	$C_{8:0}$		**73** 117 201
Decanoic acid *(Capric acid)*	$C_{10:0}$		**73** 117 229
Dodecanoic acid *(Lauric acid)*	$C_{12:0}$		**73** 117 257
Tetradecanoic acid *(Myristic acid)*	$C_{14:0}$		**73** 117 285
Hexadecanoic acid *(Palmitic acid)*	$C_{16:0}$		**73** 117 313
Octadecanoic acid *(Stearic acid)*	$C_{18:0}$		**73** 117 341
cis-9-octadecenoic acid *(Oleic acid)*	$C_{18:1}$		**73** 117 339
cis,cis-9,12-octadecadienoic acid *(Linoleic acid)*	$C_{18:2}$		**73** 117 337

6.2. Results and discussion

All the steps involving the optimization were carried out by spiking the different solutions in each stage (hydrolysis, extraction or derivatization) with 42.6 µL of a fresh stock solution of FAMEs at 20 $\mu g \cdot mL^{-1}$ each. Despite the lack of standard stock solutions for the biomarkers of interest, it should be highlighted that they have a similar chemical behavior

compared to the target compounds studied in this work (i.e. saturated and unsaturated fatty acids). Thus, the obtained results for the target compounds in the optimization and validation steps can be applicable to the biomarkers of whale oil.

6.2.1. Optimization of the derivatization step

Several parameters affecting the derivatization step were evaluated (i.e., derivatization reagent volume and solvent nature). On a first attempt, for the derivatization step different volumes (8.5 µL, 20 µL and 34 µL) of derivatizing agent (BSTFA + 1% TMCS) were tested using isooctane as the solvent to obtain a final volume of 170 µL according to literature [8]. The derivatization assays were performed in triplicate at 60 °C for 30 min. As it is shown in Figure 6.1, there are no statistical differences between the different studied volumes. Thus, 20 µL of BSTFA + 1% TMCS were used in the subsequent assays in order to: (i) minimize the introduction of large quantities of this reagent to the chromatographic system and (ii) ensure the quantitative derivatization of the target compounds. On the other hand, the effect of three different solvents (i.e., EtOAc, *n*-Hex, isooctane) in the derivatization step was tested in triplicate. Figure 6.2 clearly shows a significant increase in the chromatographic responses of the four target compounds when using EtOAc. Despite isooctane has been usually used in literature [16,17], more polar solvents seem to favor the trimethylsilylation reaction of the fatty acids. Hence, optimum derivatization conditions were fixed as 20 µL of BSTFA + 1% TMCS using 150 µL of EtOAc as derivatization solvent, at 60°C for 30 min.

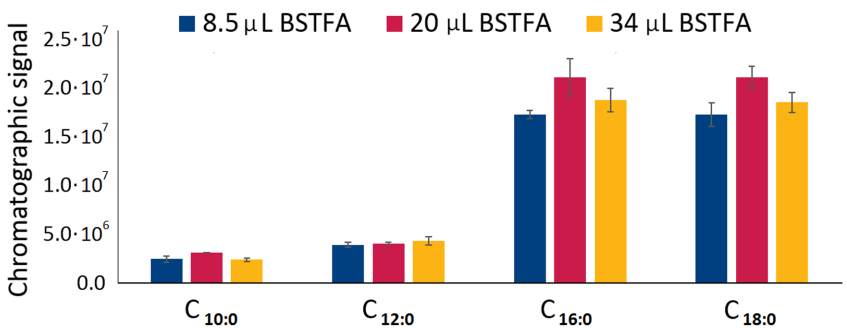


Figure 6.1: *Chromatographic response of some target compounds after their derivatization with different volumes of BSTFA + 1% TMCS using isooctane as derivatization solvent (n=3, 95% confidence level).*

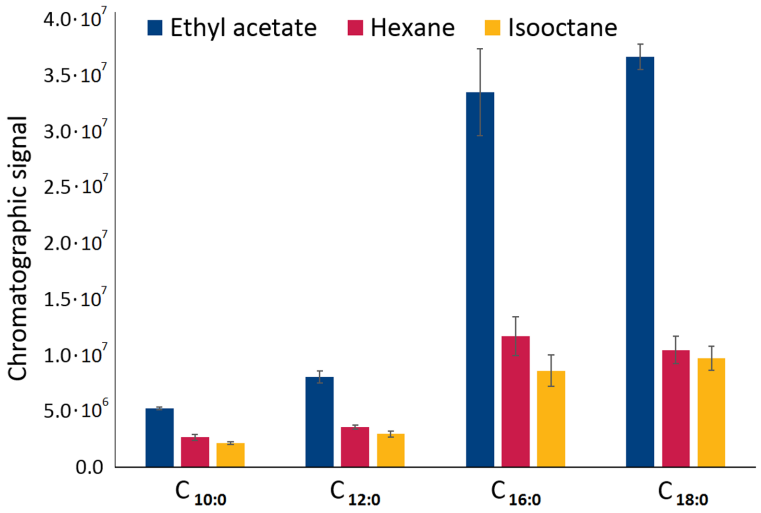


Figure 6.2: *Chromatographic response of some target compounds after their derivatization with 150 µL of different organic solvents and 20 µL of BSTFA + 1% TMCS (n=3, 95% confidence level).*

6.2.2. Optimization of the hydrolysis step

Critical factors that can affect the quantitative hydrolysis of FAMEs, such as the concentration of KOH, the hydro-alcoholic mixture ratio and the volume, were studied. In all these assays, once the FAMEs were hydrolyzed, the neutral fraction was recovered with *n*-Hex (3 x 1 mL) whereas the acidic fraction was recovered after the acidification of the aqueous media and extraction with diethyl ether. The extracts containing acid fatty acids were derivatized and analyzed by GC-MS.

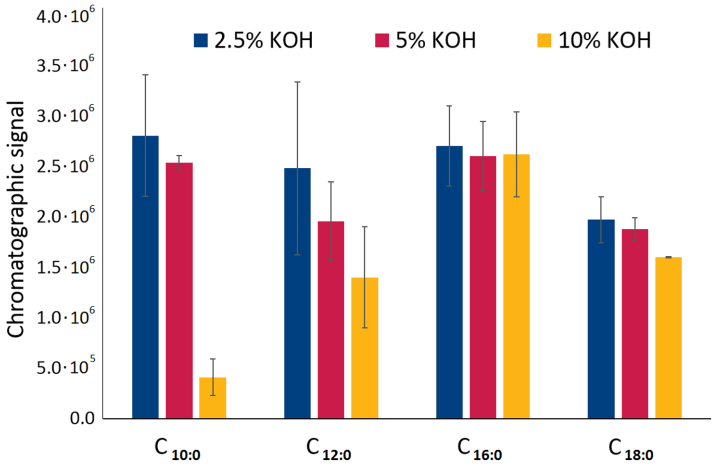


***Figure 6.3**: Comparison of the different concentrations of KOH in a mixture of 3 mL of MeOH:H_2O 2:3 (v/v) (n=3, 95% confidence level).*

Firstly, the influence of KOH % (2.5%, 5% and 10%) in the hydrolysis step was evaluated in triplicate. The different concentrations of KOH were prepared in pure MeOH and Milli-Q, and mixtures of 3 mL of alkaline MeOH:Milli-Q (2:3, *v/v*) were used to perform the hydrolysis in a water bath at 60 ^{o}C for 3 hours. Figure 6.3 shows the chromatographic signal obtained for four of the studied fatty acids at different hydrolysis conditions. The results are statistically comparable at 95% of confidence level except in the case of decanoic and octadecanoic trimethylsilyl esters when using 10% of KOH, for which a decrease in the

chromatographic signal was observed. In addition, the repeatability in terms of relative standard deviation (% RSD) was also higher than 20% when 10% KOH was used, which was attributed to the appearance of KCl salts during the extraction procedure after the acidification with HCl. The presence of this salt makes difficult the derivatization step since it is highly hygroscopic and the presence of water reverts the equilibrium of the derivatization reaction. Since the repeatability of the results was neither highly improved using the minimum amount of KOH (i.e., 2.5%), 5% of KOH was selected as optimum in order to perform an efficient and reproducible hydrolysis. This concentration value is consistent with some other procedures found in the literature [11].

In the next step different ratios of the 3 mL of hydroalcoholic mixture MeOH:H_2O were evaluated, (i.e., 0:5, 1:4, 2:3, 3:2, 4:1 and 5:0 *v/v*). During the assays, it was observed that in those replicates with high organic content (i.e., 5:0 and 4:1 *v/v*), white KCl salts appeared when adding HCl to acidify the medium, which impeded a quantitative extraction and subsequent derivatization. Thus, these ratios were directly discarded and the conclusions were taken from the chromatographic responses obtained for the rest tested ratios (see Figure 6.4). No statistical differences were observed at 95% of confidence level between the ratios that rendered higher chromatographic responses (i.e., 0:4 and 2:3 MeOH:H_2O ratios), thus, 2:3 MeOH:H_2O ratio was selected as optimum for the rest of experiments since high chromatographic responses and repeatabilities were obtained for all the target compounds, and, according to literature, alkaline saponification in hydroalcoholic KOH is the most widespread approach [15].

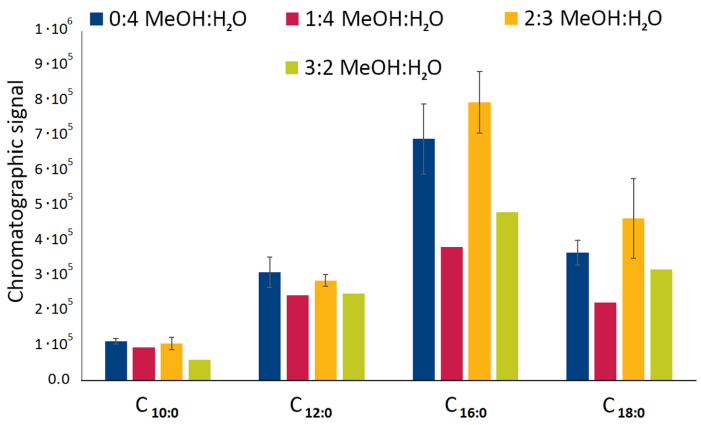


Figure 6.4: *Comparison of the ratios of MeOH: H_2O with 5 % of KOH (n=3, 95 % confidence level).*

6.2.3. Optimization of ultrasound micro bath hydrolysis

In order to accelerate the hydrolysis step, the use of ultrasound micro bath was studied and optimized. Thus, several process variables affecting the hydrolysis yield were optimized, such as ultrasound amplitude, duty cycle and hydrolysis time. In pulsed sonication the extraction time is divided in different cycles being each of these cycles the sum of the period of time that pulse is on (duty cycle) and off. In this work, cycles of 1 second were used. The influence of the first two parameters was simultaneously evaluated by means of an experimental design approach, concretely a central composite design (CCD). Thus, 20 assays (including four replicates of the central point and two replicates of the rest of the points) were performed using aliquots of 42.6 µL of containing ~0.85 µg of FAMEs in a hydroalcoholic KOH mixture and varying the amplitude of the delivered ultrasound from 10 to 30% and the duty cycle from 0.2 to 0.8 $s \cdot s^{-1}$. The studied ranges were chosen based on manufacturer's recommendations and previous knowledge [18]. The responses obtained for the CCD were analyzed by means of multiple linear regressions. ANOVA results indicated that none of the studied variables had significant effect at 95% of confidence level (p-value

> 0.05) for any of the target analytes. Consequently, the amplitude and duty cycle were fixed at intermediate values of 20% and 0.5 $s \cdot s^{-1}$.

Finally, a time profile was performed from 1 to 10 minutes in order to study the optimum hydrolysis time. Figure 6.5 shows the chromatographic signal obtained for each target compound after the application of ultrasound micro bath energy at 5 different times (i.e., 1, 2.5, 5 and 10 min) performed in triplicate. Since no statistical differences were observed among the whole studied time range, the optimum hydrolysis time was fixed at 2.5 min. This time was selected as optimum to avoid the heating of the system, which would take place with longer hydrolysis times involving the possible loss of volatile compounds or the reduction of the cavitation phenomena [19].

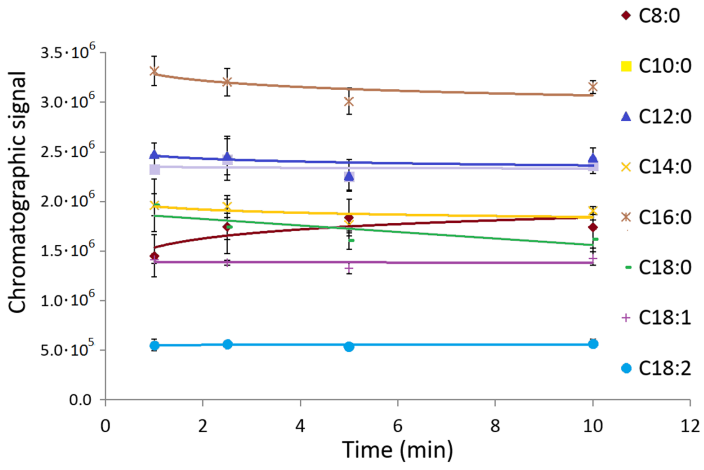


Figure 6.5: *Hydrolysis time profiles for target compounds under optimized conditions for ultrasound micro bath (amplitude at 20%, duty cycle of 0.5 $s \cdot s^{-1}$) (n=3, 95% confidence level).*

As a result, optimum extraction conditions were set as follows: amplitude at 20%, duty cycle of 0.5 s and extraction time of 2.5 minutes. This extraction time is significantly lower in comparison to other traditional hydrolysis procedures that require longer hydrolysis times (i.e., up to three hours [11,20]), and thus, the method throughput is totally assured.

6.2.4. Optimization of the extraction step

Once the conditions for the hydrolysis were fixed, the extraction of neutral and acid fraction of fatty acids from the hydroalcoholic mixture was optimized. To this aim, both extraction volume and organic solvent nature were studied in parallel. Three different volumes of organic solvent were tested: 500, 1000 and 1500 µL. The use of 500 µL was discarded due to the difficulties to separate and recover the organic phase quantitatively. Statistically comparable results were obtained with 1000 and 1500 µL of organic solvent. Hence, 1000 µL of organic solvent was used in subsequent assays.

Regarding the organic solvent, the potential of *n*-hexane, diethyl ether and dichloromethane to extract both neutral and acid fractions was tested. Dichloromethane was rapidly discarded since the organic phase remained under the aqueous one, thus, when collected water was swept along impeding the subsequent derivatization. On the other hand, during the evaporation of the acid extracts after using diethyl ether as extraction solvent, KCl salts appeared in two of the replicates impeding the consecutive derivatization. The appearance of these salts is related to the partial solubility of ethyl acetate in water (6.9 g/100 mL). In the case of *n*-hexane, no salts were observed neither in the neutral nor in the acidic fraction, and the obtained recoveries were higher than those achieved with ethyl acetate. According to literature, when using diethyl ether as extractant, just a small aliquot (100-200 µL) is evaporated to dryness and subjected to derivatization step [1]. In this last case diethyl ether works as a suitable extraction solvent since no salts appear. However, in the present work we want to determine organic biomarkers in archaeological samples without not visible residues, thus, recovering the organic fraction completely is necessary. As a result, *n*-hexane was chosen as the best extraction solvent both for the neutral and acidic fraction.

Finally, the number of consecutive extractions required for a quantitative extraction was evaluated. Three consecutive extractions were performed in triplicate under optimum

conditions adding 1 mL of fresh *n*-hexane. The different extracts were evaporated to dryness and derivatized with 20 µL of BSTFA + 1% TMCS and 150 µL of EtOAc. Above 85% of the extracted amount was recovered with one extraction for small fatty acids (from $C_{8:0}$ to $C_{16:0}$). However, in the case of the three longer fatty acids ($C_{18:0}$, $C_{18:1}$ and $C_{18:2}$) more than 20% was still recovered in the third extraction. Thus, it was decided to establish three consecutive extractions as optimum in order to ensure a quantitative extraction for all the target compounds.

6.2.5. UMB-GC-MS method validation

The figures of merit for the developed method were quantitatively calculated for 8 fatty acids (from their corresponding methyl esters) and are summarized in Table 6.2. Linearity of the external calibration curves were found to be good over the range of tested concentrations (2.5-10 $\mu g \cdot mL^{-1}$) with coefficients of determination (r^2) higher than 0.998 for all the trymethylsilylated fatty acids.

Procedural limits of detection (LOD_{proc}) were experimentally calculated from three blank samples as the average signal of blank (n=3) plus three times the standard deviation. The obtained values were in the range 0.05-0.38 $\mu g \cdot g^{-1}$ giving octanoic and hexadecanoic acid trimethylsilyl esters particular high values (0.26 and 0.38 $\mu g \cdot g^{-1}$, respectively). The main problem of blanks regarding to these compounds were attributed to the use of plastic material, which was avoided afterwards. However, as these concentrations were found to be negligible in comparison to those found in real samples, we decided to correct the concentration with the procedural blank signals.

The repeatability of the method was calculated as the relative standard deviation (RSD %). Four replicates of 5% KOH in hydroalcoholic mixture were spiked with ~0.85 µg of FAMEs and analyzed in the same day. As can be seen in Table 6.2, RSD % values were between 4% and 12% for all the target compounds. In order to establish the accuracy of the

developed method two approaches were used. On the one hand, due to the lack of a certified reference material for fatty acids in ceramic samples, ceramic models were spiked with the FAME solution and followed the whole optimized procedure. On the other hand, the methodology using UMB for the hydrolysis was compared with the classical approach based on heating the samples for 3 hours.

Table 6.2: *Main method parameters (linearity, limits of detection, repeatability and accuracy) for the developed procedure.*

Fatty acid	Linearity		Repeatability	Accuracy	
	r^2	LOD_{proc} ($\mu g \cdot g^{-1}$)	% RSD (n=3)	Recovery (%) with UMB[a]	Recovery (%) with water bath[b]
$C_{8:0}$	0.999	0.26	7	35	12
$C_{10:0}$	0.999	0.13	4	85	78
$C_{12:0}$	0.999	0.15	5	80	78
$C_{14:0}$	0.999	0.16	6	81	75
$C_{16:0}$	0.999	0.38	12	75	68
$C_{18:0}$	0.998	0.16	8	95	83
$C_{18:1}$	0.999	0.11	9	131	98
$C_{18:2}$	0.998	0.05	6	95	78

[a] Recovery estimated from the spiked synthetic ceramics using ultrasound micro bath for hydrolysis.

[b] Recovery estimated from the spiked synthetic ceramics using water bath (60^{o} C, 3 h) for hydrolysis.

For the first approach 50 mg of ceramic model was spiked with ~0.85 µg and subjected to the optimized hydrolysis (20% of amplitude, 0.5 $s \cdot s^{-1}$ of duty cycle and 2.5 min of hydrolysis time), extraction (1 mL of *n*-hexane) and derivatization (20 µL of BSTFA + 1% TMCS in 150 µL of EtOAc for 30 min). In general, good recoveries were obtained (75%-131%) after subtracting the blank signals to each target compound (see Table 6.2). However, lower values were obtained for the octanoic acid TMSE (32%), probably due to its higher volatility.

Subsequently, the optimized procedure's effectiveness was compared to the traditional approach used to determine organic compounds in archaeological ceramic samples. The classical hydrolysis is generally performed in a water bath at 60 ^{o}C for 3 hours

[8,20-23]. Thus, replicates spiked with ~0.85 µg of FAMEs were subjected to the traditional hydrolysis as well as to UMB hydrolysis under optimum conditions. The obtained results in terms of recoveries with both hydrolysis techniques were statistically comparable (t-test, p-values between 0.20 and 0.77) for all the compounds except in the case of octadecanoic and decanoic acid trimethylsilyl esters, which gave lower recoveries when performing the traditional hydrolysis (see Table 6.2). Additionally, higher % RSD values were achieved when using the water bath (1-30% RSD) in comparison with UMB hydrolysis (1-12% RSD). Taking into account these results together with the fact that ultrasound micro bath hydrolysis involves much less time (2.5 min vs. 3 hours for traditional hydrolysis), it can be concluded that hydrolysis by means of UMB is a suitable approach for the saponification of fatty acids and biomarkers of similar nature in archaeological ceramic samples.

6.2.6. Application to archaeological samples

The developed method using hydrolysis assisted by ultrasound micro bath was applied to real archaeological ceramic samples in order to detect fatty acids. Five ceramic pieces corresponding to different giant containers, in which whale oil is suspected to have been stored, were analyzed. For this purpose 0.5 g of samples were subjected to analysis (n=3).

The eight studied fatty acids were detected and quantified (see Table 6.3 and Figure 6.6) including the unsaturated ones (oleic and linoleic acids) in some samples. Among these results, it should be highlighted that, together with hexadecanoic acid (palmitic acid or $C_{16:0}$), tetradecanoic acid (myristic acid or $C_{14:0}$) showed the highest concentration values suggesting that these ceramics were used to store whale oil. Although this hypothesis cannot be consistent only with the presence of these compounds, it is widely known that these two compounds are the most characteristic ones among saturated fatty acids, both in fresh marine mammal tissues [24] and archaeological pottery related to marine commodities [25]. Additionally, the main components of the spermaceti (a waxy substance found in the head cavities of the sperm whale and, in smaller quantities, in the oils of other

whales) are cetyl palmitate ($C_{16:0}$) together with esters of myristic ($C_{14:0}$), lauric ($C_{12:0}$) and stearic ($C_{18:0}$) acids [26].

Table 6.3: *Concentrations of the studied fatty acids in $\mu g \cdot g^{-1}$ quantified in real samples from a deposit in Lekeitio (n=3, 95% confidence level).*

Fatty acids	L002	L003	L006	L016	L020
$C_{8:0}$	<LOD	1.2 ± 0.1	1.5 ± 0.1	0.94 ± 0.07	1.07 ± 0.08
$C_{10:0}$	1.4 ± 0.1	1.9 ± 0.2	2.1 ± 0.2	1.01 ± 0.04	1.9 ± 0.2
$C_{12:0}$	1.7 ± 0.2	2.0 ± 0.1	1.7 ± 0.2	0.58 ± 0.08	1.1 ± 0.1
$C_{14:0}$	6 ± 1	14 ± 2	7 ± 1	6 ± 1	3.3 ± 0.6
$C_{16:0}$	8 ± 2	20 ± 4	11 ± 2	9 ± 2	4 ± 1
$C_{18:0}$	0.53 ± 0.06	0.7 ± 0.1	0.8 ± 0.1	0.33 ± 0.06	0.35 ± 0.02
$C_{18:1}$	1.5 ± 0.2	1.8 ± 0.4	0.78 ± 0.08	0.57 ± 0.05	<LOD
$C_{18:2}$	0.42 ± 0.03	0.27 ± 0.01	0.19 ± 0.01	<LOD	<LOD

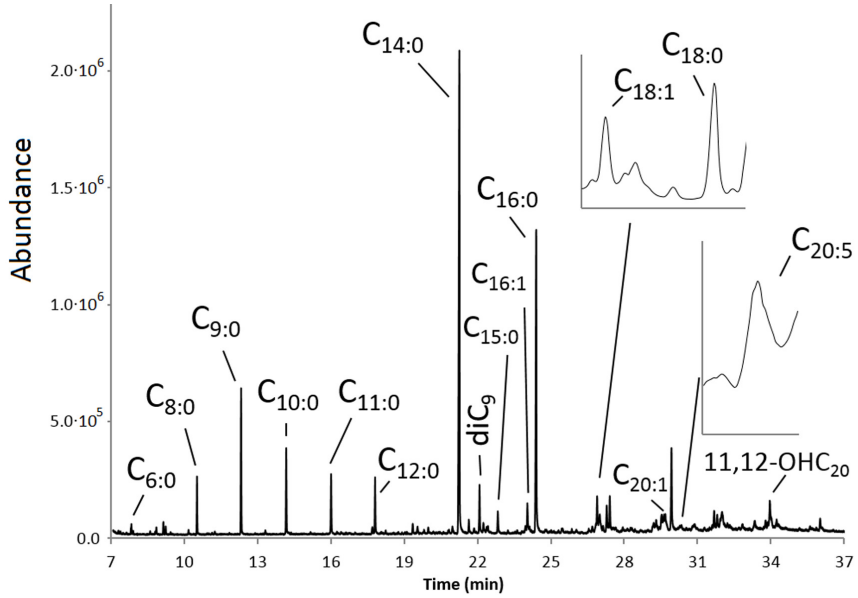


Figure 6.6: *GC–MS chromatogram obtained in SCAN mode for L016 sample showing the obtained signals for the quantified target compounds and the identified biomarkers. Cx:y represents fatty acids of carbon chain length x and level of unsaturation y; diC_9: sebacic acid; $11,12\text{-}OHC_{20}$:11,12- dihydroxyeicosanoic acid.*

Moreover, other biomarkers related to marine commodities were identified in the analyzed sample extracts (see the chromatogram in Figure 6.6). The identified compounds are listed in Table 6.4. They are mainly saturated fatty acids together with two important molecular markers of whale oil: eicosapentaenoic acid and docosahexaenoic acid [27]. Eicosapentaenoic acid (EPA, $C_{20:5\omega3}$) and docosahexaenoic acid (DHA, $C_{22:6\omega3}$) are the major polyunsaturated fatty acids in blubber and fish oil, giving evidence of their marine origin [27,28]. These two compounds were detected in trace amounts but they were identified with their characteristic *m/z* values and by comparison with mass spectral libraries (NIST 2.0) in four of the five samples (L002, L003, L016 and L020). In addition, 11-eicosenoic acid ($C_{20:1}$) was also found in three samples (L002, L003, L006). This compound is also indicative of marine organisms and has been previously found together with oleic and linoleic acids in a midden deposit used to store seal and whale oil as well as in slab-lined pits suggested as production sites for marine mammal oil [28,29].

Table 6.4: *Biomarkers found in 5 real samples from Lekeitio with their respective m/z values used for their identification (n=3, 95 % confidence level).*

		Real Samples				
Saturated fatty acids	***m/z* values**	**L002**	**L003**	**L006**	**L016**	**L020**
$C_{6:0}$	73, 117, 173				•	•
$C_{7:0}$	73, 117, 187			•	•	•
$C_{9:0}$	73, 117, 215	•	•	•	•	•
$C_{11:0}$	73, 117, 243	•	•	•	•	•
$C_{15:0}$	73, 117, 299	•	•	•	•	•
$C_{17:0}$	73, 117, 327		•	•		
Unsaturated fatty acids	***m/z* values**	**L002**	**L003**	**L006**	**L016**	**L020**
$C_{16:1}$	73, 117, 311	•	•	•	•	
$C_{20:1}$	73, 117, 327	•	•	•		
$C_{20:5}$	73, 79, 91, 117, 131, 145, 385				•	•
$C_{22:6}$	73, 79, 91, 93, 117, 119, 129, 359	•	•			

Several research works analyzing blubber or marine oils describe all the compounds previously mentioned together with other saturated, unsaturated and polyunsaturated fatty acids. In fact, most of the compounds found in those studies matches with the biomarkers identified in this work: $C_{14:0}$, $C_{16:0}$, $C_{18:1}$, $C_{20:1}$, $C_{20:5}$ and $C_{22:6}$ [30-34]. Normally, degradation products such as dihydroxy derivatives of monounsaturated fatty acids, ω-(o-alkylphenyl) alkanoic acids or isoprenoid compounds are found in archaeological samples suspected to have stored marine oil, whereas unsaturated fatty acids are absent [5]. In the present work, as can be seen in Figure 6.6, only sebacic acid (decanedioic acid) and 11,12-dihydroxyeicosanoic acid were found as degradation compounds of 11-eicosenoic acid. Nevertheless, it was checked that the extraction of the acid fraction with *n*-hexane is not quantitative for the determination of more polar compounds such as dicarboxylic acids, and consequently not suitable for the determination of dihydroxy derivatives of unsaturated fatty acids. However, we decided to use *n*-hexane instead of diethyl ether in order to avoid the salt and water problems observed using diethyl ether. In any case, other biomarkers, such as highly unsaturated compounds or even ω-(o-Alkylphenyl) alkanoic acids and isoprenoid compounds, which are more related to marine commodities, have been identified when using *n*-hexane as extraction solvent [11].

The possibility of a laboratory contamination can be ruled out since the performed blanks gave no analytical response for the mentioned biomarkers. Additionally, the hypothesis that the analyzed ceramics could have native fatty acids has also been refused since no biomarkers were found after the analysis of the external surface of the ceramics and the target compounds were below the limits of detection.

It is not usual to find unsaturated fatty acids in archaeological samples since they tend to degrade during oxidation processes faster than the saturated ones. However, according to literature, longer- (> C18) and shorter-chained (< C14) fatty acids oxidize more quickly than medium-chained compounds (C14-C18). Moreover, the oxidation rate depends significantly on the number of unsaturations increasing over ten times for each double bond

present [35]. In 1992, DeMan found that the rate of oxidation of oleic acid compared to the linoleic acid at 100 ^{o}C was 1:100:1200. By means of a simulation of decomposition of four fatty acids ($C_{18:0}$, $C_{18:1}$, $C_{18:2}$ and $C_{18:3}$) DeMan determined that after 100 decomposition steps the $C_{18:1}/C_{18}$ ratio remained almost constant, giving evidence to support the idea of the possible preservation of $C_{18:1}$ and $C_{20:1}$ [36]. Nevertheless, care must be taken when trying to establish the specific origins based on preserved fatty acid distributions alone.

6.3. Conclusions

The challenge of understanding the composition of the organic remains in archaeological ceramic samples has been taken by many researchers in the last decade. However, no special attention has been paid to the development, optimization and validation of a suitable analytical procedure. In addition, the methods used normally in this field are time consuming and have to be performed in more than one day, running the risk of losing important information. In this sense, in this work we propose a fast, eco-friendly and sensitive analytical method which allowed the detection of the target compounds and many important fatty acid biomarkers with high precision (RSD < 12 %) and accuracy (75-100 %) as well as acceptable limits of detection.

The reduction in the analysis time was mainly attributed to the use of ultrasound micro bath energy, which allowed obtaining in 2.5 minutes similar results to those achieved with traditional hydrolysis techniques that normally require at least 3 hours of heating. Hence, the developed method has provided a new approach to perform fast screening of archaeological ceramic samples in order to identify and quantify (if desired) organic biomarkers. The detection of such organic biomarkers offers a powerful tool for understanding the past use of this type of archaeological materials. In this work, we have found some evidences that suggest the use of the analyzed ceramics to store whale oil.

However, care must be taken with final conclusions about the origin of a certain archaeological sample. Marine fats and oils have remained difficult to detect in the organic residues in archaeological pottery and unsaturated compounds rarely survive as significant components due to their susceptibility to oxidation during vessel use and burial [5]. Decomposition and other confounding problems relegate these analyses more to the role of producing hypotheses or supporting information about the function of artifacts [35].

In this respect, it would be worthwhile to support fatty acid analysis with other hydrolysis and extraction techniques. Another approach, if available, is the use of a range of supplementary analytical and derivatization techniques, such as stable isotope analysis. Nevertheless, the use of these multi-analytical approaches is difficult and sometimes impossible due to the unavailability of such analytical tools.

6.4. References

[1] F. Saliu, I. Degano, M.P. Colombini, Identification of triacylglycerols in archaelogical organic residues by core-shell reversed phase liquid chromatography coupled to electrospray ionization-quadrupole-time of flight mass spectrometry, J. Chromatogr. A. 1346 (2014) 78-87.

[2] R.P. Evershed, S.N. Dudd, S. Charters, H. Mottram, A.W. Stott, A. Raven, P.F. Van Bergen, H.A. Bland, Lipids as carriers of anthropogenic signals from prehistory, Philos. Trans. R. Soc. B Biol. Sci. 354 (1999) 19-31.

[3] R.P. Evershed, C. Heron, S. Charters, L.J. Goad, The Survival of Food Residues: New Methods of Analysis, Interpretation and Application, Proc. Br. Acad. 77 (1991) 187-208.

[4] M.S. Copley, H.A. Bland, P. Rose, M. Horton, R.P. Evershed, Gas chromatographic, mass spectrometric and stable carbon isotopic investigations of organic residues of plant oils and animal fats employed as illuminants in archaeological lamps from Egypt, Analyst. 130 (2005) 860-871.

[5] R.P. Evershed, M.S. Copley, L. Dickson, F.A. Hansel, Experimental evidence for the processing of marine animal products and other commodities containing polyunsaturated fatty acids in pottery vessels, Archaeometry. 50 (2008) 101-113.

[6] R.P. Evershed, A.W. Stott, A. Raven, S.N. Dudd, S. Charters, A. Leyden, Formation of long-chain ketones in ancient pottery vessels by pyrolysis of acyl lipids, Tetrahedron Lett. 36 (1995) 8875-8878.

[7] F.A. Hansel, R.P. Evershed, Formation of dihydroxy acids from Z-monounsaturated alkenoic acids and their use as biomarkers for the processing of marine commodities in archaeological pottery vessels, Tetrahedron Lett. 50 (2009) 5562-5564.

[8] M.P. Colombini, G. Giachi, F. Modugno, E. Ribechini, Characterisation of organic residues in pottery vessels of the Roman age from Antinoe (Egypt), Microchem. J. 79 (2005) 83-90.

[9] R.P. Evershed, Experimental approaches to the interpretation of absorbed organic residues in archaeological ceramics, World Archaeol. 40 (2008) 26-47.

[10] A. Vallejo, A. Usobiaga, I. Martinez-Arkarazo, A. Prieto, N. Etxebarria, O. Zuloaga, L.A. Fernández, Ultrasonic-assisted derivatization of estrogenic compounds in a cup horn booster and determination by GC-MS, J. Sep. Sci. 33 (2010) 104-111.

[11] F.A. Hansel, M.S. Copley, L.A.S. Madureira, R.P. Evershed, Thermally produced ω-(o-alkylphenyl)alkanoic acids provide evidence for the processing of marine products in archaeological pottery vessels, Tetrahedron Lett. 45 (2004) 2999-3002.

[12] M. Olivares, A. Vallejo, M. Irazola, X. Murelaga, J.I. Baceta, A. Tarriño, N. Etxebarria, Optimisation of focused ultrasound extraction (FUSE) and microwave-assisted extraction (MAE) of hydrocarbons in geological chert samples, Talanta. 83 (2010) 605-612.

[13] S.F.-. de Vallejuelo, A. Barrena, G. Arana, A. de Diego, J.M. Madariaga, Ultrasound energy focused in a glass probe: An approach to the simultaneous and fast extraction of trace elements from sediments, Talanta. 80 (2009) 434-439.

[14] J.G. Iñañez, G. Arana-Momoitio, K. Castro, E. Calparsoro-Forcada, S. Escribano, Ceramic containers and Iberian trade: provenance of 16th - 18th century tinajas from Lekeitio (Biscay), 13th European Meeting on Ancient Ceramics, Athens 24-26 September 2015.

[15] M.P. Colombini, F. Modugno, Organic Mass Spectrometry in Art and Archaeology, John Wiley & Sons, Ltd, Pisa, 2009.

[16] E. Ribechini, S. Orsini, F. Silvano, M.P. Colombini, Py-GC/MS, GC/MS and FTIR investigations on LATE Roman-Egyptian adhesives from opus sectile: New insights into ancient recipes and technologies, Anal. Chim. Acta. 638 (2009) 79-87.

[17] M.P. Colombini, F. Modugno, E. Ribechini, Organic mass spectrometry in archaeology: Evidence for Brassicaceae seed oil in Egyptian ceramic lamps, J. Mass Spectrom. 40 (2005) 890-898.

[18] A. Errekatxo, A. Prieto, O. Zuloaga, A. Usobiaga, N. Etxebarria, L.A. Fernández, Simultaneous extraction of several persistent organic pollutants in sediment using focused ultrasonic solid-liquid extraction, Anal. Bioanal. Chem. 392 (2008) 1471-1478.

[19] J.L. Capelo, M.M. Galesio, G.M. Felisberto, C. Vaz, J.C. Pessoa, Micro-focused ultrasonic solid-liquid extraction (μFUSLE) combined with HPLC and fluorescence detection for PAHs determination in sediments: Optimization and linking with the analytical minimalism concept, Talanta. 66 (2005) 1272-1280.

[20] E. Ribechini, F. Modugno, M.P. Colombini, R.P. Evershed, Gas chromatographic and mass spectrometric investigations of organic residues from Roman glass unguentaria, J. Chromatogr. A. 1183 (2008) 158-169.

[21] M.P. Colombini, F. Modugno, M.C. Gamberini, M. Rocchi, C. Baraldi, T. Deviese, R.J. Stacey, M. Orlandi, F. Saliu, C. Riedo, O. Chiantore, G. Sciutto, E. Catelli, L. Brambilla, L. Toniolo, C. Miliani, P. Rocchi, J. Bleton, U. Baumer, P. Dietemann, G. Pojana, S. Marras, A round robin exercise in archaeometry: Analysis of a blind sample reproducing a seventeenth century pharmaceutical ointment, Anal. Bioanal. Chem. 401 (2011) 1847-1860.

[22] F. Modugno, E. Ribechini, M.P. Colombini, Aromatic resin characterisation by gas chromatography-mass spectrometry. Raw and archaeological materials, J. Chromatogr. A. 1134 (2006) 298-304.

[23] J. Pérez-Arantegui, E. Ribechini, C. Pardos, M.P. Colombini, Chemical investigation on black pigments in the carved decoration of sixteenth century alabaster tombs from Zaragoza (Spain), Anal. Bioanal. Chem. 395 (2009) 2191-2197.

[24] R. Guitart, A.M. Silvestre, X. Guerrero, R. Mateo, Comparative study on the fatty acid composition of two marine vertebrates: Striped dolphins and loggerhead turtles, Comp. Biochem. Physiol. B Biochem. Mol. Biol. 124 (1999) 439-443.

[25] O.E. Craig, H. Saul, A. Lucquin, Y. Nishida, K. Taché, L. Clarke, A. Thompson, D.T. Altoft, J. Uchiyama, M. Ajimoto, K. Gibbs, S. Isaksson, C.P. Heron, P. Jordan, Earliest evidence for the use of pottery, Nature. 496 (2013) 351-354.

[26] M. Wellendorf, Composition of Spermaceti, Nature. 198 (1963) 1086-1087.

[27] C. Solazzo, D. Erhardt, Sea Mammal Oil and Cooking Practices in the Arctic, in: H. Barnard, J.W. Eerkens (Eds.), Theory and Practice of Archaeological Residues Analysis, Archaeopress, Oxford, 2007.

[28] E.D. Morgan, The Composition of Fatty Materials from a Thule Eskimo Site on Herschel Island, Arctic. 36 (1983) 356-360.

[29] C. Heron, G. Nilsen, B. Stern, O. Craig, C. Nordby, Application of lipid biomarker analysis to evaluate the function of 'slab-lined pits' in Arctic Norway, J. Archaeol. Sci. 37 (2010) 2188-2197.

[30] P. Araujo, Y. Zeng, Z.Y. Du, T.T. Nguyen, L. Frøyland, B. Grung, Discrimination of n-3 rich oils by gas chromatography, Lipids. 45 (2010) 1147-1158.

[31] D.H. Buisson, D.R. Body, G.J. Dougherty, L. Eyres, P. Vlieg, Oil from deep water fish species as a substitute for sperm whale and jojoba oils, JAOCS J Am Oil Chem Soc. 59 (1982) 390-395.

[32] C.H. Milligan, C.A. Knuth, A.S. Richardson, The composition of whale oil, J. Am. Chem. Soc. 46 (1924) 157-166.

[33] H. Kimoto, Y. Endo, K. Fujimoto, Influence of interesterification on the oxidative stability of marine oil triacylglycerols, J. Am. Oil Chem. Soc. 71 (1994) 469-473.

[34] D. Ruchonnet, M. Boutoute, C. Guinet, P. Mayzaud, Fatty acid composition of Mediterranean fin whale Balaenoptera physalus blubber with respect to body heterogeneity and trophic interaction, Mar. Ecol. Prog. Ser. 311 (2006) 165-174.

[35] J.W. Eerkens, Organic Residue Analysis and the Decomposition of Fatty Acids in Ancient Potsherds, in: H. Barnard, J.W. Eerkens (Eds.), Theory and Practice of Archaeological Residue Analysis, Archaeopress, Oxford, 2007.

[36] J.M. DeMan, Chemical and Physical Properties of Fatty Acids, in: C.K. Chow (Ed.), Fatty Acids in Foods and their Health Implications, Marcel Dekker Inc., New York, 1992.

Chapter 7

GC-MS and HPLC-ESI-QToF characterization of organic lipid residues from ceramic vessels used by Basque whalers from 16th to 17th centuries

Microchemical Journal 137 (2018) 190–203

"Consider the subtleness of the sea; how its most dreaded creatures glide under water, unapparent for the most part, and treacherously hidden beneath the loveliest tints of azure. Consider all this; and then turn to this green, gentle , and most docile earth; consider them both, the sea and the land; and do you not find a strange analogy to something in yourself?."

Herman Melville, Moby-dick

As it has been shown in the results of the previous chapter, the unequivocal characterization of organic compounds in archaeological ceramics provides important information about their use and the history around them. Chapter 6 highlights that among thousands of organic compounds, lipid profiling is one of the main subjects of archaeological studies aiming to infer information about the use and environmental source of pottery sherds, human remains, ancient sediments, etc. [1-6]. In fact, it is known that lipids, and specially their diagenetic and catagenetic hydrocarbon products, can survive unaltered in recent and ancient archaeological remains due to their inherent resistance to decomposition in comparison to other compounds such as proteins [1]. Nevertheless, the natural degradation processes of lipids can be accelerated in oxidant environments and at high temperatures, which often occurs in the context of archaeological pottery vessels preservation [7].

Marine commodities have played a very important role in the past 16th-17th centuries in the Basque Country (northern Spain). Fishing or hunting whales was undoubtedly one of the most productive activities in the Bay of Biscay during more than half a millennium [8]. Basque whalers used to sail across the Atlantic Ocean as far as Newfoundland and Labrador, where there is archaeological evidence of more than ten Basque whaling stations located along the Gulf of Saint Lawrence (see Figure 7.1). The Red Bay whale station, which UNESCO named World Heritage Site in 2013, is the most outstanding of all the whale stations where excavations have taken place. Once the whale was captured its fat was melted in the ovens located at the stations to collect the blubber or oil, which was then stored in wooden barrels and transported back to the Basque Country in order to be sold in Spain or in different places in Europe, such as Flanders or England [8-10]. Before the fats were sold, they were stored in particular large ceramic jars in the docks of Basque ports, ready for their distribution. Recent archaeological excavations in the old quarter of the Biscayan village of Lekeitio have recorded a 16th-17th century old warehouse linked to the trade and storage of whale oil. In this archaeological site, 15 big ceramic jars laid beneath a clay floor at the same level as their mouths, serving as big deposits for liquid storage. These *tinajas* or big jars measured

between 1.30 and 1.80 m in height, and 1.10 and 1.45 m in diameter with an estimated capacity of around 1000 L. Chronologically, these big ceramic vessels made in Seville are attributed to the Modern Era, and according to the archaeological record and written sources, correspond to ceramics likely from the 2nd half of the 16th century and 1st half of the 17th century, matching the highest peak of Basque whaling activity [11,12].

Figure 7.1: *Excavation works performed in the Basque whaling station of Chateau Bay (Labrador) where the battery of ovens to melt blubber is visible. Source: Azkarate (1992) [8].*

Marine fats and oils are an important class of unsaturated lipids difficult to detect in the organic residues in archaeological pottery [13,14]. As described in previous chapters, they contain high abundances of polyunsaturated fatty acids (PUFAs), which rarely survive in the ceramic vessels in significant amounts due to degradation processes affecting their chemical composition. Despite these complications, several degradation products have been identified as biological markers in recent studies focused on marine oils and fats in archaeological ceramic samples. ω-(*o*-Alkylphenyl) alkanoic acids with 16-20 carbon atoms

are known to be formed at cooking temperatures from fatty acids (FAs) of marine sources [14-16]. Specific dihydroxy acids can also be found in extracts of archaeological pottery and are the direct degradation products of Z-monounsaturated alkenoic acids such as $C_{20:1}$ and $C_{22:1}$ fatty acids, which are suggestive of seal or whale fats [10,17]. PUFAs can also be oxidized forming dicarboxylic acids, which have been found in slab-lined pits where marine mammal oil was produced [4,18]. In addition, the isoprenoid FAs 4,8,12-trimethyltridecanoic acid (4,8,12-TMTD), 2,6,10,14-tetramethylpentadecanoic acid (2,6,10,14-TMPD, or pristanic acid) and 3,7,11,25-tetramethylhexadecanoic acid (3,7,11,15-TMHD, or phytanic acid) are as well important biomarkers of marine products in archaeological ceramics [14,15,17].

In the peer-reviewed literature, analytical methodologies based on gas chromatography-mass spectrometry (GC-MS) [2,6,19-23] and gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) [1,5,24-26] have been applied for lipid analysis of organic residues in ceramics. Nevertheless, the FA content can also be obtained with the acquisition of the entire triacylglycerol (TAG) profile of the lipid material [27]. In previous investigations, high-performance liquid chromatography - atmospheric-pressure chemical ionization - mass spectrometry (HPLC-APCI-MS) has been applied to analyze the content of pottery sherds to identify its TAG composition [28,29]. Nevertheless, high resolution chromatographic techniques such as high-performance liquid chromatography - electrospray ionization - quadrupole time-of-flight mass spectrometry (HPLC-ESI-QToF) has not been widely used for the analysis of the triglyceride profile of the lipid fraction in archaeological ceramic samples, only in archaeological ointments [30], which could provide more accurate information about the structure of the TAGs. The information obtained by HPLC-ESI-QToF, together with the complementary information obtained by more consolidated analytical techniques such as GC-MS would allow identifiying the oils and fats stored in archaeological vessels.

Specific extraction, saponification and derivatization steps are required before the chromatographic analyses. Evershed et al. used solvent extraction of the powdered potsherd with a mixture of chloroform/methanol using ultrasonication [1], Ribechini et al. performed the direct saponification of the organic residues found in ceramic pieces with a subsequent extraction step [20], whereas Correa-Ascencio et al. developed a direct acidified methanol extraction [5]. Different derivatization procedures have also been applied: i) trimethylsilylation [20] and ii) esterification with diazomethane [31] or with iii) boron trifluoride in methanol [15].

The aim of the present work was the analysis of the non-visible residual contents of five different ceramic samples belonging to five different ceramic jars found in a deposit in Lekeitio (Basque Country, northern Spain) suspected to have been used to store oil from whales from Newfoundland and Labrador, captured by Basque whalers in the period from 16th to 17th century. To fulfil this goal, fresh blubber samples, used as reference materials, and archaeological samples were analyzed by means of HPLC-ESI-QToF and GC-MS. The former was used to identify TAGs whereas the latter was mainly used to obtain the FAs profile as well as to detect new possible biomarkers of whale oil in the archaeological samples.

7.1 Materials and methods

7.1.1. Archaeological samples

The archaeological ceramic artefacts under study were sampled from the ceramic set recovered in the 16th-17th century warehouse found in the old quarter of Lekeitio (Basque Country, northern Spain). Just three out of the fifteen big ceramic containers were unearthed without any damage and are currently exhibited at the Archaeological Museum of Biscay. The rest of the ceramics were accidentally broken into several pieces during archaeological works due to their size and poor state of conservation. Thus, several

fragments without visible residues and corresponding to different ceramic vessels (n=5) were analyzed: Lekeitio 002 (L002), Lekeitio 003 (L003), Lekeitio 006 (L006), Lekeitio 016 (L016) and Lekeitio 020 (L020) (see Figure 7.2).

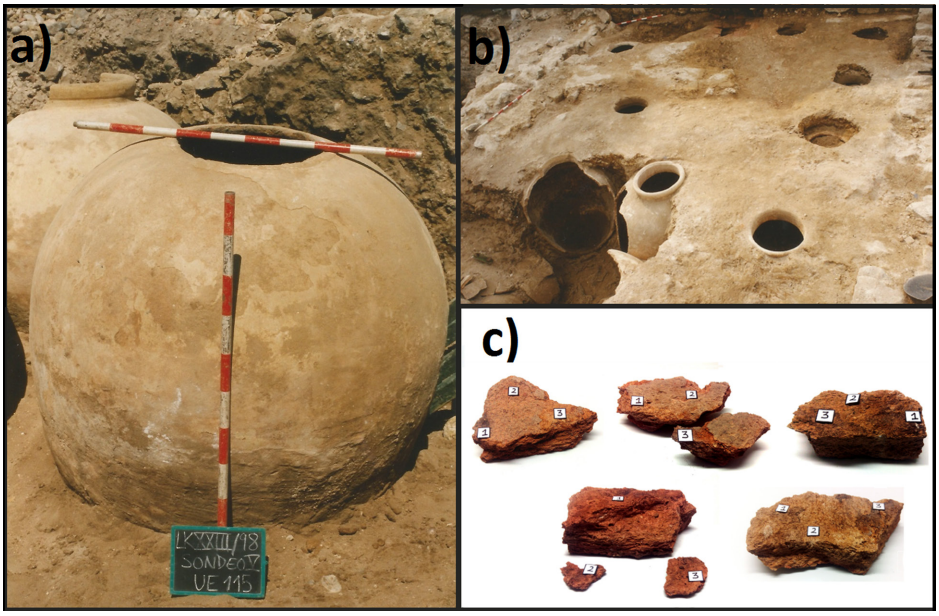


Figure 7.2: *Archaeological ceramics found in the warehouse of Lekeitio: a) entire ceramic container without neck showing the scale (10 cm per colored section), b) arrangement of the ceramic containers beneath the clay floor with the mouth visible and c) archaeological ceramic fragments under study showing the internal surface of the vessel (arranged in order as L002, L003, L006, L016 and L020). Photos a) and b): courtesy of Silvia Cajigas.*

7.1.2. Fresh blubber and whale oil samples

Due to the difficulty to obtain ancient whale oil samples, four fresh whale blubber samples and a whale oil sample were used as reference materials (see Table 7.1).

Table 7.1: *Description of the whale blubbers and oil used as reference materials.*

Sample type	Whale sub-order	Genus	Specie	Source
Whale Blubber	Mysticete	*Balaenoptera*	Fin Whale (*Balaenoptera physalus*)	Swedish Museum of Natural History in Stockholm
			Minke Whale (*Balaenoptera acutorostrata*)	
			Sei Whale (*Balaenoptera borealis*)	
		Megaptera	Humpback Whale (*Megaptera novaeangliae*)	Australia
	Odontoncete	*Phocoena*	Harbour Porpoise (*Phocoena phocoena*)	Swedish Museum of Natural History in Stockholm
Whale Oil	Unknown	Unknown	Unknown	Association for the Study and Conservation of Marine Fauna (AMBAR)

Four fresh whale blubber samples belonging to different whale species were provided by the Swedish Museum of Natural History in Stockholm. Three different blubber samples from three different whale species belonging to the sub-order mysticete and genus *Balaenoptera* were characterized: (i) fin whale (*Balaenoptera physalus*), (ii) minke whale (*Balaenoptera acutorostrata*) and (iii) sei whale (*Balaenoptera borealis*). The fourth blubber sample was extracted from harbour porpoise specie (*Phocoena phocoena*) belonging to the

sub-order odontocete and genus *Phocoena*. The last specie was obtained from whale blubber sampled from a humpback whale (*Megaptera novaeangliae*) in Australia belonging to the sub-order mysticete and genus *Megaptera*.

Besides the whale blubber samples, a sample of liquid whale oil extracted from fat of unknown whale species in Asturias (Northern Spain) supplied by the Association for the Study and Conservation of Marine Fauna (AMBAR) was characterized.

7.1.3. Reagents and materials

Laboratory material was carefully cleaned with abundant pure water (<0.2 $\mu S \cdot cm^{-1}$, Millipore, USA) and without using detergent to avoid possible contamination produced by detergent residues. The material was sonicated under clean acetone (Q.P., Panreac Química, Spain) for an hour and then rinsed with ultrapure water (<0.057 $\mu S \cdot cm^{-1}$, Milli-Q model, Millipore, USA). Finally, the glass material was dried in an oven at 400 °C for 4 h.

The mix solution of mono and dicarboxylic fatty acids (FAs) in acetone and purchased from Sigma-Aldrich (Milan, Italy) contained lauric acid ($C_{8:0}$; 0.24 $mg \cdot g^{-1}$), suberic acid (C8 diacid; 0.27 $mg \cdot g^{-1}$), azelaic acid (C9 diacid; 0.28 $mg \cdot g^{-1}$), myristic acid ($C_{14:0}$; 0.25 $mg \cdot g^{-1}$), sebacic acid (C10 diacid; 0.30 $mg \cdot g^{-1}$), palmitic acid ($C_{16:0}$; 0.25 $mg \cdot g^{-1}$), oleic acid ($C_{18:1}$; 0.51 $mg \cdot g^{-1}$) and stearic acid ($C_{18:0}$; 0.51 $mg \cdot g^{-1}$). Tridecanoic acid solution in isooctane (118.3 $\mu g \cdot g^{-1}$) and hexadecane solution in isooctane (133.3 $\mu g \cdot g^{-1}$) were both supplied by Sigma-Aldrich (USA) and used as internal standards (ISs).

The used solvents *n*-hexane (Hex), ethyl acetate (EtOAc), methanol (MeOH), ethanol (EtOH), chloroform ($CHCl_3$), dichloromethane (DCM) and 2-propanol (all HPLC grade, 99.8%) were purchased from Sigma-Aldrich (Milan, Italy). Hydrochloric acid (HCl), diethyl ether, potassium hydroxide pellets and *N,O*-bis(trimethyl)silyltrifluoro-acetamide (BSTFA) containing 1 % trimethylchlorosilane, were purchased from Sigma-Aldrich (Milan, Italy).

7.1.4. Sample preparation

Special care was taken in all analytical steps (sample crushing, fractionation and extraction) in order to minimize cross-contamination. Ceramic samples (1 g) were collected with a scalpel, both from the external and internal part of the archaeological pieces, weighed and stored in glass vessels until their analysis (see Figure 7.2). Fresh whale blubber and oil samples were stored in the supplied glass vials before taking 1-2 mg for analysis.

7.1.5. Sample treatment

7.1.5.1. Solvent soluble fraction: isolation of triacylglycerols (TAGs)

Microwave assisted extraction (Milestone microwaves Ethos One, USA) was used to characterize the solvent soluble fraction. The extraction conditions were fixed according to the previous works found in the literature [32,33]. In this work, the extractions were performed at 80 ^{o}C and 600 W for 25 min using 300 µL and 600 µL of a $CHCl_3$:Hex mixture (3:2, *v/v*) to extract 1-2 mg of fresh whale oil samples and 1 g of ceramic archaeological samples, respectively. The extracts were completely dried under a nitrogen stream, diluted with 600 µL of MeOH: 2-propanol (90:10, *v/v*) and filtered through 0.45 µm PTFE filters (Grace Davison Discovery Sciences, USA) just before the analysis by HPLC-ESI-QToF.

7.1.5.2. Saponified fraction: isolation of fatty acids (FAs)

The extraction of the acidic and neutral compounds was performed according to the methodology described by Colombini et al. [7]. Briefly, 1 g of each archaeological sample was directly subjected to microwave assisted saponification (Milestone microwaves Ethos One, USA) with 600 µL of 10% KOH in EtOH at 80 °C and 200 W for 60 min. In the case of the fresh samples (i.e., whale blubber and oil samples) used as reference materials 0.5-1 mg were immersed in 300 µL of the previously mentioned saponification solution.

After saponification, the alcoholic solutions were diluted in bi-distilled water and the neutral fractions were extracted with Hex (3 x 400 µL). Subsequently, the aqueous phases were acidified with HCl (6 M to pH=2) and re-extracted with diethyl ether (3 x 400 µL). 5 µL of tridecanoic acid solution (118.3 $\mu g \cdot g^{-1}$) used as IS was added to the 200 µL of the final EtOAc extract and evaporated to dryness under a gentle stream of nitrogen. Derivatization was performed with 20 µL of BSTFA and 150 µL of isooctane (derivatization solvent) at 60 °C for 30 min in a water bath. 5 µL of hexadecane solution (133.3 $\mu g \cdot g^{-1}$) used as IS were added to the derivatized extract before the analysis by means of GC-MS.

7.1.6. Instruments and methods

7.1.6.1. High performance liquid chromatography/mass spectrometry

HPLC-ESI-QToF analyses were carried out using a 1200 Infinity HPLC, coupled with a Quadrupole-Time of Flight tandem mass spectrometer 6530 Infinity QToF detector by a Jet Stream ESI interface (Agilent Technologies). The chromatographic separation was carried out using a Poroshell 120 EC-C18 column (3.0 mm x 5.0 mm, 2.7 µm particle size) with a Zorbax eclipse plus C-18 guard column (4.6 mm x 12.5 mm, 5 µm particle size) at a flow rate of 0.3 $mL \cdot min^{-1}$ and at 45 °C. Aliquots of 10 µL were injected and the elution gradient was programmed using methanol/water 85:15 (eluent A) and *iso*-propanol (eluent B) as follows: 90 % A for 5 min, followed by a linear gradient to 90 % B in 30 min (held for 10 min). Re-equilibration time for each analysis run was 10 min.

The ESI operating conditions were: drying gas (N_2, purity > 98 %): 350 °C and 10 $L \cdot min^{-1}$; capillary voltage 4.5 KV; nebulizer gas 35 psig; sheath gas (N_2, purity > 98 %): 375 °C and 11 $L \cdot min^{-1}$. High resolution MS and MS/MS spectra were acquired in positive mode in the range 100-1700 *m/z*. The fragmentor was kept at 200 V, nozzle voltage 1000 V, skimmer 65 V, octapole RF 750 V. The MS/MS spectra were obtained at 50 V. The collision gas was nitrogen (purity 99.999%). The data were collected by auto MS/MS acquisition with an MS

scan rate of 1.03 spectra·sec^{-1} and an MS/MS scan rate of 1.05 spectra·sec^{-1}; only one precursor was acquired per cycle (relative threshold 0.010%). The mass axis was calibrated daily using the Agilent tuning mix HP0321 properly diluted in water and acetonitrile (Agilent Technologies). MassHunter® Workstation Software (B.04.00) was used to carry out mass spectrometer control, data acquisition and data analysis.

The structures of the TAGs were identified by the evaluation of their exact mass, the interpretation of their tandem mass spectra and by comparison with previously published mass spectra data [34-38]. TAGs were named by means of the three abbreviations corresponding to the nature of the acyl chains (FAs) linked to the glycerol backbone: C, capric acid ($C_{10:0}$); La, lauric acid ($C_{12:0}$); M, myristic acid ($C_{14:0}$); P, palmitic acid ($C_{16:0}$); Po, palmitoleic acid ($C_{16:1}$); S, stearic acid ($C_{18:0}$); O, oleic acid ($C_{18:1}$); L, linoleic acid ($C_{18:2}$); Ln, linolenic acid ($C_{18:3}$); S_4, stearidonic acid ($C_{18:4}$); E, eicosenoic acid ($C_{20:1}$); E_2, eicosadienoic acid ($C_{20:2}$); E_3, eicosatrienoic acid ($C_{20:3}$); E_4, eicosatetraenoic acid ($C_{20:4}$); Epa, eicosapentaenoic acid ($C_{20:5}$); A, arachidic acid ($C_{20:0}$); D, docosenoic acid ($C_{22:1}$); Dte, docosatetraenoic acid ($C_{22:4}$); Dpa, docosapentaenoic acid ($C_{22:5}$); Dha, docosahexaenoic acid ($C_{22:6}$); B, behenic acid ($C_{22:0}$); T, tetracosenoic acid ($C_{24:1}$).

7.1.6.2. Gas chromatography/mass spectrometry

The gas chromatographic system 6890N (Agilent Technologies, Palo Alto, CA, USA) was coupled to a 5975 single quadrupole mass spectrometer (Agilent Technologies, Palo Alto, CA, USA). 2 µL of the derivatized extract were injected in splitless mode at 280 ^{o}C into the GC-MS system. For the gas chromatographic separation, a HP-5MS fused silica capillary column (5% diphenyl- 95% dimethyl-polysiloxane, 30 m × 0.25 mm i.d., 0.25 µm film thickness, J&W Scientific Agilent Technologies, USA) with a de-activated silica pre-column (2 m × 0.32 mm i.d., J&W Scientific Agilent Technologies, USA) was used. The carrier gas (He, purity 99.9995%) was used in the constant flow mode at 1.2 mL·min^{-1}. The GC-MS oven

program temperature was as follows: 80 ^{o}C (2 min), a temperature increase of 10 ^{o}C·min^{-1} up to 200 ^{o}C (3 min) to continue rising at 10 ^{o}C·min^{-1} up to 280 ^{o}C (40 min).

Measurements were performed in SCAN mode (*m/z* 50–650) and peak assignments were performed using standard solutions of FAs, mass spectra interpretation, comparison with mass spectral libraries (NIST 2.0) and with mass spectra reported in the literature [4,7].

7.1.7. Semi-quantitative analysis

For the HPLC-ESI-QToF analysis, relative abundances were calculated from extract ion chromatograms, considering the isotopic distribution of the molecular species. To establish the TAG profiles in both archaeological and fresh reference samples the signals were normalized to 100% for each chromatogram.

For the GC-MS analysis, the chromatographic peak areas of the extract ion chromatograms were integrated and corrected with the signal of *n*-tridecanoic acid and hexadecane internal standards. The data were normalized to 100% for each chromatogram in both archaeological and fresh materials.

7.2. Results and discussion

7.2.1. Characterization of blubber and whale oil reference samples

Due to the lack of certified reference material of whale oil, the main characteristic fingerprints of this type of samples were first identified in fresh blubber samples, which were used as reference materials (see section 7.1.2.).

7.2.1.1. HPLC-ESI-QToF triglycerides profiling

HPLC-ESI-QToF was applied for the characterization of the TAG profiles of the reference materials. Auto MS-MS acquisition mode in HPLC-ESI-QToF allows the non-target screening of as many TAGs as possible in the sample. The identification of each acyl substituent was carried out considering the detected fragment ions and searching for *m/z* values corresponding to the loss of acyl substituents in positions sn-1, sn-2 and sn-3. According to La Nasa et al. [39], several fragmentation studies in the literature show that the losses of acylic chain in position sn-1 and sn-3 are energetically more favored than the loss in the sn-2 position in the case of sodiated adducts. Consequently, it is expected that diacylglycerol ions of sn-1 and sn-3 position fragments in the MS-MS spectra will be the more abundant ones [39,40], provided that no coelution of isobaric species occurs.

Figure 7.3 shows the mass spectrum of PPoO as an example: the relative abundances of both the sodiated and proton adducts resulting from the losses in position sn-1 ($[PoO]^+$ and $[PoO+Na]^+$) and sn-3 ($[PPo]^+$ and $[PPo+Na]^+$) are higher than the corresponding ones for the adducts $[PO]^+$ and $[PO+Na]^+$ that result from the loss of the acyl group in the sn-2 position. However, this interpretation is not always useful for the identification of those complex oils with TAGs containing high amounts of omega-3 long chain polyunsaturated FAs (PUFAs). Baiocchi et al. [35] observed an inversion of abundance profiles when comparing thermal- and collision- induced fragmentations for this kind of TAGs when studying samples of tuna oil. In the latter case, the most abundant peaks are due to fragment ions corresponding to the loss of the acyl chain from the sn-2 position. Consequently, in the present work the first mentioned fragmentation pattern was used for the saturated or modestly unsaturated FAs, whereas for the TAGs containing omega-3 PUFA the collisionally-induced fragmentation observed in the literature was considered predominant.

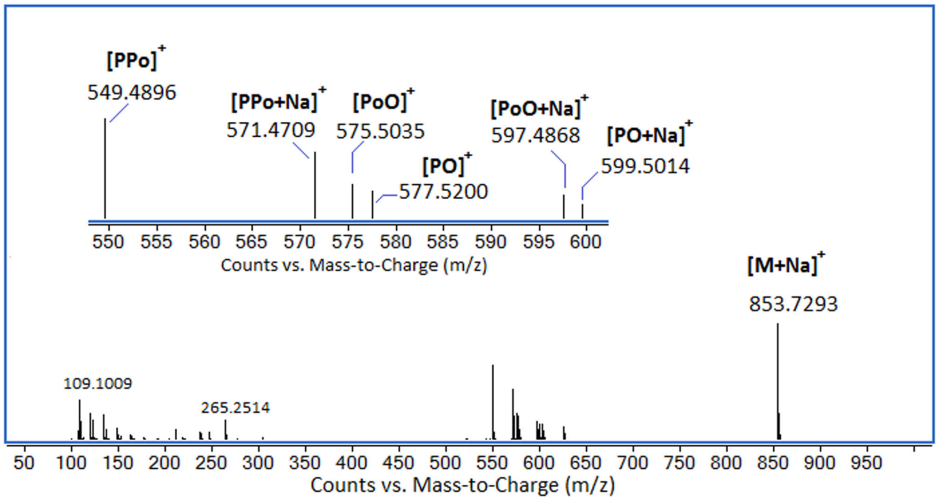


Figure 7.3: *MS/MS spectra for the PPoO.*

Table 7.2 shows the 91 TAGs identified in fresh blubber and whale oil samples used as reference materials. Their relative abundance was obtained based on the peak area of their precursor ion in the extracted ion chromatograms. As it can be seen, most of them are characterized by the presence of the two main omega-3 long PUFAs: acyl chains docosahexaenoic acid and eicosapentaenoic acid.

Table 7.2: *Triglycerides (TAGs) determined by HPLC-ESI-QToF in the reference whale blubbers and oil with formula, parent ions, daughter ions and proposed fragmentation of the mass spectra and tandem mass spectra for all the identified species.*

MS¹			MS²					
			Major fragments				Minor fragments	
TAG	Formula	[M+Na]⁺	Product ion	Fragment. pattern	Product ion	Fragment. pattern	Product ion	Fragment. pattern
M-P-La	$C_{45}H_{86}O_6$	745.6	545.4 523.4	$[MP+Na]^+$ $[MP]^+$	517.4 495.4	$[PLa+Na]^+$ $[PLa]^+$	489.4 467.4	$[MLa+Na]^+$ $[MLa]^+$
M-La-L	$C_{47}H_{86}O_6$	769.6	541.4 519.4	$[LaL+Na]^+$ $[LaL]^+$	489.4 467.4	$[MLa+Na]^+$ $[MLa]^+$	569.5 547.5	$[ML+Na]^+$ $[ML]^+$
M-O-La	$C_{47}H_{88}O_6$	771.6	571.4 549.4	$[MO+Na]^+$ $[MO]^+$	543.4 521.4	$[OLa+Na]^+$ $[OLa]^+$	489.4 467.4	$[MLa+Na]^+$ $[MLa]^+$
La-P-L	$C_{49}H_{90}O_6$	797.6	517.4 495.4	$[LaP+Na]^+$ $[LaP]^+$	597.5 575.5	$[PL+Na]^+$ $[PL]^+$	541.4 519.4	$[LaL+Na]^+$ $[LaL]^+$
O-C-O	$C_{49}H_{90}O_6$	797.6	515.4 493.4	$[OC+Na]^+$ $[OC]^+$			625.5 603.5	$[OO+Na]^+$ $[OO]^+$
O-La-P	$C_{49}H_{92}O_6$	799.6	543.4 521.4	$[OLa+Na]^+$ [OLa]+	517.4 495.4	$[LaP+Na]^+$ $[LaP]^+$	599.5 577.5	$[OP+Na]^+$ $[OP]^+$
M-P-P	$C_{49}H_{94}O_6$	801.7	545.4 523.4	$[MP+Na]^+$ $[MP]^+$	573.5 551.5	$[PP+Na]^+$ $[PP]^+$		
Po-O-M	$C_{51}H_{96}O_6$	825.7	597.5 575.5	$[PoO+Na]^+$ $[PoO]^+$	571.5 549.5	$[MO+Na]^+$ $[MO]^+$	543.4 521.4	$[PoM+Na]^+$ $[PoM]^+$
M-O-P	$C_{51}H_{96}O_6$	827.7	571.5 549.5	$[MO+Na]^+$ $[MO]^+$	599.5 577.5	$[OP+Na]^+$ $[OP]^+$	545.4 523.4	$[MP+Na]^+$ $[MP]^+$
P-P-P	$C_{51}H_{98}O_6$	829.7	573.5	$[PP+Na]^+$	551.5	$[PP]^+$		
M-Po-Epa	$C_{55}H_{90}O_6$	845.6	543.4 521.4 591.4	$[MPo+Na]^+$ $[MPo]^+$ $[MEpa+Na]^+$	569.4 617.4 595.4	$[MEpa]^+$ $[PoEPA+Na]^+$ $[PoEPA]^+$		
M-O-Ln	$C_{53}H_{94}O_6$	849.7	571.5 549.5 567.5	$[MO+Na]^+$ $[MO]^+$ $[MLn+Na]^+$	621.5 599.5 545.5	$[OLn+Na]^+$ $[OLn]^+$ $[MLn]^+$		
P-Po-Ln	$C_{53}H_{94}O_6$	849.7	571.5 549.5 593.5	$[PPo+Na]^+$ $[PPo]^+$ $[PoLn+Na]^+$	571.5 595.7 573.5	$[PoLn]^+$ $[LnP+Na]^+$ $[LnP]^+$		
M-L-L	$C_{53}H_{94}O_6$	849.7	569.5 547.5	$[ML+Na]^+$ $[ML]^+$	621.5 599.5	$[LL+Na]^+$ $[LL]^+$		
Ln-P-P	$C_{53}H_{96}O_6$	851.7	595.7 573.5	$[LnP+Na]^+$ $[LnP]^+$	573.5 551.5	$[PP+Na]^+$ $[PP]^+$		

Table 7.2: *Continuation.*

MS¹			MS²					
			Major fragments				Minor fragments	
TAG	Formula	[M+Na]⁺	Product ion	Fragment. pattern	Product ion	Fragment. pattern	Product ion	Fragment. pattern
P-Po-O	$C_{53}H_{98}O_6$	853.7	571.5 549.5	[PPo+Na]⁺ [PPo]⁺	597.5 575.5	[PoO+Na]⁺ [PoO]⁺	599.5 577.5	[PO+Na]⁺ [PO]⁺
P-O-P	$C_{53}H_{100}O_6$	855.7	599.5	[PO+Na]⁺	577.5	[PO]⁺		
P-P-O	$C_{53}H_{100}O_6$	855.7	573.5 551.5	[PP+Na]⁺ [PP]⁺	599.5 577.5	[PO+Na]⁺ [PO]⁺		
P-P-S	$C_{53}H_{102}O_6$	857.7	573.5 551.5	[PP+Na]⁺ [PP]⁺	601.5 579.5	[PS+Na]⁺ [PS]⁺		
P-Po-Epa	$C_{55}H_{94}O_6$	873.7	571.5 549.5	[PPo+Na]⁺ [PPo]⁺	597.5 617.4	[PEpa]⁺ [PoEPA+Na]⁺		
P-O-Ln	$C_{55}H_{98}O_6$	877.7	619.5 599.5 577.5 621.5	[PEpa+Na]+ [PO+Na]⁺ [PO]⁺ [OLn+Na]⁺	595.4 599.5 595.7 573.5	[PoEPA]⁺ [OLn]⁺ [PLn+Na]⁺ [PLn]⁺		
Po-O-L	$C_{55}H_{98}O_6$	877.7	597.5 575.5	[PoO+Na]⁺ [PoO]⁺	623.5 601.5	[OL+Na]⁺ [OL]⁺	595.5 573.5	[PoL+Na]⁺ [PoL]⁺
PLL	$C_{55}H_{98}O_6$	877.7	597.5 575.5	[PL+Na]⁺ [PL]⁺	621.5 599.5	[LL+Na]⁺ [LL]⁺		
Po-O-O	$C_{55}H_{100}O_6$	879.7	597.5 575.5	[PoO+Na]⁺ [PoO]⁺	625.5 603.5	[OO+Na]⁺ [OO]⁺		
O-P-O	$C_{55}H_{102}O_6$	881.7	599.5	[OP+Na]⁺	577.5	[OP]⁺		
P-O-O	$C_{55}H_{102}O_6$	881.7	599.5 577.5	[PO+Na]⁺ [PO]⁺	625.5 603.5	[OO+Na]⁺ [OO]⁺		
P-O-S	$C_{55}H_{104}O_6$	883.7	599.5 577.5	[PO+Na]⁺ [PO]⁺	627.5 605.5	[OS+Na]⁺ [OS]+	601.5 579.5	[SP+Na]+ [SP]+
P-S-S	$C_{55}H_{106}O_6$	885.7	601.5 579.5	[PS+Na]⁺ [PS]⁺	629.5 607.5	[SS+Na]⁺ [SS]⁺		
Ln-Ln-Ln	$C_{57}H_{92}O_6$	895.7	617.5	[LnLn+Na]⁺	595.5	[LnLn]⁺		
Epa-O-Po	$C_{59}H_{100}O_6$	899.7	617.5 645.5	[EpaPo+Na]⁺ [OEpa+Na]⁺	597.5 575.5	[PoO+Na]⁺ [PoO]⁺	595.5 623.5	[EpaPo]+ [OEpa]+
Epa-O-P	$C_{57}H_{98}O_6$	901.7	645.5 619.5	[OEpa+Na]⁺ [EpaP+Na]⁺	599.5 577.5	[PO+Na]⁺ [PO]⁺	623.5	[OEpa]+
L-L-L	$C_{57}H_{98}O_6$	901.7	621.5	[LL+Na]⁺	599.5	[LL]⁺		
Ln-L-O	$C_{57}H_{98}O_6$	901.7	619.5 597.5	[LnL+Na]⁺ [LnL]⁺	623.5 601.5	[LO+Na]⁺ [LO]⁺		

Table 7.2: *Continuation.*

MS¹			MS²					
			Major fragments				Minor fragments	
TAG	Formula	$[M+Na]^+$	Product ion	Fragment. pattern	Product ion	Fragment. pattern	Product ion	Fragment. pattern
			621.5	$[OLn+Na]^+$	599.5	$[OLn]^+$		
P-O-E_4	$C_{57}H_{100}O_6$	903.7	599.5 577.5	$[PO+Na]^+$ $[PO]^+$	621.5 647.5	$[PE_4+Na]^+$ $[OE4+Na]^+$	625.5 625.5	$[OE_4]^+$ $[OO+Na]^+$
O-Ln-O	$C_{57}H_{100}O_6$	903.7	621.5 599.5	$[OLn+Na]^+$ $[OLn]^+$	625.5 603.5	$[OO+Na]^+$ $[OO]^+$	603.5	$[OO]^+$
L-L-O	$C_{57}H_{100}O_6$	903.7	621.5 599.5	$[LL+Na]^+$ $[LL]^+$	623.5 601.5	$[LO+Na]^+$ $[LO]^+$		
O-O-L	$C_{57}H_{102}O_6$	905.7	625.5 603.5	$[OO+Na]^+$ $[OO]^+$	623.5 601.5	$[LO+Na]^+$ $[LO]^+$		
O-L-O	$C_{57}H_{102}O_6$	905.7	623.5 601.5	$[LO+Na]^+$ $[LO]^+$	601.5	$[LO]^+$	625.5 603.5	$[OO+Na]^+$ $[OO]^+$
S-O-Ln	$C_{57}H_{102}O_6$	905.7	627.5 605.5 623.5	$[SO+Na]^+$ $[SO]^+$ $[SLn+Na]^+$	621.5 599.5 601.5	$[OLn+Na]^+$ $[OLn]^+$ $[SLn]^+$		
O-O-O	$C_{57}H_{104}O_6$	907.7	625.5	$[OO+Na]^+$	603.5	$[OO]^+$		
S-L-O	$C_{57}H_{104}O_6$	907.7	625.5 603.5	$[SL+Na]^+$ $[SL]^+$	623.5 601.5	$[LO+Na]^+$ $[LO]^+$	627.5 605.5	$[SO+Na]^+$ $[SO]^+$
O-O-S	$C_{57}H_{106}O_6$	909.7	625.5 603.5	$[OO+Na]^+$ $[OO]^+$	627.5 605.5	$[OS+Na]^+$ $[OS]^+$		
E-P-O	$C_{57}H_{106}O_6$	909.8	627.5 605.5	$[EP+Na]^+$ $[EP]^+$	599.5 577.5	$[PO+Na]^+$ $[PO]^+$	653.5 631.5	$[EO+Na]^+$ $[EO]^+$
S-S-O	$C_{57}H_{108}O_6$	911.8	629.5 607.5	$[SS+Na]^+$ $[SS]^+$	627.5 605.5	$[SO+Na]^+$ $[SO]^+$		
Po-Epa-Epa	$C_{59}H_{92}O_6$	919.6	617.5 665.5	$[PoEpa+Na]^+$ $[EpaEpa+Na]^+$	643.5	$[EpaEpa]^+$	595.5	[EpaPo]+
M-Epa-Dha	$C_{59}H_{92}O_6$	919.6	591.5 617.5	$[MEpa+Na]^+$ $[DhaM+Na]^+$	691.5	$[DhaEpa]^+$	569.5 595.5	$[MEpa]^+$ $[DhaM]^+$
Dha-O-Po	$C_{59}H_{98}O_6$	925.7	643.5 597.5	$[DhaPo+Na]^+$ $[OPo+Na]^+$	575.5 671.5	$[OPo]^+$ $[DhaO+Na]^+$	649.5 621.5	$[DhaO]^+$ $[DhaPo]^+$
Epa-O-O	$C_{59}H_{100}O_6$	927.7	645.5 625.5	$[EpaO+Na]^+$ $[OO+Na]^+$	603.5	$[OO]^+$	623.5	$[EpaO]^+$
Dha-P-O	$C_{59}H_{100}O_6$	927.7	645.5 599.5	$[DhaP+Na]^+$ $[PO+Na]^+$	577.5 671.5	$[PO]^+$ $[DhaO+Na]^+$		

Table 7.2: *Continuation.*

MS¹			MS²					
			Major fragments				Minor fragments	
TAG	Formula	[M+Na]⁺	Product ion	Fragment. pattern	Product ion	Fragment. pattern	Product ion	Fragment. pattern
Dpa-O-P	$C_{59}H_{102}O_6$	929.7	647.5 673.5	[DpaP+Na]+ [DpaO+Na]+	599.5 577.5	[OP+Na]⁺ [OP]⁺		
E_4-O-S	$C_{59}H_{104}O_6$	931.7	649.5 627.5	[E_4S+Na]+ [OS+Na]⁺	605.5	[OS]⁺		
Dte-O-P	$C_{59}H_{104}O_6$	931.7	675.5 599.5 649.5	[DteO+Na]⁺ [OP+Na]⁺ [DteP+Na]⁺	653.5 577.5 627.5	[DteO]⁺ [OP]⁺ [DteP]⁺		
L-E_2-O	$C_{59}H_{104}O_6$	931.7	651.5 629.5	[E_2O+Na]⁺ [E_2O]⁺	649.5 627.5	[LE_2+Na]⁺ [LE_2]⁺	623.5 601.5	[LO+Na]⁺ [LO]⁺
E-L-O	$C_{59}H_{106}O_6$	933.7	651.5 629.5	[EL+Na]⁺ [EL]⁺	623.5 601.5	[LO+Na]⁺ [LO]⁺	653.5 631.5	[EO+Na]⁺ [EO]⁺
E-O-O	$C_{59}H_{108}O_6$	935.8	653.5 631.5	[EO+Na]⁺ [EO]⁺	625.5 603.5	[OO+Na]⁺ [OO]⁺		
A-O-O	$C_{59}H_{110}O_6$	937.8	655.5 633.5	[AO+Na]⁺ [AO]⁺	625.5 603.5	[OO+Na]⁺ [OO]⁺		
Dha-Dha-Mo	$C_{61}H_{92}O_6$	943.7	717.5 615.4	[DhaDha+Na]⁺ [DhaMo+Na]⁺	615.4	[DhaMo+Na]⁺	695.5 593.5	[DhaDha]⁺ [DhaMo]⁺
Po-Epa-Dha	$C_{61}H_{94}O_6$	945.7	617.5 643.5	[PoEpa+Na]⁺ [DhaPo+Na]⁺	691.5	[DhaEpa+Na]⁺	595.5 621.5	[PoEpa]⁺ [DhaPo]⁺
Dha-Dha-M	$C_{61}H_{94}O_6$	945.7	617.5 717.5	[DhaM+Na]⁺ [DhaDha+Na]⁺			595.5 695.5	[DhaM]⁺ [DhaDha]⁺
Dha-S_4-O	$C_{61}H_{96}O_6$	947.7	619.5 665.5	[DhaS_4+Na]⁺ [S_4O+Na]⁺	671.5	[DhaO+Na]⁺	597.5 643.5	[DhaS_4]⁺ [S4O]⁺
Dha-Dpa-M	$C_{61}H_{96}O_6$	947.7	619.5 617.5	[DpaM+Na]⁺ [DhaM+Na]⁺	719.5	[DhaDpa+Na]⁺	597.5 595.5	[DpaM]⁺ [DhaM]⁺
P-E_4-Dha	$C_{61}H_{98}O_6$	949.7	621.5 645.5	[PE_4+Na]⁺ [PDha+Na]⁺	693.5	[A4Dha+Na]⁺	671.5 599.5	[E_4Dha]⁺ [PE_4]⁺
P-E_3-Dha	$C_{61}H_{100}O_6$	951.7	623.5 695.5	[PE_3+Na]⁺ [E_3Dha+Na]⁺	645.5	[PDha+Na]⁺	601.5 673.5	[PE_3]⁺ [E_3Dha]⁺
Dha-O-O	$C_{61}H_{102}O_6$	953.7	671.5 625.5	[DhaO+Na]⁺ [OO+Na]⁺	603.5	[OO]⁺	649.5	[DhaO]⁺
Dha-S-O	$C_{61}H_{104}O_6$	955.7	673.5	[DhaS+Na]⁺	605.5	[SO]⁺	651.5	[DhaS]⁺

Table 7.2: *Continuation.*

MS¹			MS²					
			Major fragments				Minor fragments	
TAG	**Formula**	**[M+Na]⁺**	**Product ion**	**Fragment. pattern**	**Product ion**	**Fragment. pattern**	**Product ion**	**Fragment. pattern**
			627.5	[SO+Na]⁺	671.5	[DhaO+Na]⁺		
Epa-O-E	$C_{61}H_{104}O_6$	955.7	645.5 673.5	[EpaO+Na]⁺ [EpaE+Na]⁺	653.5 631.5	[EO+Na]⁺ [EO]⁺	623.5 651.5	[EpaO]⁺ [EpaE]⁺
Dpa-O-S	$C_{61}H_{104}O_6$	957.7	673.5 675.5	[DpaO+Na]⁺ [DpaS+Na]⁺	627.5 605.5	[SO+Na]⁺ [SO]⁺	651.5 653.5	[DpaO]⁺ [DpaS]⁺
O-E_2-E	$C_{61}H_{110}O_6$	961.8	651.5 629.5	[OE_2+Na]⁺ [OE_2]⁺	679.5 657.5	[E_2E_1+Na]⁺ [E_2E_1]⁺	653.5 631.5	[OE+Na]⁺ [OE]⁺
O-D-L	$C_{61}H_{110}O_6$	961.8	679.5 657.5	[DL+Na]⁺ [DL]⁺	681.5 659.5	[OD+Na]⁺ [OD]⁺	623.5 601.5	[LO+Na]⁺ [LO]⁺
T-P-O	$C_{61}H_{114}O_6$	965.8	683.6 661.6	[TP+Na]⁺ [TP]⁺	599.5 577.5	[PO+Na]⁺ [PO]⁺	709.6 687.6	[TO+Na]⁺ [TO]⁺
P-E-D	$C_{61}H_{114}O_6$	965.8	627.5 605.5	[PE+Na]⁺ [PE]⁺	709.6 687.6	[ED+Na]⁺ [ED]⁺	655.6 633.5	[PD+Na]⁺ [PD]⁺
B-O-O	$C_{61}H_{114}O_6$	965.8	683.6 661.6	[BO+Na]⁺ [BO]⁺	625.5 603.5	[OO+Na]⁺ [OO]⁺		
Dha-Dha-Po	$C_{63}H_{96}O_6$	971.7	643.5 717.5	[DhaPo+Na]⁺ [DhaDha+Na]⁺	621.5	[DhaPo]⁺	695.5	[DhaDha]⁺
O-Epa-Dha	$C_{63}H_{98}O_6$	973.7	645.5 691.5	[EpaO+Na]⁺ [DhaEpa+Na]⁺	671.5	[DhaO+Na]⁺	623.5 649.5	[EpaO]⁺ [DhaO]⁺
P-Dpa-Dha	$C_{63}H_{100}O_6$	975.7	647.5 645.5	[DpaP+Na]⁺ [DhaP+Na]⁺	719.5	[DpaDha+Na]⁺		
Dpa-O-E	$C_{63}H_{108}O_6$	983.8	673.5 653.5	[DpaO+Na]⁺ [OE+Na]⁺	631.5 701.5	[OE]⁺ [DpaE+Na]⁺	679.5 651.5	[DpaE]⁺ [DpaO]⁺
S-Epa-D	$C_{63}H_{110}O_6$	985.8	647.5 701.5	[SEpa+Na]+ [EpaD+Na]+	683.5	[SD+Na]+		
E-D-O	$C_{63}H_{116}O_6$	991.8	709.6 687.6	[ED+Na]⁺ [ED]⁺	681.5 659.5	[OD+Na]⁺ [OD]⁺	653.5 631.5	[OE+Na]⁺ [OE]⁺
D-Po-D	$C_{63}H_{116}O_6$	991.8	653.5 631.5	[PoD+Na]⁺ [PoD]⁺			737.6 715.6	[DD+Na]⁺ [DD]⁺
Dha-Epa-Epa	$C_{65}H_{94}O_6$	993.7	665.5 691.5	[EpaEpa+Na]⁺ [DhaEpa+Na]⁺			643.5 669.5	[EpaEpa]⁺ [DhaEpa]⁺

Table 7.2: *Continuation.*

MS¹			MS²					
			Major fragments				Minor fragments	
TAG	Formula	[M+Na]⁺	Product ion	Fragment. pattern	Product ion	Fragment. pattern	Product ion	Fragment. pattern
P-E-T	$C_{65}H_{94}O_6$	993.8	627.5 605.5	[PE+Na]⁺ [PE]⁺	709.6 687.6	[ET+Na]⁺ [ET]⁺	683.6 661.6	[PT+Na]⁺ [PT]⁺
Dha-Dha-O	$C_{65}H_{100}O_6$	999.7	671.5 717.5	[DhaO+Na]⁺ [DhaDha+Na]⁺	649.5	[DhaO]⁺	695.5	[DhaDha]⁺
O-Dpa-Dha	$C_{65}H_{102}O_6$	1001.7	673.5 671.5	[DpaO+Na]⁺ [DhaO+Na]⁺	719.5	[DpaDha+Na]⁺	653.5	[OE+Na]⁺
D-Epa-E	$C_{65}H_{112}O_6$	1011.8	673.5 701.5	[EpaE+Na]⁺ [DEpa+Na]⁺	709.6	[DE+Na]⁺	651.5 679.5	[EpaE]⁺ [DEpa]⁺
Dha-Epa-Dha	$C_{67}H_{96}O_6$	1019.7	691.5 717.5	[DhaEpa+Na]⁺ [DhaDha+Na]⁺			669.5 695.5	[DhaEpa]⁺ [DhaDha]⁺
O-D-D	$C_{65}H_{120}O_6$	1019.8	681.5 659.5	[OD+Na]⁺ [OD]⁺	737.6 715.6	[DD+Na]⁺ [DD]⁺		
T-O-E	$C_{65}H_{120}O_6$	1019.8	653.6 631.6	[OE+Na]⁺ [OE]⁺	709.6 687.6	[TO+Na]⁺ [TO]⁺	737.6 715.6	[TE+Na]⁺ [TE]⁺
Dha-E4-Dha	$C_{67}H_{98}O_6$	1021.7	693.5 717.5	[DhaE4+Na]⁺ [DhaDha+Na]⁺			671.5 695.5	[DhaE4]⁺ [DhaDha]⁺
E-D-Dha	$C_{67}H_{114}O_6$	1037.8	709.6 699.5	[ED+Na]⁺ [DhaE+Na]⁺	727.6 687.6	[DhaD+Na]⁺ [ED]⁺	705.6 677.5	[DhaD]⁺ [DhaE]⁺
D-D-Dha	$C_{69}H_{118}O_6$	1065.8	727.6 737.6	[DhaD+Na]⁺ [DD+Na]⁺	[DD+Na]⁺		705.6 715.6	[DhaD]⁺ [DD]⁺
D-D-D	$C_{69}H_{128}O_6$	1075.9	737.6 715.6	[DD+Na]⁺ [DD]⁺				

[M+Na]⁺: Precursor ion

Fragment. pattern: fragmentation pattern

Some of the most abundant TAGs in all the characterized whale blubber samples are Epa-O-P, Dha-P-O, Epa-O-O, P-Po-Epa, and E-O-O, A-O-O. In concordance with other works studying TAGs in other marine species [37], the omega-3 FAs Dha and Epa were preferentially linked to the backbones of the TAGs with higher level of monounsaturated fatty acids (MUFAs) such as palmitoleic (Po), oleic (O), eicosenoic (E) and docosenoic (D) acids than saturated FAs.

As mentioned above, the adopted strategy fails in the identification of the compounds when chromatographic coelution of isobaric TAGs occur, since the MS/MS spectra is due to the mixture of product ions corresponding to different TAGs. Exemplarily, this happens for the precursor ion 991.8 (Figure 7.4) which, according to the product ions, can correspond to E-D-O or D-Po-D. Due to the homogeneity of their structure and the absence of PUFAs, the fragmentation behavior for both TAGs is explained by the energetically more favored losses in positions sn-1 and sn-3 (ions 709.6 for $[ED+Na]^+$ and 681.5 for $[DO+Na]^+$ or ion 653.5 for $[PoD+Na]^+$). Most probably, the most abundant of the two coeluting compounds is E-D-O due to the higher abundance of the product 709.6 ($[ED+Na]^+$) with respect to the 653.5 ($[PoD+Na]^+$ or $[EO+Na]^+$). Nevertheless, the occurrence of the product with *m/z* 737.6 ($[DD+Na]^+$) confirms the presence of D-Po-D.

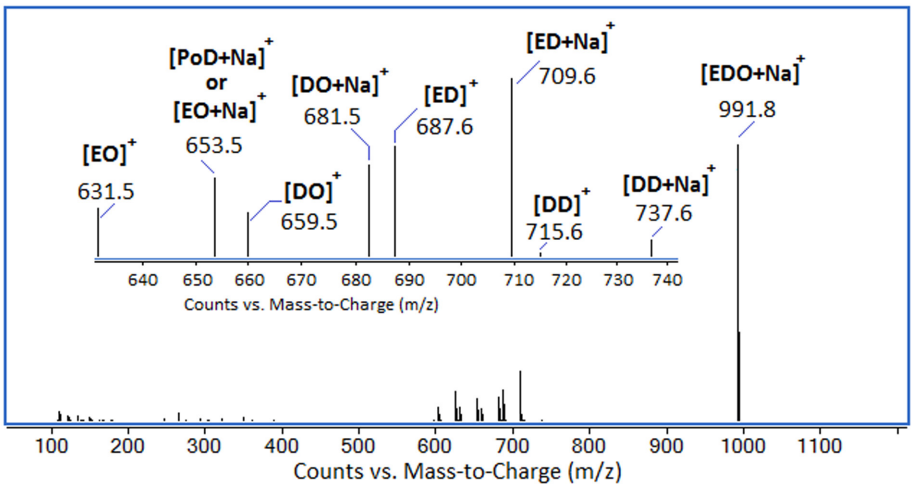


Figure 7.4: *MS/MS spectra for the two possible compounds with the same fragments and parent ion E-D-O and D-Po-D.*

The obtained TAG profiles in fresh material were compared amongst the different whale species in order to study possible similarities or differences that could be used as

markers. According to the results shown in Figure 7.5, it can be clearly observed that there is a close TAG distribution profile for all the blubber samples belonging to *Balaenoptera* genus (i.e., sei whale, fin whale and minke whale) whereas totally different distributions were found for the rest of the studied genus (i.e., *Phocoena* and *Megaptera*). Thus, it can be suggested that the TAG profile could be related to the genus of the whale from which oil or blubber has been extracted. Due to the faster degradation of the polyunsaturated TAGs with respect to the saturated or modestly unsaturated ones, it is highly unlikely to find them preserved in the archaeological ceramic samples. Nevertheless, the TAGs written in blue in Table 7.2 were found in archaeological samples (see below) and thus, they were considered to study trends and ratios in the fresh material as well.

Figure 7.6. shows the relative abundances of the previously mentioned TAGs in the reference blubber samples, taking into account only the modestly unsaturated TAGs (Figure 9.1 in the annexes shows the HPLC-ESI-Q-ToF extract ion chromatograms referring to the mentioned TAGs in fresh samples). Five groups of triglycerides, highlighted in boxes and numbered 1-5 in the figure, can be considered significant in discriminating between samples belonging to the same or different whale genus. The species belonging to *Balaenoptera* genus show similar tendencies for the TAGs highlighted in the five groups, whereas they are different in the cases of *Phocoena* and *Megaptera*. Additionally, observing these profiles, we might hypothesize that the whale from which blubber was extracted and transformed into the unknown whale oil sample belonged to the *Balaenoptera* genus.

In order to add a statistical value to the observation of the trends of the TAGs in the five boxes, four ratios were defined and calculated for the blubber samples: LaPO/LaPL, PPoO/POP, MOP/SPoM and MOLa/MLaL. Those groups involving TAGs with a higher number of total unsaturations (i.e. groups 4 and 5 in Figure 7.6) were not taken into account in order to establish the ratios due to their different degradation rate with time. As it can be seen in Table 7.3, there are no statistical differences among the four ratios of TAGs for

sei, fin and minke whales from *Balaenoptera* genus, whereas statistically different results were found for humpback whale (*Phocoena* genus) and harbour porpoise (*Megaptera* genus) whales. The ratios calculated with the TAGs detected in the unknown whale oil match-up those obtained for *Balaenoptera* species, thus confirming our hypothesis.

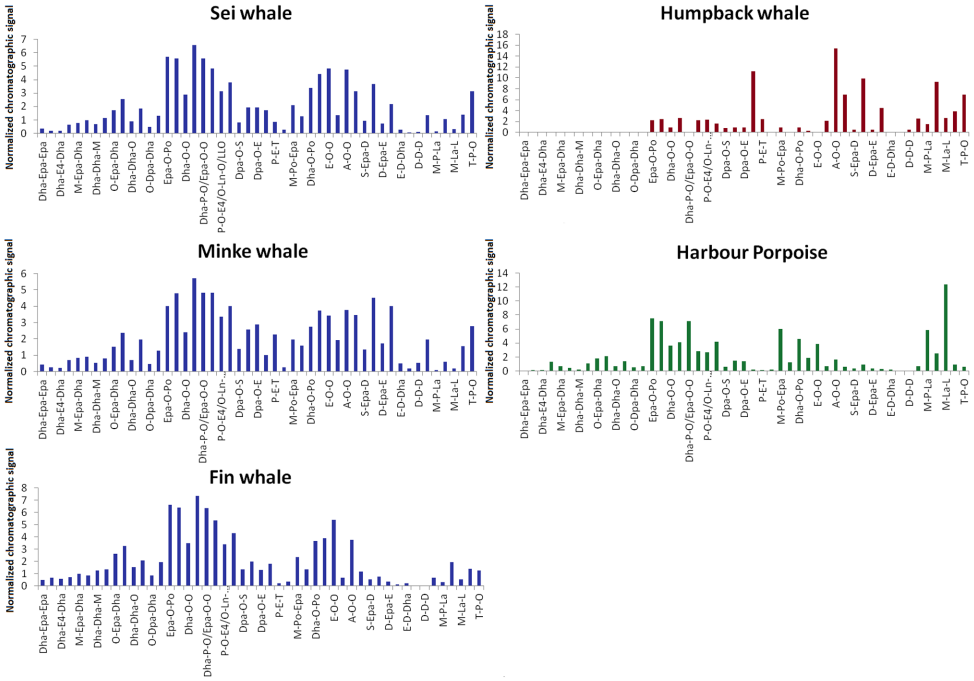


Figure 7.5: *Obtained profiles of TAGs in different whale blubber samples used as reference materials. Profiles in blue correspond to whale species from genus Balaenoptera; maroon profile to Megaptera and profile in green to Phocoena.*

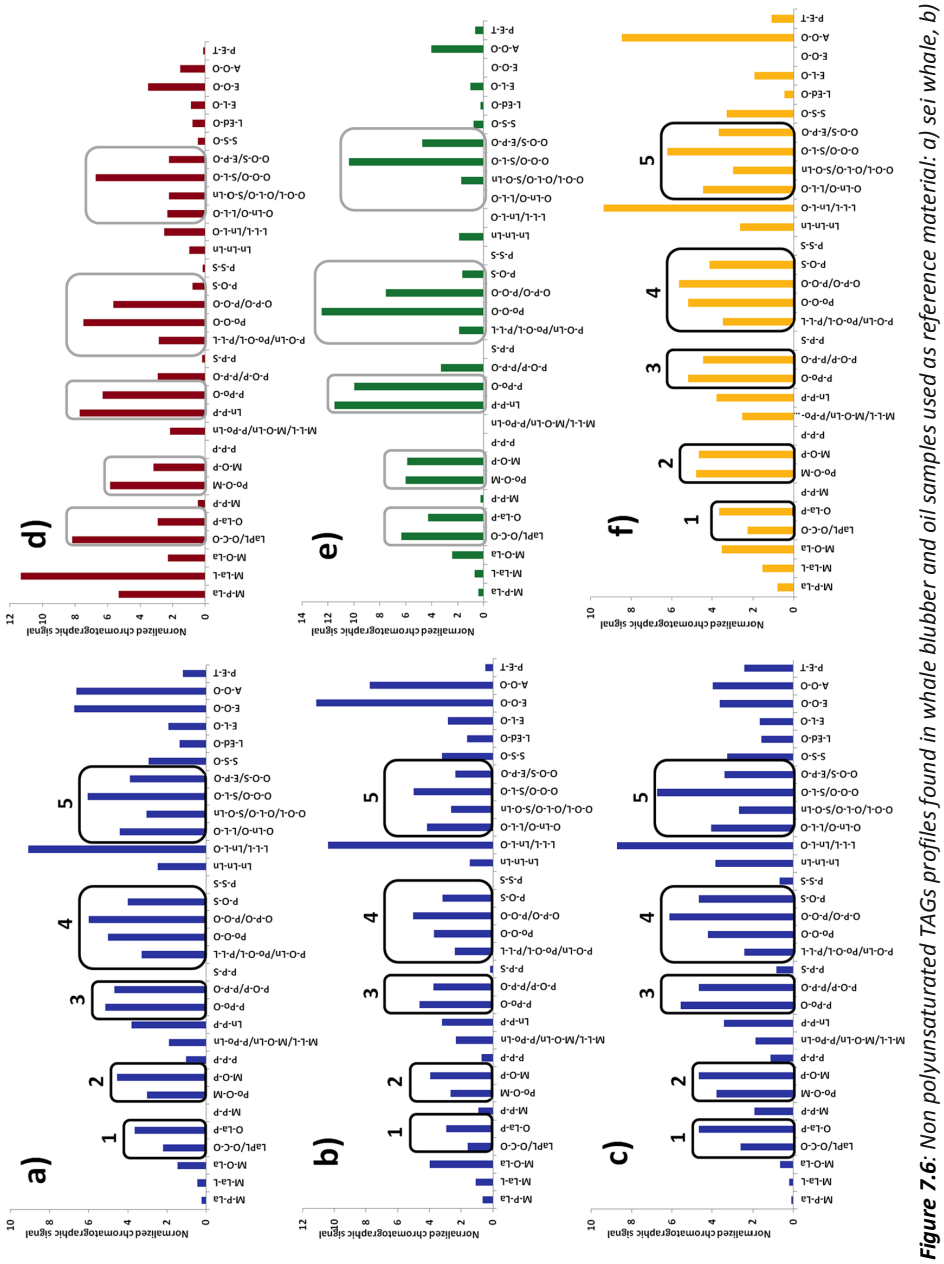


***Figure 7.6**: Non polyunsaturated TAGs profiles found in whale blubber and oil samples used as reference material: a) sei whale, b) fin whale, c) minke whale, d) harbour porpoise, e) humpback whale and d) whale oil from unknown origin.*

Table 7.3: *TAG ratios calculated for the reference materials and the archaeological samples.*

	Fresh reference materials						Archaeological ceramic samples
	Balaenoptera			*Megaptera*	*Phocoena*	Unknown whale oil sample	
Ratio	Sei whale	Fin whale	Minke whale	Humpback whale	Harbour porpoise		
$\frac{\mathbf{LaPO}}{\mathbf{LaPL}}$	1.65	1.87	1.80	0.67	0.36	1.62	1.64
$\frac{\mathbf{PPoO}}{\mathbf{POP}}$	1.10	1.23	1.20	3.02	2.15	1.17	0.82
$\frac{\mathbf{MOP}}{\mathbf{SPoM}}$	1.51	1.49	1.24	0.98	0.54	0.97	1.64
$\frac{\mathbf{MOLa}}{\mathbf{MLaL}}$	3.35	3.64	3.41	3.57	0.20	2.29	2.69

7.2.1.2. GC-MS fatty acid analysis

With the aim to obtain complementary information on the composition of the reference materials, fresh blubber samples were also analyzed by means of GC-MS. As expected, the identified FAs in the blubber and whale oil were in agreement with the lipid profile identified by HPLC-MS analysis. In this way, all the PUFAs, MUFAs and saturated FAs that form the acyl chains of the listed TAGs in Table 7.2 were found in all the blubber and whale oil samples being oleic ($C_{18:1}$), palmitoleic ($C_{16:1}$), palmitic ($c_{16:0}$), myristic ($C_{14:0}$), gondoic ($C_{20:1}$) and cetoleic ($C_{22:1}$) acids the most abundant ones, which is in agreement with the literature [41-46].

Similarly to the results obtained by HPLC-QToF analyses, the distributions of the FAs in the different reference materials were compared and very similar trends were found for all the studied whale samples. Some ratios of FAs were also calculated and palmitic to palmitoleic ratio showed different values according to the genus of the analyzed whale oil. In fact, higher concentrations of palmitic acid were found in all the species of *Balaenoptera*

genus whereas palmitoleic acid was found as the main FA in the rest of studied whales from different genus (i.e., *Phocoena* and *Megaptera*). These results are in concordance with the obtained results by means of HPLC-ESI-QToF presented in Table 7.3. The ratio PPoO/POP, which roughly corresponds to the ratio between palamitoleic and palmitic acids, is significantly higher in the case of the whales of genus *Megaptera* and *Phocoena* (3.02 and 2.15, respectively) whereas for *Balaenoptera* is lower (<1.23), as it was previously concluded from GC-MS analysis. This phenomenon is also observed for the MOP/SPoM ratio roughly corresponding to the palmitic to palmitoleic acid ratio. In the latter, values lower than 1 are obtained for *Megaptera* and *Phocoena* (0.98 and 0.54, respectively) and they are higher than 1 for *Balaenoptera* (> 1.24). The whale oil from unknown origin also matches *Balaenoptera* genus according to the GC-MS analysis. Consequently, the combination of GC-MS and HPLC-ESI-QToF is adequate to distinguish the genus of the blubber under study.

According to the literature, in addition to the already mentioned FAs, other biomarkers must be detected to meet the criteria established by Evershed et al. to enable the unambiguous identification of marine lipid [14]. These compounds are isoprenoid acids, particularly 4,8,12-trimethyltridecanoic acid (4,8,12-TMTD), 2,6,10,14-tetramethylpentadecanoic acid (pristanic acid) and 3,7,11,25-tetramethylhexadecanoic acid (phytanic acid), which are found at high abundance in aquatic oils and marine tissues (specially whale oils) but are absent in plants, since they originate from ingested phytoplankton [47]. The three isoprenoid FAs were detected in all the fresh whale blubber and oil samples.

7.2.2. Characterization of organic residues from archaeological ceramic samples

7.2.2.2. TAGs

The archaeological ceramics were subjected to the same treatment as the fresh samples used as reference material in order to determine the content in TAGs. As it has

been previously mentioned, no PUFAs were found in the chains of the identified TAGs due to their higher susceptibility to degrade with time with respect to the less unsaturated species. Nevertheless, all the modestly unsaturated TAGs found in fresh materials shown in Figure 7.6 were found in the ancient materials (Figure 9.2 in the annexes shows the HPLC-ESI-Q-ToF extract ion chromatograms referring to the mentioned TAGs in the archaeological samples). Figure 7.7 shows the distributions of the TAGs found in archaeological samples. In general terms, they match with the TAG profiles in boxes observed for the blubber samples from the whales of *Balaenoptera* genus (i.e., sei, fin and minke whales). However, some TAGs in the archaeological samples are lower with respect to the fresh material, as it is the case of the isobaric TAGs P-O-Ln + Po-O-L + L-L-P and O-Ln-O + L-L-O (marked with an asterisk in boxes 4 and 5 in Figure 7.7). This decay could be attributed to the larger number of unsaturations present in P-O-Ln + Po-O-L + L-L-P (5 or 4 unsaturations) and O-Ln-O + L-L-O (5 unsaturations) in comparison to the rest of TAGs (< 4 unsaturations).

As a matter of fact, the compounds with the highest degree of unsaturations (Ln-Ln-Ln and L-L-L + Ln-L-O with 9 and 6 unsaturations, respectively) (see Figure 7.7) were not identified in all the archaeological ceramics. It is known that not only the total number of unsaturations influences the degradation of the TAGs but also the unsaturation degree of each acyl chain that forms the TAG, since the oxidation rate depends significantly on the number of unsaturations per chain, increasing over ten times for each double bond present [48,49]. According to DeMan et al. [50], the oxidation rate of stearic acid compared to oleic (O), linoleic acid (L) and linolenic acid at 100°C is 1:100:1200:2500, but the precise relative rate at which this type of fats is decomposed depends on the temperature, and on oxygen and water availability. In any case, both linoleic and linolenic acids are often found at lower concentrations in comparison to the rest of FAs due to their faster degradation rate [50], as observed in the present work.

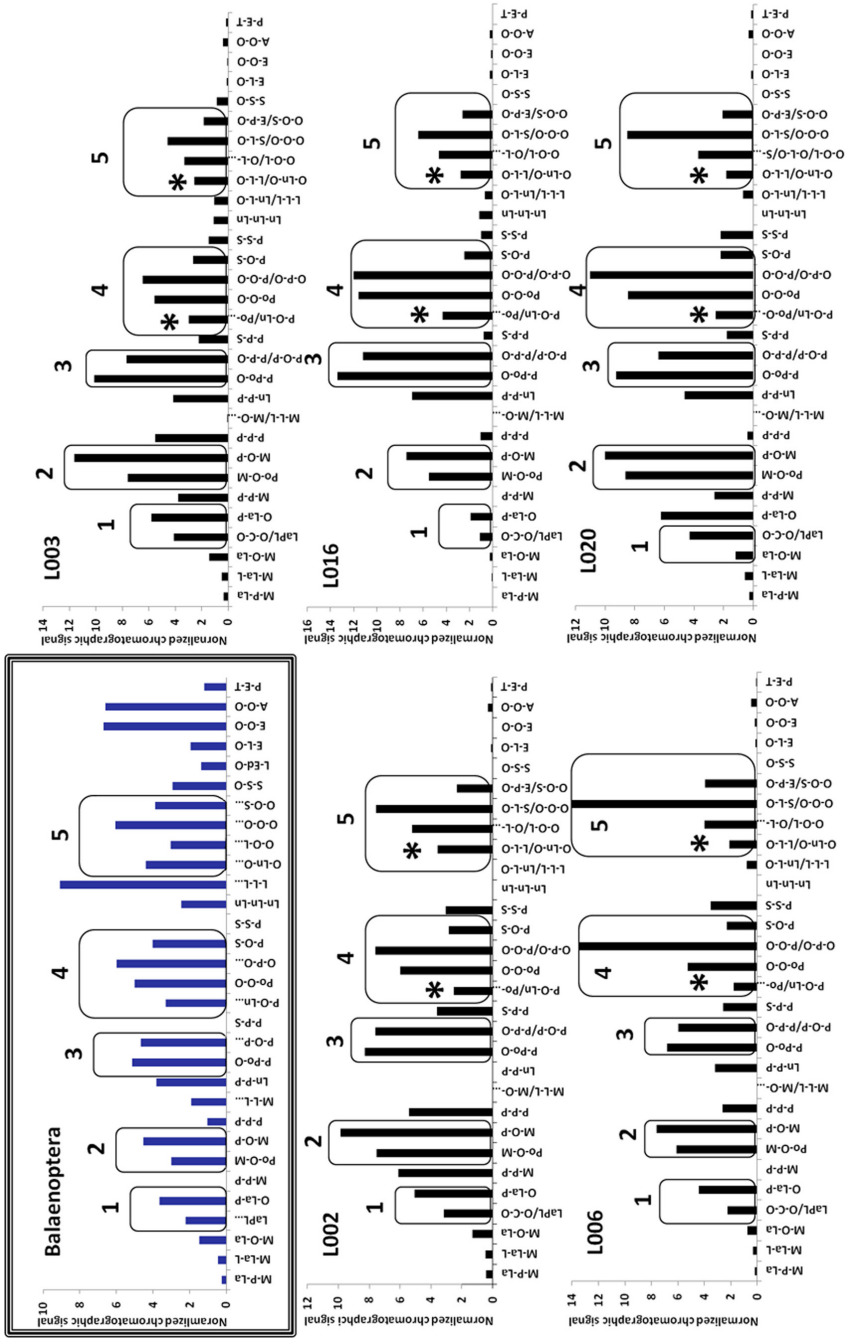


Figure 7.7: *Non polyunsaturated TAGs profiles for the archaeological ceramic samples in comparison with one of the Balaenoptera species (sei whale).*

Finally, as it can be seen in Table 7.3, there are no statistical differences between the ratios of LaPO/LaPL, PPoO/POP, MOP/SPoM and MOLa/MLaL for the blubber of *Balaenoptera* (sei, fin and minke whales) and those calculated for the archaeological materials. Thus, these ratios can be applied in the future both to fresh and archaeological materials in order to establish the source or the possible genus of whale from which oil and blubber have been extracted.

External surface of the ceramics was only analyzed by means of HPLC-ESI-QToF. The results showed that the TAG content in the external surface was scarce in comparison to that found in the internal part, especially for those TAGs containing a higher degree of unsaturation per chain (such as Ln-P-P) and a higher number of unsaturations (O-O-O and O-O-S). Some of these compounds were not identified in the external part of the vessels whereas they were present in the internal one (i.e., O-Ln-O + L-L-O and O-P-O). Thus, it could be concluded that the TAGs in the external surface have been partially or totally oxidized, whereas those in the internal surfaces were better preserved, as it is often reported in the literature [51]. Nevertheless, the absence of these compounds in the external surface may also be related to the lack of diffusion of the TAGs through the ceramics. Similar results were obtained in the previous chapter, where organic biomarkers related to whale oils were only detected in the inner part of the studied ceramic samples [52].

7.2.2.3. FAs and degradation products

The organic residues preserved in all the archaeological ceramic samples were mainly characterized by the presence, after saponification, of saturated FAs such as, lauric ($C_{12:0}$), myristic ($C_{14:0}$), palmitic ($C_{16:0}$), stearic ($C_{18:0}$), arachidic ($C_{20:0}$) and behenic ($C_{22:0}$) acids together with several MUFAs (oleic ($C_{18:1}$), palmitoleic ($C_{16:1}$), gondoic ($C_{20:1}$), cetoleic ($C_{22:1}$) and nervonic ($C_{24:1}$) acids). Diunsaturated FAs (linoleic ($C_{18:2}$) and eicosadienoic acid ($C_{20:2}$)) were only identified in sample L016 (see Figure 7.8).

Additionally, degradation of FAs during the burial period led to the appearance of several organic products used in other works as lipid biomarkers. For example, the oxidative cleavage of PUFAs can form dicarboxylic acids (diacids) [18,53]. Different mechanisms lead to the formation of these diacids being the oxidation of any FA with a double bond in position 9 (e.g. oleic acid) the most common transformation, generating azelaic acid (C9 diacid) that has been detected in samples L003, L006, L016 and L020. Azelaic acid or pimelic acid (C7 diacid found in L003, L016 and L020) can be formed from PUFAs, such as linoleic and linolenic acids or oleic and palmitoleic acids, respectively [4,6]. On the other hand, succinic acid (C4 diacid) and glutaric acid (C5 diacid), found in sample L016, can be formed from Epa and Dha, respectively [54]. Long-chain MUFAs such as gondoic ($C_{20:1}$) and cetoleic acids ($C_{22:1}$) (found in all the archaeological ceramic samples) with double bonds at position 11 are the primary source for diacids with more than 9 carbons, such as undecanedioic acid [6], which was also found in all the archaeological samples (see Figure 7.8).

Dihydroxy fatty acids (DHAs) were also detected in high abundance levels, especially in sample L016. The hydroxyl groups in these compounds are located in the position of the double bond in the precursor Z-monounsaturated fatty acids ($C_{14:0}$) [17,55,56]. Thus, selected DHAs are recognized biomarkers for detecting processed marine animal products [17,57]. Furthermore, strong alkaline treatments (such as the microwave assisted saponification used in this work) allow the complete release of these target compounds from potsherds by favoring the cleavage of ester linkages of the compounds with the matrix [17]. In all the ceramic samples (except in sample L006), several DHAs such as 9,10-dihydroxyhexadecanoic acid, 9,10- dihydroxyoctadecanoic acid, 9,10-dihydroxyeicosanoic acid, 11,12-dihydroxyeicosanoic acid and 11,12-dihydroxydocosanoic acid were detected (see Figure 7.8). They derived from the precursors palmitoleic, oleic, gadoleic, gondoic and cetoleic acids, respectively. Amongst the detected DHAs, the high abundance of 11,12-dihydroxydocosanoic acid (deriving from cetoleic acid) supports the marine origin of the lipid residues preserved in the studied archaeological samples [4].

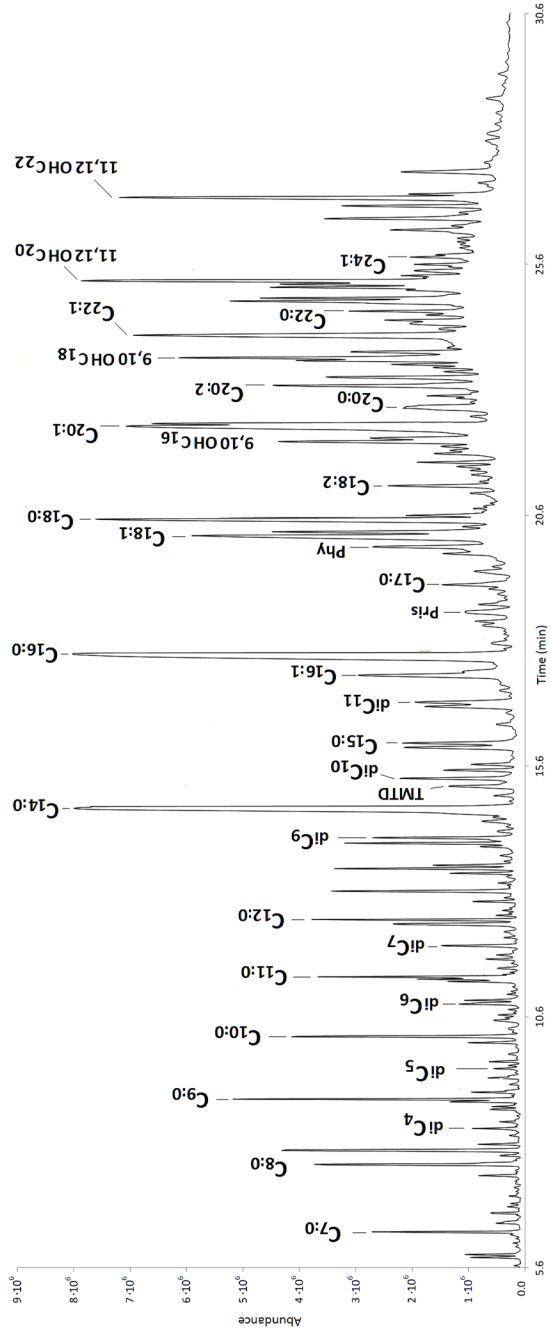


Figure 7.8: *TIC of the trimethylsilylated acid fraction of sample L016 obtained by GC–MS. Cx:y are fatty acids of chain length x and degree of unsaturation y; diCx are α,ω-dicarboxylic fatty acids of chain length x; 9,10OHC16 is 9,10-dihydroxyhexadecanoic acid; 9,10OHC18 is 9,10-dihydroxyoctadecanoic acid; 11,12OHC20 is 11,12-dihydroxyeicosanoic acid; 11,12OHC22 is 11,12-dihydroxydocosanoic acid; TMTD is 3,8,12-trimethyltridecanoic acid; Pris is pristanic acid; Phy is phytanic acid.*

The profile of the FAs found in both the fresh reference blubber samples and the archaeological ceramics was compared to study possible similarities. The FA profiles found for archaeological samples were comparable to those found for sei whale, fin whale and minke whale, all belonging to *Balaenoptera* genus (see Figure 7.9.a). All the detected compounds followed the same trend in all the analyzed samples except for the unsaturated FAs (i.e., palmitoleic, oleic, gondoic and cetoleic acids), which showed decay due to the ageing of the samples. This is evidenced by the appearance of degradation products such as pimelic, azelaic, sebacic and undecanedioic acid. Moreover, the comparison of the profiles obtained for the archaeological ceramics to the ones obtained for *Phocoena* and *Megaptera* fresh blubber samples (see Figure 7.9.b) showed a pronounced difference for palmitoleic acid as well as for palmitic and stearic acids.

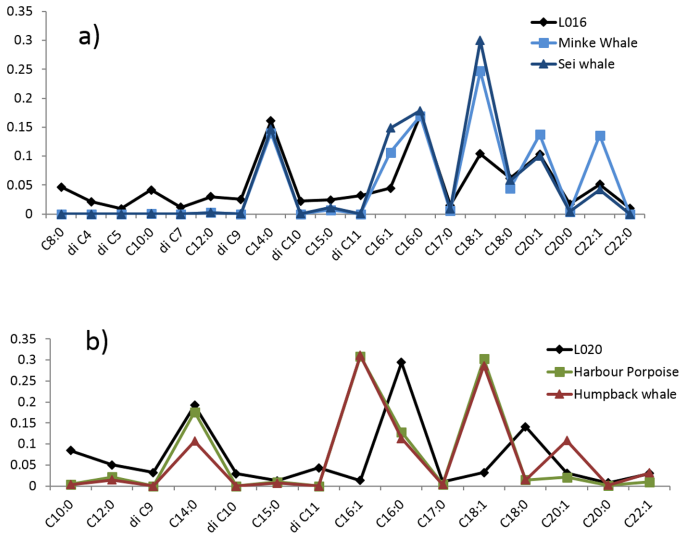


Figure 7.9: *Comparison of the FA profile between the fresh reference materials and the archaeological ceramics: a) sample L016 vs. whale species of Balaenoptera genus and b) sample L020 vs. Phocoena and Megaptera.* Cx:y are fatty acids of chain length x and degree of unsaturation y; diCx are α,ω-dicarboxylic fatty acids of chain length x.

7.2.2.4. Isoprenoid compounds and sterols

The isoprenoid biomarkers 4,8,12-TMTD, pristanic acid and phytanic acid were all detected in all the archaeological ceramic samples except for sample L002. In this way, the marine lipid identification (see Figure 7.8) was accomplished using the already mentioned criteria (see section 7.2.1.2.).

Cholesterol and squalene were found in all the blubber samples. Due to the higher stability with time of the former, it can survive in the walls of the archaeological ceramics for long periods. In fact, cholesterol, which is the main biomarker of animal fats, was detected in the neutral fraction of four of the five samples (L002, L003, L006, and L016), together with its oxidation product, cholesta-3,5-dien7-one (only found in sample L016). Squalene was not found in ancient samples, since it rapidly degrades and its detection could be the result of handling contamination [4].

Pristane is another isoprenoid that was detected in the neutral fraction of both fresh and archaeological samples and has been used in the present work as a marine biomarker in archaeological ceramics for the first time. Blumer et al. isolated pristane in high concentrations from the copepod *Calanus finmarchicus,* small crustaceans found in the sea, and after subsequent analysis on a large variety of marine organisms it appeared likely that *Calanus* was the main source of pristane for marine predators such as sharks or whales [58]. Blumer pinpointed that phytol can be converted into pristane by these copepods and this way it becomes part of the marine lipid pool [58,59]. Additionally, pristane is a specific product of organisms of limited geographical occurrence. Large populations of *Calanus finmarchicus* are present in the coasts of Labrador and Newfoundland due to the large amount of phytoplankton accumulated in these regions [60]. Thus, it can be hypothesized that pristane coming from these crustaceans was accumulated in the fat of the whales and survived in the walls of the ceramic containers due to its high stability.

7.3. Conclusions

To our knowledge this is the first time that HPLC-ESI-QToF is applied to the analysis of whale blubber and oil to characterize the structure of the TAGs. Despite not being able to assign the exact position of each acyl chain in each TAG due to the impossibility to perform MS^3 experiments, the obtained results allowed us to distinguish among whales of different genus by using their TAG profiles as fingerprints. In fact, it has been possible to identify the most likely genus (*Balaenoptera*) of the unknown whale from which a reference oil sample was extracted. These results could be complemented in future works by MS^n or even ^{13}C-NMR analyses to study the regioisomeric distribution of the FAs forming the complex TAG profile in whale oil.

On the other hand, this work presents also the determination of the TAG profile of organic residues preserved in archaeological ceramic samples. The use of TAG distributions as well as specific TAG ratios (i.e., PPoO/POP and MOP/SPoM) can provide clues to identify the genus of the analyzed organic residue. In this work, the profile and ratios of these TAGs agree with those found for the fresh blubber samples of the whale species of *Balaenoptera* genus (fin whale, sei whale and minke whale) used as reference materials. Thus, it can be suggested the possible genus of some of the whales that Basque whalers used to capture. In any case, this analysis should be expanded to a higher number of samples of blubber from different species (of different genus) as well as to other archaeological ceramic samples, since the range of whales that Basque people used to hunt is wider. McLeod et al. analyzed whale bones from several Basque whaling stations in the Strait of Belle Isle and Gulf of St. Lawrence by extracting their DNA [61]. In that work, a single right whale (genus *Eubalaena*) bone and 203 bowhead whale (genus *Balaena*) bones from at least 72 individuals were identified. Thus, blubber samples from these species should be analyzed in subsequent experiments in order to study differences and similarities with species from *Balaenoptera* genus. Although archaeological and historical data strongly suggest that during this period

Basque whalers sailed to Canada to bring the whale oil back to Biscay [9], it could be also hypothesized that the oil in the analyzed archaeological ceramics corresponds to whales captured in the Biscayan coasts.

The application of GC-MS analysis to both fresh and archaeological materials confirmed the obtained information by HPLC-ESI-QToF using the profiles of the detected FAs. Additionally, the identified isoprenoid biomarkers (pristanic, phytanic and 4,8,12-TMTD acids) together with the degradation products (dicarboxylic fatty acids and dihydroxy fatty acids) meet the criteria established in the literature for the confirmation of the presence of marine lipids in ceramic vessels. Finally, pristane has been suggested as a possible new marine biomarker due to its presence in fresh material and its high stability in archaeological ceramics.

The analytical approach that combines the use of many analytical techniques was essential in order to get information about known and unknown organic biomarkers. According to the results obtained in this work using complementary analytical approaches, we found that the large ceramic jars found in Lekeitio were used as whale oil containers.

7.4. References

[1] R.P. Evershed, S.N. Dudd, S. Charters, H. Mottram, A.W. Stott, A. Raven, P.F. Van Bergen, H.A. Bland, Lipids as carriers of anthropogenic signals from prehistory, Philos. Trans. R. Soc. B Biol. Sci. 354 (1999) 19-31.

[2] J. Baeten, B. Jervis, D. De Vos, M. Waelkens, Molecular evidence for the mixing of Meat, Fish and Vegetables in Anglo-Saxon coarseware from Hamwic, UK, Archaeometry. 55 (2013) 1150-1174.

[3] R.P. Evershed, A.W. Stott, A. Raven, S.N. Dudd, S. Charters, A. Leyden, Formation of long-chain ketones in ancient pottery vessels by pyrolysis of acyl lipids, Tetrahedron Lett. 36 (1995) 8875-8878.

[4] C. Heron, G. Nilsen, B. Stern, O. Craig, C. Nordby, Application of lipid biomarker analysis to evaluate the function of 'slab-lined pits' in Arctic Norway, J. Archaeol. Sci. 37 (2010) 2188-2197.

[5] M. Correa-Ascencio, R.P. Evershed, High throughput screening of organic residues in archaeological potsherds using direct acidified methanol extraction, Anal. Methods. 6 (2014) 1330-1340.

[6] C. Solazzo, D. Erhardt, Sea Mammal Oil and Cooking Practices in the Arctic, in: H. Barnard, J.W. Eerkens (Eds.), Theory and Practice of Archaeological Residues Analysis, Archaeopress, Oxford, 2007, 161-178.

[7] M.P. Colombini, F. Modugno, E. Ribechini, Organic mass spectrometry in archaeology: Evidence for Brassicaceae seed oil in Egyptian ceramic lamps, J. Mass Spectrom. 40 (2005) 890-898.

[8] J.A. Hernandez, J. Nuñez, Balleneros Vascos Del Siglo XVI. Estudio Arqueológico Y Contexto Histórico (Chateau Bay, Labrador, Canadá), 1ª ed., Servicio Central de Publicación del Gobierno Vasco, Vitoria-Gasteiz, 1992.

[9] A. Azkarate, S. Escribano-Ruiz, Basque Fisheries in Eastern Canada, a Special Case of Cultural Encounter in the Colonizing of North America, in: Archaeology of Culture Contact and Colonialism in Spanish and Portuguese America, Springer, Switzerland, 2015, 239-256.

[10] E.D. Morgan, The Composition of Fatty Materials from a Thule Eskimo Site on Herschel Island, Arctic. 36 (1983) 356-360.

[11] J.A. Azpiazu, La Empresa Vasca De Terranova : Entre El Mito Y La Realidad, Donostia Ttarttalo, Donostia, 2008.

[12] A. Azkarate, S. Escribano-Ruiz, La pesca transatlántica vasca en el universo colonial: un ejemplo paradigmático de interacción multicultural. Revista de arqueología americana. 32 (2014) 209-228.

[13] M. Patrick, A.J. de Koning, A.B. Smith, Gas liquid chromatographic analysis of fatty acids in food residues from ceramics found in the southwestern Cape, South Africa, Archaeometry. 27 (1985) 231-236.

[14] R.P. Evershed, M.S. Copley, L. Dickson, F.A. Hansel, Experimental evidence for the processing of marine animal products and other commodities containing polyunsaturated fatty acids in pottery vessels, Archaeometry. 50 (2008) 101-113.

[15] F.A. Hansel, M.S. Copley, L.A.S. Madureira, R.P. Evershed, Thermally produced ω-(o-alkylphenyl)alkanoic acids provide evidence for the processing of marine products in archaeological pottery vessels, Tetrahedron Lett. 45 (2004) 2999-3002.

[16] O.E. Craig, H. Saul, A. Lucquin, Y. Nishida, K. Taché, L. Clarke, A. Thompson, D.T. Altoft, J. Uchiyama, M. Ajimoto, K. Gibbs, S. Isaksson, C.P. Heron, P. Jordan, Earliest evidence for the use of pottery, Nature. 496 (2013) 351-354.

[17] F.A. Hansel, R.P. Evershed, Formation of dihydroxy acids from Z-monounsaturated alkenoic acids and their use as biomarkers for the processing of marine commodities in archaeological pottery vessels, Tetrahedron Lett. 50 (2009) 5562-5564.

[18] M.S. Copley, H.A. Bland, P. Rose, M. Horton, R.P. Evershed, Gas chromatographic, mass spectrometric and stable carbon isotopic investigations of organic residues of plant oils and animal fats employed as illuminants in archaeological lamps from Egypt, Analyst. 130 (2005) 860-871.

[19] O.E. Craig, M. Forster, S.H. Andersen, E. Koch, P. Crombé, N.J. Milner, B. Stern, G.N. Bailey, C.P. Heron, Molecular and isotopic demonstration of the processing of aquatic prooducts in northern European prehistoric pottery, Archaeometry. 49 (2007) 135-152.

[20] E. Ribechini, F. Modugno, M.P. Colombini, R.P. Evershed, Gas chromatographic and mass spectrometric investigations of organic residues from Roman glass unguentaria, J. Chromatogr. A. 1183 (2008) 158-169.

[21] E. Ribechini, F. Modugno, C. Baraldi, P. Baraldi, M.P. Colombini, An integrated analytical approach for characterizing an organic residue from an archaeological glass bottle recovered in Pompeii (Naples, Italy), Talanta. 74 (2008) 555-561.

[22] M.P. Colombini, F. Modugno, E. Ribechini, Direct exposure electron ionization mass spectrometry and gas chromatography/mass spectrometry techniques to study organic coatings on archaeological amphorae, J. Mass Spectrom. 40 (2005) 675-687.

[23] R.P. Evershed, Biomolecular Archaeology and Lipids, World Archaeology. 25 (1993) 74-93.

[24] I. Lantos, J.E. Spangenberg, M.A. Giovannetti, N. Ratto, M.S. Maier, Maize consumption in pre-Hispanic south-central Andes: Chemical and microscopic evidence from organic residues in archaeological pottery from western Tinogasta (Catamarca, Argentina), J. Archaeol. Sci. 55 (2015) 83-99.

[25] V. Papakosta, R.H. Smittenberg, K. Gibbs, P. Jordan, S. Isaksson, Extraction and derivatization of absorbed lipid residues from very small and very old samples of ceramic potsherds for molecular analysis by gas chromatography-mass spectrometry (GC-MS) and single compound stable carbon isotope analysis by gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS), Microchem. J. 123 (2015) 196-200.

[26] E. Manzano, A. García, E. Alarcón, S. Cantarero, F. Contreras, J.L. Vílchez, An integrated multianalytical approach to the reconstruction of daily activities at the Bronze Age settlement in Peñalosa (Jaén, Spain), Microchem. J. 122 (2015) 127-136.

[27] J. La Nasa, M. Zanaboni, D. Uldanck, I. Degano, F. Modugno, H. Kutzke, E.S. Tveit, B. Topalova-Casadiego, M.P. Colombini, Novel application of liquid chromatography/mass spectrometry for the characterization of drying oils in art: Elucidation on the composition of original paint materials used by Edvard Munch (1863–1944), Anal. Chim. Acta. 896 (2015) 177-189.

[28] K. Romanus, J. Poblome, K. Verbeke, A. Luypaerts, P. Jacobs, D. De Vos, M. Waelkens, An evaluation of analytical and interpretative methodologies for the extraction and identification of lipids associated with pottery sherds from the site of Sagalossos, Turkey, Archaeometry. 49 (2007) 729-747.

[29] K. Romanus, J. Baeten, J. Poblome, S. Accardo, P. Degryse, P. Jacobs, D. De Vos, M. Waelkens, Wine and olive oil permeation in pitched and non-pitched ceramics: relation with results from archaeological amphorae from Sagalassos, Turkey, J. Archaeol. Sci. 36 (2009) 900-909.

[30] F. Saliu, I. Degano, M.P. Colombini, Identification of triacylglycerols in archaelogical organic residues by core-shell reversed phase liquid chromatography coupled to electrospray ionization-quadrupole-time of flight mass spectrometry, J. Chromatogr. A. 1346 (2014) 78-87.

[31] B. Stern, C. Heron, M. Serpico, J. Bourriau, A comparison of methods for establishing fatty acid concentration gradients across potsherds: a case study using Late Bronze Age Canaanite amphorae, Archaeometry. 42 (2000) 399-414.

[32] F. Saliu, F. Modugno, M. Orlandi, M.P. Colombini, HPLC-APCI-MS analysis of triacylglycerols (TAGs) in historical pharmaceutical ointments from the eighteenth century, Anal. Bioanal. Chem. 401 (2011) 1785-1800.

[33] S. Balasubramanian, J.D. Allen, A. Kanitkar, D. Boldor, Oil extraction from Scenedesmus obliquus using a continuous microwave system – design, optimization, and quality characterization, Bioresour. Technol. 102 (2011) 3396-3403.

[34] P. Dugo, T. Kumm, B. Chiofalo, A. Cotroneo, L. Mondello, Separation of triacylglycerols in a complex lipidic matrix by using comprehensive two-dimensional liquid chromatography coupled with atmosheric pressure chemical ionization mass spectrometric detection, J. Sep. Sci. 29 (2006) 1146-1154.

[35] C. Baiocchi, C. Medana, F. Dal Bello, V. Giancotti, R. Aigotti, D. Gastaldi, Analysis of regioisomers of polyunsaturated triacylglycerols in marine matrices by HPLC/HRMS, Food Chem. 166 (2015) 551-560.

[36] P. Dugo, T. Kumm, M.L. Crupi, A. Cotroneo, L. Mondello, Comprehensive two-dimensional liquid chromatography combined with mass spectrometric detection in the analyses of triacylglycerols in natural lipidic matrixes, J. Chromatogr. A. 1112 (2006) 269-275.

[37] G. Jiao, J.P.M. Hui, I.W. Burton, M.-. Thibault, C. Pelletier, J. Boudreau, N. Tchoukanova, B. Subramanian, Y. Djaoued, S. Ewart, J. Gagnon, K.V. Ewart, J. Zhang, Characterization of shrimp oil from Pandalus borealis by high performance liquid chromatography and high resolution mass spectrometry, Mar. Drugs. 13 (2015) 3849-3876.

[38] A. Tu, Q. Ma, H. Bai, Z. Du, A comparative study of triacylglycerol composition in Chinese human milk within different lactation stages and imported infant formula by SFC coupled with Q-TOF-MS, Food Chem. 221 (2017) 555-567.

[39] J. La Nasa, E. Ghelardi, I. Degano, F. Modugno, M.P. Colombini, Core shell stationary phases for a novel separation of triglycerides in plant oils by high performance liquid chromatography with electrospray-quadrupole-time of flight mass spectrometer, J. Chromatogr. A. 1308 (2013) 114-124.

[40] L.C. Herrera, M.A. Potvin, J.E. Melanson, Quantitative analysis of positional isomers of triacylglycerols via electrospray ionization tandem mass spectrometry of sodiated adducts, Rapid Commun. Mass Spectrom. 24 (2010) 2745-2752.

[41] M. Yamato, H. Koopman, M. Niemeyer, D. Ketten, Characterization of lipids in adipose depots associated with minke and fin whale ears: Comparison with "acoustic fats" of toothed whales, Mari. Mamm. Sci. 30 (2014) 1549-1563.

[42] D. Ruchonnet, M. Boutoute, C. Guinet, P. Mayzaud, Fatty acid composition of Mediterranean fin whale Balaenoptera physalus blubber with respect to body heterogeneity and trophic interaction, Mar. Ecol. Prog. Ser. 311 (2006) 165-174.

[43] E.D. Morgan, C. Edwards, S.A. Pepper, Analysis of the fatty debris from the wreck of a Basque whaling ship at red bay, Labrador, Archaeometry. 34 (1992) 129-133.

[44] H.N. Koopman, Phylogenetic, ecological, and ontogenetic factors influencing the biochemical structure of the blubber of odontocetes, Mar. Biol. 151 (2007) 277-291.

[45] M. Linder, N. Belhaj, P. Sautot, E.A. Tehrany, From krill to whale: An overview of marine fatty acids and lipid compositions, OCL Ol. Corps Gras Lipides. 17 (2010) 194-204.

[46] E. Olsen, O. Grahl-Nielsen, Blubber fatty acids of minke whales: Stratification, population identification and relation to diet, Mar. Biol. 142 (2003) 13-24.

[47] R.G. Ackman, S.N. Hooper, Examination of isoprenoid fatty acids as distinguishing characteristics of specific marine oils with particular reference to whale oils, Comparative Biochemistry and Physiology. 24 (1968) 549-565.

[48] J.W. Eerkens, Organic Residue Analysis and the Decomposition of Fatty Acids in Ancient Potsherds, in: H. Barnard, J.W. Eerkens (Eds.), Theory and Practice of Archaeological Residue Analysis, Archaeopress, Oxford, 2007, 90-96.

[49] I. Degano, J. La Nasa, E. Ghelardi, F. Modugno, M.P. Colombini, Model study of modern oil-based paint media by triacylglycerol profiling in positive and negative ionization modes, Talanta. 161 (2016) 62-70.

[50] J.M. DeMan, Chemical and Physical Properties of Fatty Acids, in: C.K. Chow (Ed.), Fatty Acids in Foods and their Health Implications, Marcel Dekker Inc., New York, 1992, 17-45.

[51] A. Charrié-Duhaut, J. Connan, M. Darnell, J. Spangenberg, E. Szymczyk, A. Bissada, P. Albrecht, Molecular and isotopic characterization of organic samples from the wreck of the Saint-Etienne merchant ship (XVIIIth century): Identification of pitch, fat, hair and sulfur, Org. Geochem. 40 (2009) 647-665.

[52] L. Blanco-Zubiaguirre, M. Olivares, K. Castro, J.G. Iñañez, J.M. Madariaga, An alternative analytical method based on ultrasound micro bath hydrolysis and GC-MS analysis for the characterization of organic biomarkers in archaeological ceramics, Anal. Bioanal. Chem. 408 (2016) 8001-8012.

[53] M.P. Colombini, G. Giachi, F. Modugno, E. Ribechini, Characterisation of organic residues in pottery vessels of the Roman age from Antinoe (Egypt), Microchem. J. 79 (2005) 83-90.

[54] S. Passi, M. Picardo, C. De Luca, M. Nazzaro-Porro, L. Rossi, G. Rotilio, Saturated dicarboxylic acids as products of unsaturated fatty acid oxidation, Biochimica et Biophysica Acta (BBA) - Lipids and Lipid Metabolism. 1168 (1993) 190-198.

[55] R.P. Evershed, Organic residue analysis in archaeology: The archaeological biomarker revolution, Archaeometry. 50 (2008) 895-924.

[56] A.J. Mukherjee, A.M. Gibson, R.P. Evershed, Trends in pig product processing at British Neolithic Grooved Ware sites traced through organic residues in potsherds, J. Archaeol. Sci. 35 (2008) 2059-2073.

[57] M. Roffet-Salque, J. Dunne, D.T. Altoft, E. Casanova, L.J.E. Cramp, J. Smyth, H.L. Whelton, R.P. Evershed, From the inside out: Upscaling organic residue analyses of archaeological ceramics, Journal of Archaeological Science: Reports. (2016).

[58] M.A.X. Blumer, M.M. Mullin, D.W. Thomas, Pristane in zooplankton, Science. 140 (1963) 974.

[59] M. Blumer, M.M. Mullin, D.W. Thomas, Pristane in the marine environment, Helgolander Wiss. Meeresunters. 10 (1964) 187-201.

[60] W. Melle, J. Runge, E. Head, S. Plourde, C. Castellani, P. Licandro, J. Pierson, S. Jonasdottir, C. Johnson, C. Broms, H. Debes, T. Falkenhaug, E. Gaard, A. Gislason, M. Heath, B. Niehoff, T.G. Nielsen, P. Pepin, E.K. Stenevik, G. Chust, The North Atlantic Ocean as habitat for Calanus finmarchicus: Environmental factors and life history traits, Prog. Oceanogr. 129 (2014) 244-284.

[61] B.A. McLeod, M.W. Brown, M.J. Moore, W. Stevens, S.H. Barkham, M. Barkham, B.N. White, Bowhead whales, and not right whales, were the primary target of 16th- to 17th-century Basque whalers in the western North Atlantic, Arctic. 61 (2008) 61-75.

Chapter 8

Conclusions

"Don't adventures ever have an end? I suppose not. Someone else always has to carry on the story."

J.R.R. Tolkien – The Lord of the Rings

In order to have another view of the work described in this PhD Thesis, it is time to look back to see the main achievements and to discuss them critically. Although the first checklist will be to verify the accomplishment of the main objectives of this work, we would like to share as well those items that we learned along this work as well as some others that would be adequate to be taken into account in further works.

The two main objectives included in this PhD Thesis, namely the development of analytical procedures for the analysis of organic markers in several landscape and heritage samples and their application to some real cases to find out chemical evidences, were in general terms completed. Therefore, we will summarize the most relevant conclusions obtained in each topic.

The first tasks were based on the development and validation of a set of methods for quantifying organic biomarkers in several matrices. Concretely, four analytical methods were developed for the determination of a wide range of organic biomarkers in samples belonging to different fields of application (i.e., geological, environmental and archaeological samples). All the methods were based mainly on the use of GC-MS to identify known and unknown biomarkers. Adequate sensitivity was achieved to determine dicarboxylic acids in mortars or in wine residues as well as fatty acids and derivatives in archaeological ceramics, after their sylilation using BSTFA. The possibility of using large volume injection coupled to GC-MS allowed also to get good detection limits to quantify PAHs in beachrock samples but also to find other organic biomarkers related to beachrock formation.

Sonication is widely accepted in the literature to extract a wide range of organic biomarkers from solid matrices, but according to the observations of this work, focused ultrasound solid liquid extraction (FUSLE) has shown up as an efficient alternative to that aim. In fact, this approach allows the isolation of the target compounds using short

extraction periods and low amounts of solvent (7 mL for FUSLE and 3 mL for UMB). In any case, quantitative results are only guaranteed if appropriate solvents are used. Although, in a first approach, chloroform: methanol mixtures can be used as adequate extraction solvent, we observed that the optimization of extraction solvent nature is a must in order to get quantitative results. We concluded that pure chlorinated solvents (i.e., dichloromethane) were required to isolate PAHs from carbonate matrices (i.e., beachrock samples), more polar mixtures (i.e., AcN:H_2O 2:1, *v/v*) were needed to extract short-chain dicarboxylic acids from mortars and wine residues, whereas hydro-alcoholic mixtures (5 % KOH in MeOH:H_2O, 2:3 *v/v*) were required to hydrolyze fatty acids and promote their extraction from archaeological ceramic samples. It is noteworthy that the quantitative cleavage of organic biomarkers, especially those that can have ionic interactions with the clay of archaeological ceramics, is often difficult; but we were able to obtain accurate concentrations using appropriate surrogates in each case.

Later on, we found the first hurdle in our way: the preconcentration of the target analytes in presence of chemically-similar interfering compounds. LLE is by far the most employed extraction technique to this aim, and we used it successfully to preconcentrate fatty acids and related compounds using *n*-hexane as extraction solvent in order to isolate both neutral and acid fractions. The use of *n*-hexane avoided the problems of salt appearance after the evaporation of the extracts when using diethyl ether as extraction solvent. However, LLE largely failed when we aimed to preconcentrate short-chain dicarboxylic acids from aqueous matrices such as marine aerosol or FUSLE extracts containing these compounds. We tried several options to enhance the efficiency of LLE (i.e., ion-pair formation, changing the ionic strength of the extraction media, etc.), but the results were not satisfactory enough to detect the organic markers at trace levels. Hopefully, we could deal with it using solid phase extraction, concretely, using mixed-mode cartridges. Strong anion exchange-mixed-mode sorbents allow selective extractions of acid compounds with a wide range of polarities through a dual mechanism (ion-exchange and reversed-

phase mode). Although we tested different reversed phase sorbents, only those assays performed with mixed-mode sorbents rendered quantitative results to isolate short-chain dicarboxylic acids in marine aerosol and mortar samples (from C2 oxalic acid to C10 sebacic acid) and in wine residues (including tartaric, malic and citric acid) even in presence of interfering salts.

This was not the first time that scientist tackled the development of analytical methods to determine organic markers, but, eventually in this PhD thesis we wanted to find out the optimum working conditions and to highlight the importance of method validation in terms of accuracy, precision and limits of detection. Therefore, we were able to propose new accurate, precise and sensitive methods, as other alternative to the established in the literature, to determine organic biomarkers in geological, environmental and archaeological samples.

Once we gained knowledge about the properties of the target organic compounds and the samples we were analyzing, and once we got reliable methods for the analysis of organic markers at trace levels, we faced our second challenge: to identify known and unknown organic markers in real samples and to relate them with the "history" hidden in the analyzed samples.

In this sense, we were able to detect 16 EPA priority PAHs in beachrock samples collected in the coast of Biscay. The occurrence of this phenomena in the sampled location is quite weird since they are mainly formed in warm climates. Hence, the identification of organic biomarkers was crucial in order to have more knowledge about the past environments and the biophysical-chemical conditions in which the beachrock occurred. The occurrence of gammacerane, 17α(H),21β(H),28,30-bisnorhopane, 17α(H),21β(H)-norhopane, 22S-17α(H),21β(H)-homohopane, 22R-17α(H),21β(H)-homohopane, 22S-17α(H),21β(H)-bishomohopane and 22R-17α(H),21β(H)-bishomohopane indicated that the

microbial activity and suboxic depositional environments, together with the high input of organic contaminants, may have promoted the accelerated formation of beachrock at this unusual temperate latitude setting. These preliminary results allowed starting new research lines of investigation based on the characterization of the organic fraction of beachrock samples from different origins, aiming to get more insights into the beachrock formation phenomena.

The development of mixed-mode SPE coupled to GC-MS aimed us to determine if short-chain organic compounds present in marine aerosol samples can also affect the mortars of a 20th century historical building (Punta Begoña Galleries, Getxo) located in a coastal environment. According to our observations, it was concluded that harbor activities in the surrounding area may be the responsible of the concentrations of dicarboxylic acids found in both matrices. In fact, higher concentrations of short-chain dicarboxylic acids (especially oxalic acid) were found in the mortars and marine aerosols sampled in the zone of the galleries more exposed to the industrial harbor, whereas lower concentrations (values < LOD for oxalic acid) were found in samples oriented to the less exposed sampling point. It is also known that these compounds can react with carbonaceous building materials once deposited. In the specific case of oxalic acid, a reaction takes place with the calcium carbonate to form the highly insoluble calcium oxalate, causing damage to the building material. Hence, taking into account that the mentioned reaction takes place in not more than two days, the detection of oxalic acid in its free form reveals a daily input of WSOCs. After these results, we think that a long-term monitoring of the zone to study the long-term impact of those activities on the galleries of Punta Begoña seems necessary.

We cannot hide that the analysis of small organic molecules was a great analytical challenge for us. However, the efforts made to develop an efficient analytical strategy to determine these compounds even in presence of interfering salts, rendered very interesting information in some archaeological remains. We were lucky to analyze archaeological

potsherds dating back to II-I BCE that were supposed to be used as wine containers. To that aim, we used the developed FUSLE-mixed mode SPE-GC-MS procedure. Despite the antiquity of the archaeological samples, tartaric, malic, succinic, fumaric, citric and syringic acids were detected in the analyzed samples. Moreover, the presence of dehydroabietic and 7-oxo-dehydroabietic acids was also confirmed in the organic residues. These two biomarkers highlight the presence of pine resin, used in order to coat the interior surface of the ceramic artifacts or to act as antioxidant and preserve wine. But besides the wine-related organic markers, we detected some other markers more related with the presence of vegetable oil: oleic and linoleic acid. These chemical evidences suggested us to know that the analyzed amphora were used for multiple purposes. The archaeological conclusions extracted from these chemical evidences should be interpreted by specialist scientist, but we were happy to be able to detect them even in archaeological ceramic remains where the residue was not visible.

To analyze ceramic samples without visible residues is the most common scenario that we need to handle. Moreover, most of the archaeological ceramics are cleaned before arriving to the lab. In fact, we dealt with these two problems when we analyzed archaeological ceramic remains from 16^{th} to 17^{th} centuries suspected to have been used by Basque whalers to store whale oil. We wanted to find chemical evidences that supported the archaeological hypothesis: did the ceramic vessels contain whale oil?. By means of a target approach based on FUSLE-LLE-GC-MS, we were able to detect some biomarkers of marine commodities, such as $C_{14:0}$, $C_{16:0}$, $C_{20:1}$, $C_{22:1}$, $C_{18:1}$ and even trace levels of $C_{20:5}$ and $C_{22:6}$ were detected. Only sebacic and 11,12-dithydroxyeicosanoic acids were detected as degradation compounds of $C_{20:1}$. Thus, despite it must be stated with caution, there was evidence to suggest that the studied potsherds belonged to ceramics destined to store whale oil.

The developed procedure resulted in a fast analytical method applicable to any kind of archaeological ceramic sample as a first target screening tool of lipidic markers. However, we needed to extend the analytical approach to a non-target screening method in order to find unknown organic biomarkers that would be specific of whale oil species.

With the collaboration of the colleagues from the University of Pisa, we were able to go further in the characterization of ceramic remains by using two complementary analytical approaches based on HPLC-ESI-QToF and GC-MS. Although with some difficulties, we finally managed to get fresh whale oil samples belonging to different species from the Swedish Museum of National History in Stockholm and we analyzed them in order to find out each characteristic lipidic profile as well as unknown biomarkers. The distribution of triacylglycerols (TAGs) was determined by means of HPLC-ESI-QToF, and we discovered some interesting profiles depending on the whale specie analyzed. Similar TAG distributions were observed in fresh reference materials for whale species belonging to the same genus (*Balaenoptera*), whereas clear differences were noticeable between species of different genus. Additionally, some TAG ratios were established for each genus under study. Using this approach we were able to find chemical evidences of the presence of whale oil traces in the analyzed ceramics, but also to identify the genus of the whale: *Balaenoptera*. GC-MS analysis provided the fatty acid (FA) profile and allowed the detection of degradation compounds (i.e. diacids and DHAs) and biomarkers related to marine commodities (i.e. Pri, phy and 4,8,12-TMTD) not detected with the previous method. The results were promising and, in future works, this analysis should be expanded to other archaeological ceramic samples as well as to a higher number of samples of blubber from species of different genus. According to archaeological and historical data, right whale (*Eubalaena* genus) and bowhead whale (*Balaena* genus) were the main whales captured by Basque whalers in Labrador and Newfoundland during that period, thus, species belonging to these two genus should be studied.

Despite all the performed research and the obtained satisfactory results, taking a look into the future, there is still a lot of work to do regarding biomarker analysis. As experience has shown us, the use of chromatographic techniques coupled to high resolution mass spectrometry leads to the unequivocal characterization of specific compounds. Thus, their use to detect previously unknown biomarkers results essential for future experiments in the field of landscape and heritage. Additionally, experiments based on artificial accelerated ageing of fresh reference samples could also be very useful and interesting in order to understand the formation of these new biomarkers. On the other hand, more emphasis on the application of non-destructive analytical techniques should be promoted, since in many cases, valuable materials from cultural heritage cannot be destroyed, even if the required amount of sample is minimum. During the development of the present PhD thesis, expertise on Surface Enhanced Raman Spectroscopy (SERS) was acquired with the collaboration of the research group from the Institute of the Structure of Matter in the Superior Council of Scientific Research (CSIC). Hence, with an eye to the future, the application of this technique for the characterization of organic remains without damaging the original sample could result in a very attractive project.

Bearing in mind all the results obtained in the present PhD thesis, it can be concluded that the main aims and objectives have been accomplished. All the analytical methods were successfully developed and applied to real samples from different fields, obtaining interesting and valuable results. It is at this moment, writing the concluding remarks, when one becomes aware of the overcome obstacles and difficulties and really values all the work carried out. And of course, it is at this point when one feels proud of the mistakes, the effort and both the unsuccessful and successful results. In the end, it is all about learning.

Chapter 9

Annexes

Table 9.1: *Maturity related biomarker parameters arranged by families of related compounds.*

Biomarker parameter	Examples	Specificity	References
Terpanes			
22S/(22S+22R)	Relation of 22S and 22R diastereoisomers at C-22 in the C_{31}-C_{35} 17α-hopanes	High specificity for immature to early oil generation	[1]
Ts/(Ts+Tm)	Relation of C_{27} 18α-22,29,30-trisnorhopane (Ts)and C_{27} 17α-22,29,30-trisnorhopane (Tm)	Thermal parameter applicable over the range immature to mature and postmature, but strong dependence on source	[2]
Ts/Hopane	Relation of C_{27} 18α-22,29,30-trisnorhopane (Ts) and 17α-hopane	Mature to postmature range	[3]
(BNH+TNH)/hopanes	Ratio defined as (28,30-bisnorhopanes or BNH + 25,28,30-trisnorhopanesor TNH)/(C_{29}+C_{30} 17α-hopanes)	Highly specificity for immature to mature range when BNH and TNH are present	[4]
Polycadinenes and related products			
Bicadinane maturity indexes and diaromatic secobicadinane and tricadinane ratios	Relation of bicadinane isomers and ratio nof diaromatics in bicadinane-rich oils	Immature to early oil window maturity parameter for Tertiary sequences with *Dipterocarpaceae* higher plant input. Useful when other maturity ratios based on steranes have been altered	[5-7]
Isocadalene/(isocadalene+cadalene)	Relation of 1,6-dimethyl-4-isoprophylnaphtalene (cadalene) with 1,6-3- isoprophylnaphtalene (isocadalene)	Oil-window maturity parameter for sequences with higher-plant input. Broader application than bicadiene maturity indexes	[8]
Steranes			
20S/(20S+20R)	Relation of 20S and 20R diastereoisomers at C-20 in the C_{29} 5α,14α,17α(H)-steranes	Highly specific for immature to mature range	[9]
20S/(20S+20R) 13β,17α(H)-diasteranes	Relation of 20S and 20R diastereoisomers at C-20 in C_{27} - C_{29} diasteranes	Specific for mature to highly mature range	[10]
Aromatic steroids			
TA/(MA+TA)	Relation of C-ring monoaromatic (MA) with ABC-ring triaromatic (TA) steroids	Highly specific for immature to mature range	[11]
20S/(20S+20R)	Relation of 20S and 20R diastereoisomers at C-20 in C_{26} triaromatic steroids	Specific for mature to highly mature range	[12]
Porphyrins			
DPEP/etio	Relation of deoxophylloerythroetio porphyrins and etio porphyrins	Immaature to mature range	[13]
Porphyrin maturity parameter	Relation of two vanadyl porphyrins, C_{28} ethyoporphyrin and C_{32} DPEP porpyrin	Highly specific for immature to early mature range	[14]

9.1. References in Table 9.1

[1] W.K. Seifert, J.M. Moldowan, The effect of thermal stress on source-rock quality as measured by hopane stereochemistry, Phys. Chem. Earth. 12 (1980) 229-237.

[2] J.M. Moldowan, P. Sundararaman, M. Schoell, Sensitivity of biomarker properties to depositional environment and/or source input in the Lower Toarcian of SW-Germany, Org. Geochem. 10 (1986) 915-926.

[3] J.K. Volkman, Lipid markers for marine organic matter, Handb. Environ. Chem. Vol. 2 React. Processes. 2 N (2006) 27-70.

[4] J.M. Moldowan, W.K. Seifert, E. Arnold, J. Clardy, Structure proof and significance of stereoisomeric 28,30-bisnorhopanes in petroleum and petroleum source rocks, Geochim. et Cosmochim. Acta. 48 (1984) 1651-1661.

[5] H.C. Cox, J.W. De Leeuw, P.A. Schenck, H. Van Koningsveld, J.C. Jansen, B. Van De Graaf, V.J. Van Geerestein, J.A. Kanters, C. Kruk, A.W.H. Jans, Bicadinane, a C30 pentacyclic isoprenoid hydrocarbon found in crude oil, Nature. 319 (1986) 316-318.

[6] A.P. Murray, R.E. Summons, C.J. Boreham, L.M. Dowling, Biomarker and n-alkane isotope profiles for Tertiary oils: relationship to source rock depositional setting, Org. Geochem. 22 (1994) 521-542.

[7] B.G.K. van Aarssen, Z. Quanxing, J.W. de Leeuw, An unusual distribution of bicadinanes, tricadinanes and oligocadinanes in sediments from the Yacheng gasfield, China, Org. Geochem. 18 (1992) 805-812.

[8] R. Alexander, R.I. Kagi, R.K. Singh, I.B. Sosrowidjojo, The effect of maturity on the relative abundances of cadalene and isocadalene in sediments from the Gippsland Basin, Australia, Org. Geochem. 21 (1994) 115-120.

[9] W.K. Seifert, J.M. Moldowan, Use of biological markers in petroleum exploration. Biological markers in the sedimentary record. (1986) 261-290.

[10] A.S. Mackenzie, R.L. Patience, J.R. Maxwell, M. Vandenbroucke, B. Durand, Molecular parameters of maturation in the Toarcian shales, Paris Basin, France—I. Changes in the configurations of acyclic isoprenoid alkanes, steranes and triterpanes, Geochim. Cosmochim. Acta. 44 (1980) 1709-1721.

[11] A.S. Mackenzie, C. Beaumont, D.P. McKenzie, Estimation of the kinetics of geochemical reactions with geophysical models of sedimentary basins and applications, Org. Geochem. 6 (1984) 875-884.

[12] K.E. Peters, Geochemistry of selected oils and rocks from the central portion of the West Siberian Basin, Russia, Am. Assoc. Pet. Geol. Bull. 77 (1993) 863-887.

[13] E.W. Baker, J.W. Louda, Porphyrins in the geological record. Biological markers in the sedimentary record. (1986) 125-225.

[14] P. Sundararaman, W.R. Biggs, J.G. Reynolds, J.C. Fetzer, Vanadylporphyrins, indicators of kerogen breakdown and generation of petroleum, Geochim. Cosmochim. Acta. 52 (1988) 2337-2341.

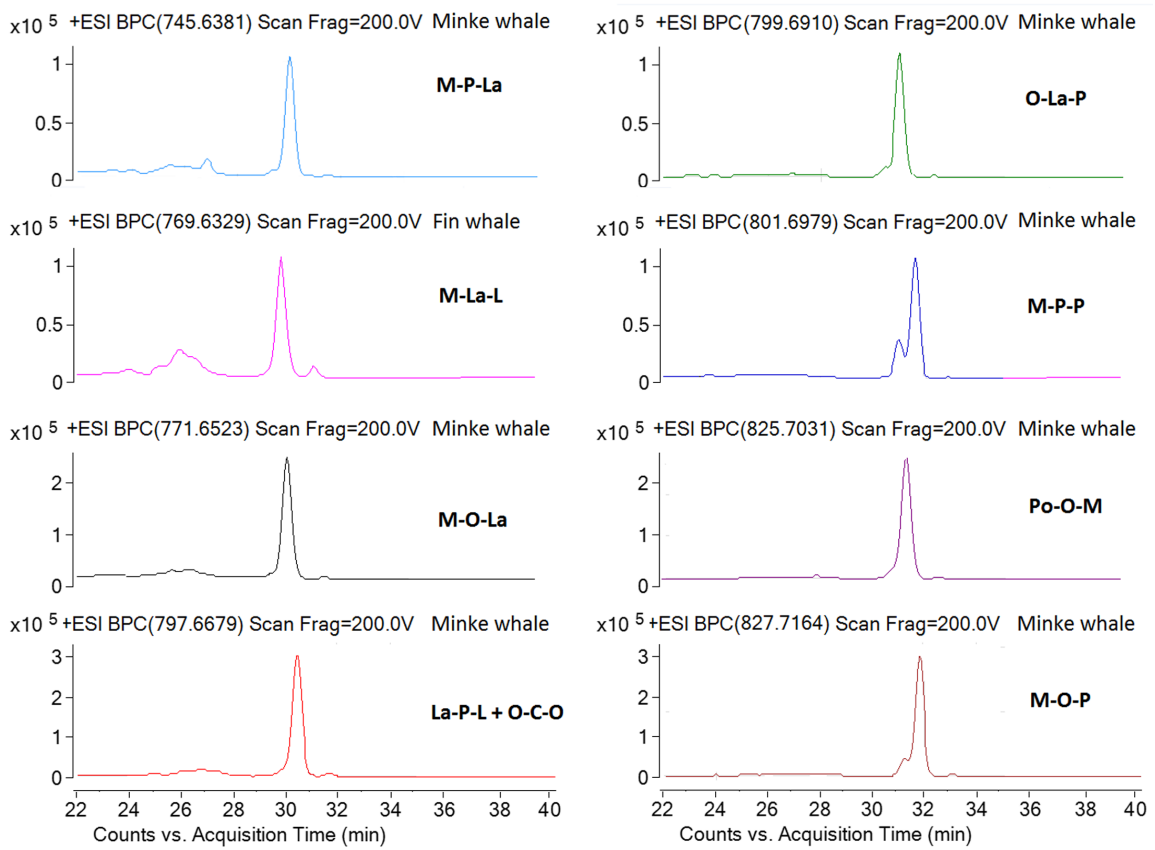


***Figure 9.1**: HPLC-ESI-QToF extract ion chromatograms of the parent ions of TAGs found both in fresh reference blubber samples and in archaeological ceramics*

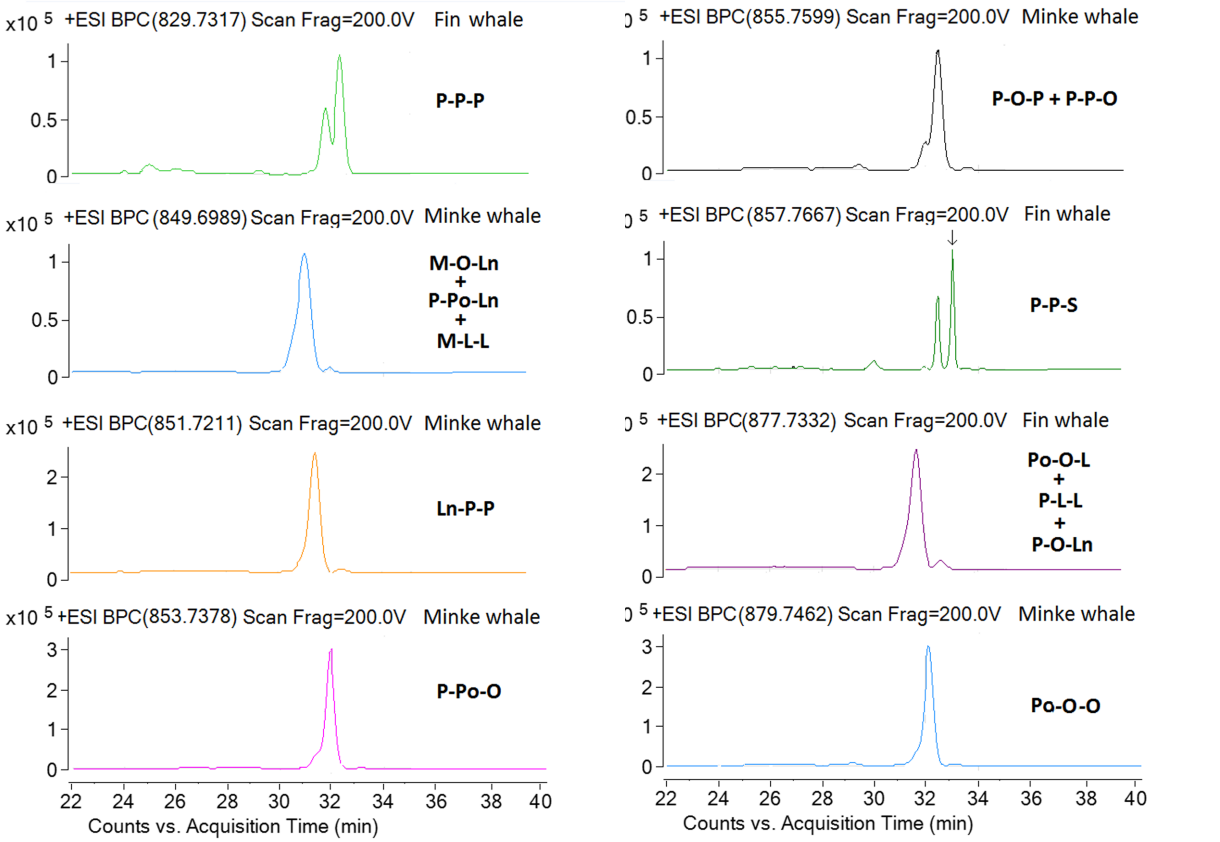


***Figure 9.1**: Continuation*

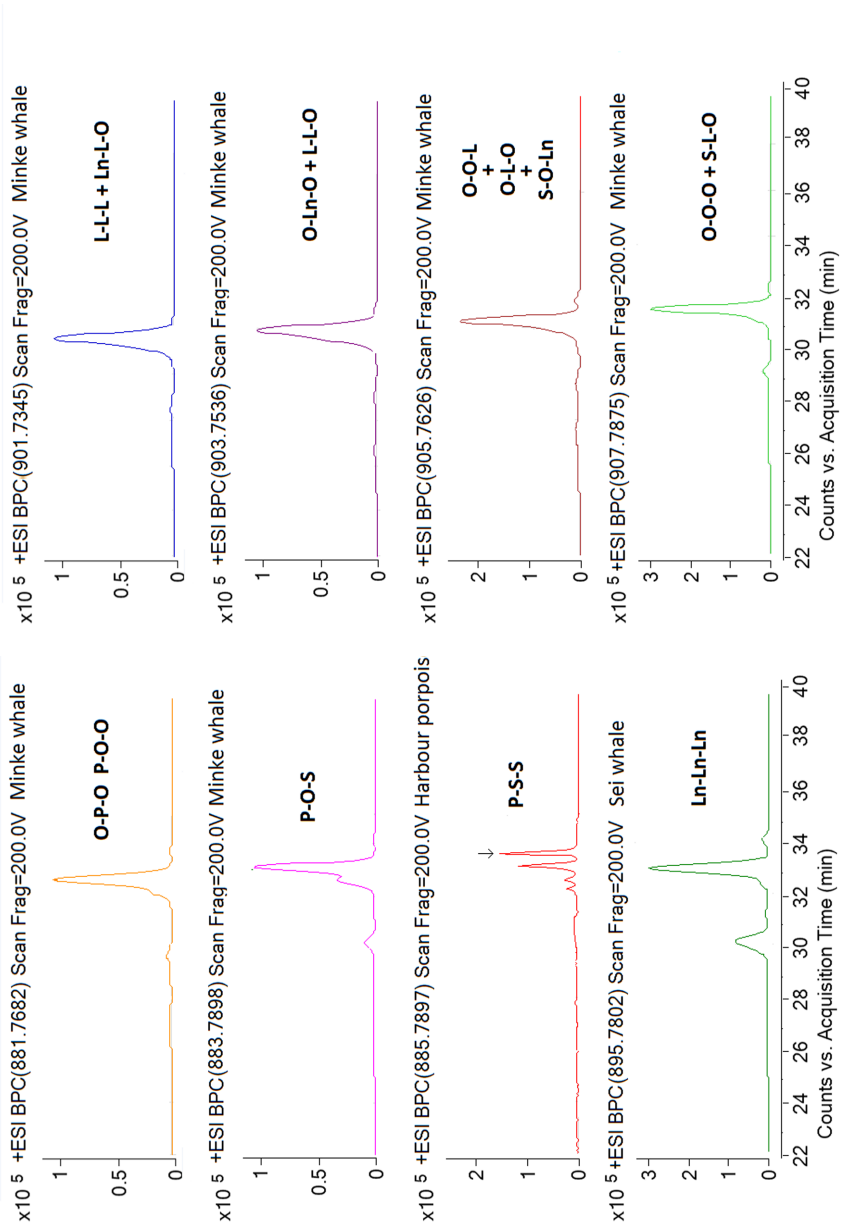


***Figure 9.1**: Continuation*

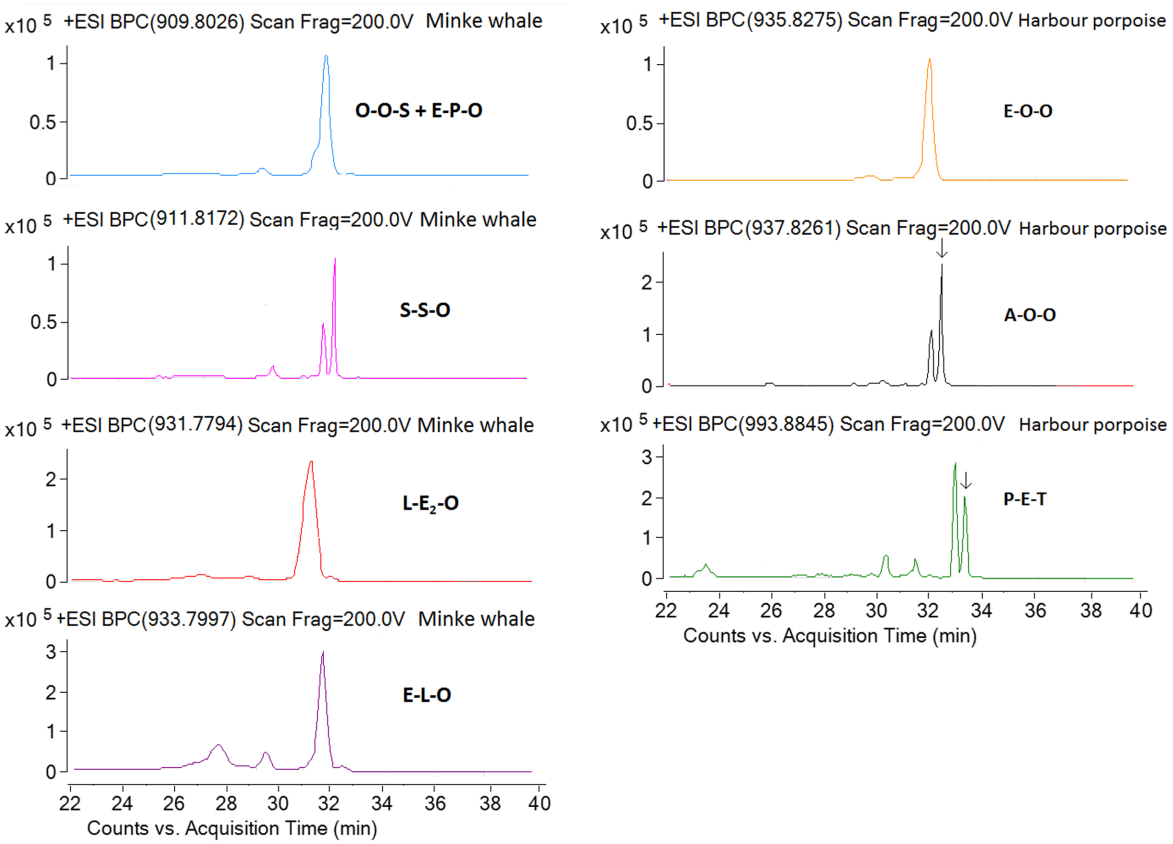


***Figure 9.1**: Continuation*

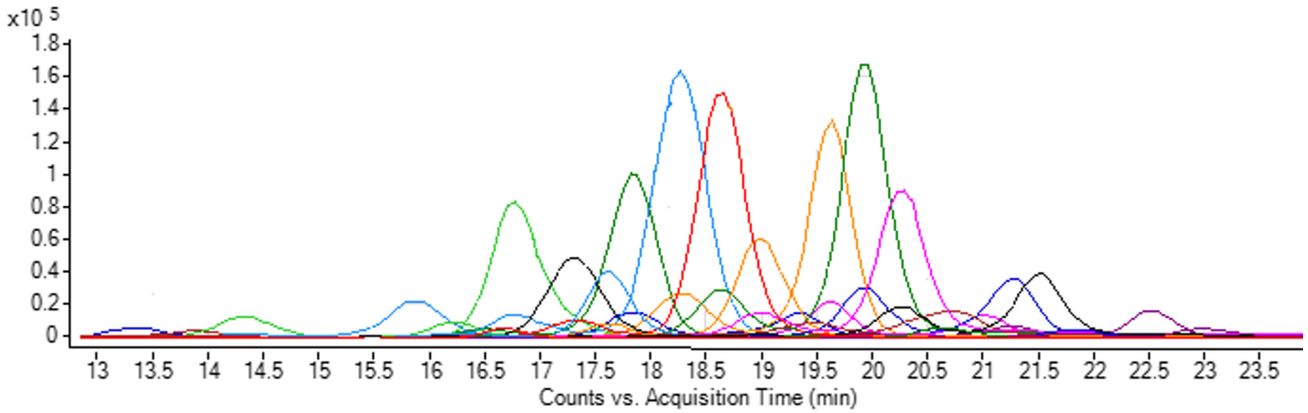


Figure 9.2: *HPLC-ESI-QToF extract ion chromatograms of the parent ions of TAGs found in archaeological ceramic L016. In order of appearance: M-P-La, M-La-L, M-O-La, La-P-L + O-C-O, O-La-P, Po-O-M, M-O-P, P-P-P, Ln-P-P, P-Po-O, P-O-P + P-P-O, P-P-S, P-O-Ln + Po-O-L + P-L-L, Po-O-O, O-P-O + P-O-O, P-O-S + P-S-S, Ln-Ln-Ln, L-L-L + Ln-L-O, O-Ln-O + L-L-O, O-O-L + O-L-O + S-O-Ln, O-O-O+ S-L-O, O-O-S + E-P-O, S-S-O, E-L-O, E-O-O, A-O-O.*

**Retention times do not match with those corresponding to the fresh blubber due to the use of a different chromatographic method in which TAGs appeared earlier.*


Geochemistry
Beachrock
Polycyclic aromatic hydrocarbons
Environment
PAH
FUSLE
Cu*
LVT
PTV
WSOCs
Built heritage
Organic biomarkers
Gas Chromatography-Mass Spectrometry
Solid Phase Extraction
Marine aerosol
Archaeological ceramics
Triacylglycerols
Fatty acid
Liquid-liquid extraction
Ultrasound
micro bath
Optimization
Strong anion exchange
Basque whalers
HPLC-ESI-QToF
Validation
Ancient wine
Blubber
Limit of detection
4,8,12-TMTD
Pristane
Landscape
Tandem mass spectrometry
Tartaric acid
Malvidin
Dihydroxy fatty acid
Focused Ultrasound Solid Liquid Extraction
Cholesterol
High Performance Liquid Chromatography
Derivatization
Preconcentration
Precision
Unknown biomarkers
Sample preparation
Punta Begoña
Mortar