LIÈGE université
Gembloux
Agro-Bio Tech

DEVELOPMENT OF GREEN AND WHITE ANALYTICAL METHODS FOR FOOD QUALITY AND SAFETY WITH MICROWAVE-ASSISTED SAMPLE PREPARATION

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Development of Green and White analytical methods for food quality and safety with microwave-assisted sample preparation

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Dissertation originale présentée (ou essai présenté) en vue de l'obtention du grade de doctorat en sciences agronomiques et ingénierie biologique

Promoteurs : Giorgia Purcaro
Année civile (= année du dépôt) : 2026

Abstract

Imagine coming home after a long and exhausting day at work, with only one thought in mind: the leftovers waiting in the fridge. You reheat them in the microwave, why? Because it is easy, fast, and efficient. This simple reasoning was the same intuition that inspired Samra et al. when they first introduced microwave technology into the laboratory. The same advantages that make the microwave indispensable in the kitchen can be effectively translated into scientific workflows.

Building on this concept, this thesis explores the application of microwave-assisted techniques for sample preparation to address different analytical challenges in food chemistry.

This research highlights the potential of microwaves as a versatile tool that bridges everyday problems with advanced analytical chemistry solutions, opening new perspectives for laboratory applications. In particular, this research is situated within the framework of food chemistry, a field that plays a vital role in understanding the composition, transformation, and stability of food, especially in relation to safety, quality, and nutritional value. With increasing global demand for food traceability, health-conscious products, and sustainable practices, the need for accurate, rapid, and environmentally friendly analytical methods has become more urgent than ever. In this context, the adoption of microwave technology represents a promising step forward.

This thesis reports optimized microwave-assisted sample preparation protocols for a variety of matrices and target analytes, demonstrating the versatility and efficiency of microwave-assisted methods compared to conventional ones. The results consistently show that microwave-assisted techniques not only reduce processing times and energy consumption but also improve extraction efficiency, reproducibility, and overall analytical throughput. Furthermore, the integration of microwave technology aligns with the principles of green chemistry, minimizing the use of hazardous solvents and lowering environmental impact. Together, these findings underscore the potential of microwaves to transform routine laboratory workflows, offering a fast, reliable, and sustainable alternative for modern food analysis.

Résumé

Imaginez rentrer chez vous après une longue et épuisante journée de travail, avec une seule pensée en tête : le reste qui vous attend dans le réfrigérateur. Vous le réchauffez au micro-ondes — pourquoi ? Parce que c'est simple, rapide et efficace. Ce raisonnement élémentaire est la même intuition qui a inspiré Samra et ses collaborateurs lorsqu'ils ont introduit pour la première fois la technologie des micro-ondes dans le laboratoire. Les mêmes avantages qui rendent le micro-ondes indispensable en cuisine peuvent être efficacement transposés aux processus scientifiques.

S'appuyant sur ce concept, la présente thèse explore l'application de techniques assistées par micro-ondes pour la préparation d'échantillons dans divers contextes analytiques de la chimie alimentaire.

Cette recherche met en évidence le potentiel des micro-ondes en tant qu'outil polyvalent reliant les problèmes du quotidien aux solutions de la chimie analytique avancée, ouvrant ainsi de nouvelles perspectives pour les applications en laboratoire. Plus particulièrement, elle s'inscrit dans le cadre plus large de la chimie des aliments, un domaine essentiel pour comprendre la composition, la transformation et la stabilité des denrées alimentaires, notamment en lien avec leur sécurité, leur qualité et leur valeur nutritionnelle.

Face à la demande mondiale croissante en matière de traçabilité alimentaire, de produits sains et de pratiques durables, la nécessité de disposer de méthodes analytiques précises, rapides et respectueuses de l'environnement est plus pressante que jamais. Dans ce contexte, l'adoption de la technologie micro-ondes représente une avancée prometteuse.

Cette thèse présente différents protocoles optimisés de préparation d'échantillons assistée par micro-ondes pour une variété de matrices et d'analytes cibles, démontrant la polyvalence et l'efficacité de ces approches par rapport aux méthodes conventionnelles. Les résultats montrent de manière constante que les techniques assistées par micro-ondes réduisent non seulement les temps de traitement et la consommation d'énergie, mais améliorent également l'efficacité d'extraction, la reproductibilité et le rendement analytique global.

De plus, l'intégration de la technologie micro-ondes s'inscrit dans les principes de la chimie verte, en réduisant l'utilisation de solvants dangereux et en limitant l'impact environnemental. Ensemble, ces résultats soulignent le potentiel des micro-ondes pour transformer les pratiques de laboratoire courantes, offrant une alternative rapide, fiable et durable pour l'analyse moderne des aliments.

Acknowledgements

These acknowledgements risk being longer than the manuscript itself, as I have come to value every single person I encountered during these years. Every interaction, every conversation, and every shared moment helped to build—brick by brick—the person I am today, so different from the one who started this journey. Each of you, in their own way, has inspired me, guided me, supported me, challenged me, and entertained me throughout this journey. This work is not the result of an individual effort, but of a shared experience made of people and moments that shaped both the research and the researcher.

I would like to express my deepest gratitude to my two supervisors. **Chiara Emilia Cordero**, for believing in me from the very beginning, for guiding me throughout this journey and beyond, and for her determination and unwavering commitment to those she mentors. **Giorgia Purcaro**, for her scientific spirit, her ideas, her constant availability toward everyone, and for always pushing me to go further, challenge my limits, and step out of my cocoon, allowing me to grow in every aspect. Her guidance went far beyond supervision!

I would also like to thank **Marco Beccaria**, who became a sort of acquired supervisor over the years, constantly bringing new ideas for projects

I am also sincerely grateful to **Diego Carnaroglio, Matteo Volpi, Matteo Remondini, Luca Rota and the entire Milestone team** for welcoming me into their "family." This acknowledgment feels almost insufficient to express my gratitude: they always responded promptly to every request, offering constant help, support, and availability, and creating a truly stimulating and supportive working environment.

My thanks also go to all the members of the committee meeting, **Cecilia Cagliero**, **Pierre-Hugues Stefanuto**, and **Hans Gerd Jansen** for their time and valuable discussions.

Thanks to my family for their constant support and for being proud of me, even if they probably understood less than 10% of what I was actually doing.

I would like to thank my in-laws for their kindness and the warmth of their support.

To **Marica**, a constant presence, who is sometimes simply there to endure my ten-minute voice messages in which I complain about absolutely everything.

I would also like to thank my colleagues at the University of Turin, who over time have become true friends, and with whom I have shared so many moments that are precious to me. I thank **Gaia, Giulia S., Giulia M., Giulia M., Marta, Eloisa, Andrea, Simone, and Fulvia** for the laughter, and memories we built together.

A special thanks goes to **Angelica**, the angel and the warrior in the same small body. The one with whom I started this journey. The one I shared the beauty and the difficulties of these years. Thanks for our escapes once a year around the world, a tradition that will continue also after the PhD.

I would also like to thank my colleagues and friends in Gembloux. My sincere gratitude goes to the Belgian— **Damien, Aleksandra, and DJ.**

I would also like to express my heartfelt thanks to **Sophie**, the person we all rely on. She keeps the laboratory running every day with dedication and care, and she always looks after us, both professionally and personally.

I would also like to thank **Philippe**, whose constant smile and positive energy always brightened the lab. He somehow managed to stop by just to make us laugh, lift our spirits, and cheer us up with his colorful ties, reminding us not to take ourselves too seriously.

To **Andrea (aka Skinki),** to whom I owe my ability to even open R. From the very first moment, I knew I had found a true and sincere friend. Our flatemate moments with **Alessio, Manu** and **Guillaume** are so precious to me.

To **Giulia**, a true concentration of determination—you often make me wonder how so much strength can fit into such a small person. She has honestly been a great source of inspiration and thank you for teaching me how to roll *braciole messinesi*.

Thanks to **Gregory** and **Paula and Silvia**. I am deeply grateful for your friendship, and kindness—also for always being willing to babysit Nori whenever I was away.

I am also thankful to those who were part of this journey, even if only for a while: **Nathalia S., Nemagna, Nicolò, Francesca, Giovanna, Natasha, Clement and Funny, Irene, Pedro, Stefano, Lulu, Andrea, Joachim, Alfred, Bryan, Jérôme** and **Marin**. Each of you contributed in a meaningful way to this experience.

To the Italian resistance, **Nicola and Carlo**: your macabre sense of humor and self-irony made me burst into laughter even in the darkest moments.

To **Mattia**, who arrived when I was nearing the end of my PhD, at one of its most demanding moments, yet never became another burden to carry. Instead, he was an equal with whom I could share responsibilities and stimulating scientific perspectives. I am truly grateful for the help and support.

Finally, I want to thank **Steven**. My home far from home. My partner in life, my greatest supporter, and the one who always stood by me with pride and care. My joy in the hardest moments, and the calm to my overthinking mind.

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List of acronyms

AA – Arachidonic acid
ALA – α-Linolenic acid
ANOVA – Analysis of variance
AOCS – American Oil Chemists' Society
BF_3 – Boron trifluoride
BIPEA – Bureau Interprofessionnel d'Études Analytiques
BSTFA – N,O-Bis(trimethylsilyl)trifluoroacetamide
CCD – Central composite design
CO_2 – Carbon dioxide
COPs – Cholesterol and cholesterol oxides
Cyclohex – Cyclohexane
DAK – Dialkyl ketones
DES – Deep eutectic solvents
DHA – Docosahexaenoic acid
DPA – Docosapentaenoic acid
EI – Electron ionization
EPA – Eicosapentaenoic acid
EPA – Environmental Protection Agency
ESI – Electrospray ionization
Et_2O – Diethyl ether

EVOO – Extra virgin olive oil
FA – Fatty acids
FAMEs – Fatty acid methyl esters
FID – Flame ionization detection
FFF – Forward fill/flush
FLQ – Fish lipid quality index
GAC – Green Analytical Chemistry
GC – Gas chromatography
GC×GC – Comprehensive two-dimensional gas chromatography
GLA – γ-Linolenic acid
HCl/MeOH – Hydrochloric acid in methanol
HDL – High-density lipoprotein
HH – Hypocholesterolemic/hypercholesterolemic ratio
HPI – Health-promoting index
HPLC – High-performance liquid chromatography
IA – Index of atherogenicity
ILs – Ionic liquids
IOC – International Olive Council
IT – Index of thrombogenicity
IUPAC – International Union of Pure and Applied Chemistry
KOH/EtOH – Potassium hydroxide in ethanol
LDL – Low-density lipoprotein
LLE – Liquid–liquid extraction
LOO – Lampante olive oil
LRIs – Linear retention indices
MAAE – Microwave-assisted aqueous extraction
MAE – Microwave-assisted extraction
MAED – Microwave-assisted extraction and derivatization
MAEH – Microwave-assisted extraction with hydrolysis
MAS – Microwave-assisted saponification
MASE – Microwave-assisted saponification and extraction
MOAH – Mineral oil aromatic hydrocarbons
MOH – Mineral oil hydrocarbons
MOSH – Mineral oil saturated hydrocarbons
MS – Mass spectrometry
MUFA – Monounsaturated fatty acids
MW – Microwave
MWE – Microwave-assisted water extraction
PAHs – Polycyclic aromatic hydrocarbons
PFAS – Per- and polyfluoroalkyl substances
PLE – Pressurized liquid extraction

PTFE – Polytetrafluoroethylene
PUFA – Polyunsaturated fatty acids
QC – Quality control
QuEChERS – Quick, Easy, Cheap, Effective, Rugged and Safe
RFF – Reverse fill/flush
RGB – Red, Green and Blue (White Analytical Chemistry model)
RSM – Response Surface Methodology
SC-CO_2-MW – Supercritical carbon dioxide–microwave
SDA – Stearidonic acid
SFA – Saturated fatty acids
SFE – Supercritical fluid extraction
SIGNIFICANCE – Sample minimization, Instrumental efficiency, Green solvents, No derivatization, In-line/real-time monitoring, Functional automation, Inherent safety, Continuous processes, Automation/miniaturization, Non-disruptive methods, Circular economy, Energy efficiency
S.I.C. – Sites of European Community Interest
SPE – Solid-phase extraction
SRC – Single Reaction Chamber
TAGs – Triacylglycerols
TFA – Trans fatty acids
TLC – Thin-layer chromatography
TOF – Time of flight
UAE – Ultrasound-assisted extraction
UFAs – Unsaturated fatty acids
UHPLC – Ultra-high-performance liquid chromatography
UI – Unsaturation index
VOO – Virgin olive oil
WAC–WhiteAnalyticalChemi

PREAMBLE

Thesis Introduction

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As global food chains expand and consumer expectations increase, analytical chemistry plays an essential role in guaranteeing that food products are safe, authentic, and compliant with regulatory requirements. However, traditional analytical processes used in food control often involve lengthy workflows, heavy solvent consumption, high energy demand, and significant environmental impacts. These challenges have created an urgent need for analytical methodologies that are not only scientifically sound but also sustainable, efficient, and aligned with the principles of responsible innovation. Within this context, green analytical chemistry (GAC) has become a driving force for rethinking laboratory workflows [1]. GAC promotes reducing hazardous reagents, minimizing waste, lowering energy consumption, and safer working conditions. It encourages the design of methods that achieve comparable or even higher analytical performance to the routine ones, while reducing their environmental footprint. However, green considerations alone are not sufficient to capture the complete picture of a method's value. White analytical chemistry (WAC) [2] expands this framework. WAC highlights the coherence and synergy of the analytical, green and practical attributes. It proposes an extended evaluation framework in which an analytical method is assessed not only in terms of environmentally friendly (green) but also performant (red), and practical (blue), i.e., reliable, accessible, and suitable for real-world applications. All together contribute to the whiteness of the analytical method.

Within this framework, microwave-assisted sample preparation emerges as a powerful and versatile tool capable of fulfilling the requirements of both GAC and WAC while still providing the analytical reliability demanded by food safety and food quality assessments. By exploiting the unique ability of microwaves to deliver rapid, uniform, and highly efficient heating, these techniques significantly reduce extraction times and solvent consumption, thereby enhancing method performance without compromising results. The precise and controlled interaction between microwave (MW) energy and the sample matrix enables more reproducible and selective processes, making MW approaches an exemplary model of how technological innovation can support greener yet high-quality analytical workflows.

In summary, this thesis focuses on the development and application of microwave-assisted analytical approaches within the framework of green and white analytical chemistry, with particular attention to food safety and quality assessment.

While green and white chemistry provide the theoretical and ethical framework for sustainable method design, MW technologies offer a practical, efficient, and environmentally friendly tool for sample preparation for food safety and

quality. For completeness, a brief overview of Green Analytical Chemistry and White Analytical Chemistry is provided.

Green Chemistry

The concept of green chemistry emerged as a response to the growing need for sustainable development, with early applications primarily focused on industrial processes, particularly within the pharmaceutical sector. From the early 2000s onward, these principles progressively expanded into the field of chemical analysis, giving rise to Green Analytical Chemistry (GAC). The main objective of GAC is to make laboratory practices more sustainable by reducing the environmental impact of analytical procedures without compromising the reliability and accuracy of the results. Analytical scientists, therefore, work toward optimizing methodologies and workflows to achieve an effective balance between efficiency, analytical performance, and minimization of waste. The principles of green chemistry formulated by Anastas and Warner (1998) provide an essential reference framework for developing more sustainable analytical methods, although not all of them can be directly applied to analytical chemistry. Among the most relevant principles for this field are waste prevention, the use of safer solvents and reagents, energy efficiency, and the reduction of derivatization steps. Gałuszka et al. (2013) proposed a specific mnemonic — SIGNIFICANCE — to summarize twelve guiding principles of GAC, highlighting the themes of Sample minimization, Instrumental efficiency, Green solvents, No derivatization, In-line/real-time monitoring, Functional automation, Inherent safety, Continuous processes, Automation/miniaturization, Non-disruptive methods, Circular economy and Energy efficiency. Nevertheless, to fully meet the requirements of modern analytical science, these principles must be adapted and expanded, creating a broader framework capable of promoting genuinely sustainable practices within analytical laboratories.

These principles were intended to improve the analytical process as a whole, with particular emphasis on minimizing or even eliminating sample preparation, which is widely recognized as one of the major contributors to environmental impact in chemical analysis. Sample preparation frequently dominates both solvent usage and energy consumption, and Gałuszka et al. emphasise that the implementation of GAC must address every stage of an analytical workflow—from sampling through to detection—with special attention to steps such as derivatisation, waste generation, and energy demand.

Moreover, to quantitatively evaluate the sustainability of sample preparation and analytical practices, several metrics and assessment tools have been developed. These tools support researchers in assessing parameters like energy consumption, solvent volumes, waste generation, as well as the real-time monitoring of processes, thereby aligning with the GREEN Analytical principles set out by Gałuszka et al.[1] These programs employ advanced algorithms to quantify parameters such as energy consumption, solvent use,

waste generation, and other resource-related metrics, offering detailed insight into the environmental efficiency of different sample preparation strategies. Their use facilitates process optimization, reduction of environmental impact, and improvement of laboratory safety, thus promoting more sustainable and eco-friendly analytical workflows [1]. A practical view of this tool will be given in the most applicative part of the thesis.

White chemistry

Green Analytical Chemistry (GAC) has significantly advanced the sustainability of analytical practices over the past two decades, its exclusive focus on environmental and safety-related criteria has also highlighted important limitations when methods must remain analytically robust and practically feasible. To address this gap, Nowak, Wietecha-Posłuszny and Pawliszyn introduced the concept of White Analytical Chemistry (WAC), a holistic framework designed to reconcile the ecological goals of GAC with the essential analytical and practical requirements of modern laboratories. WAC builds upon the RGB color model, where green represents environmental friendliness, red denotes analytical performance (e.g., sensitivity, accuracy, precision, applicability), and blue reflects practical and economic aspects (e.g., cost efficiency, time efficiency, operational simplicity). In this model, a "white" method is one that achieves an optimal and well-balanced integration of these three components, mirroring the way white light results from the combination of red, green, and blue. This perspective shifts analytical method development from a single-criterion evaluation—focused solely on greenness—to a multidimensional assessment of sustainability, in which no individual attribute is allowed to dominate at the expense of the others.

Importantly, WAC recognizes that an unconditional pursuit of greenness can sometimes degrade method functionality, for example, by reducing sensitivity or increasing analytical complexity—and therefore promote methodological choices that reflect a balanced compromise aligned with the principles of sustainable development. In WAC, a method is considered truly sustainable when it achieves high performance across all three dimensions, ensuring environmental responsibility without sacrificing analytical quality or operational practicality.

To support this comprehensive evaluation, the authors further introduced the RGB 12 algorithm, a quantitative assessment tool that scores a method according to each of the 12 WAC principles and expresses its global sustainability as a "whiteness" value. This approach facilitates transparent comparison of analytical strategies, highlights methodological strengths and bottlenecks, and encourages continuous improvement guided by sustainability principles. By integrating ecological, analytical, and practical criteria, White Analytical Chemistry thus represents an essential evolution of GAC—one that is better aligned with the multidimensional nature of modern analytical

challenges and the broader goals of sustainable development. This framework is particularly relevant in the context of advanced sample preparation techniques, such as microwave-assisted extraction, where performance, efficiency, and environmental considerations must be simultaneously optimized [2].

Structure of the thesis

This thesis investigates the application of microwave (MW) technology to improve sample preparation strategies in food analysis, with a particular focus on lipid determination. After an introductory chapter outlining the fundamental principles of microwave technology (Chapter 1), the thesis is structured to progressively guide the reader from theoretical background to experimental method development and, finally, to innovative proof-of-concept applications. Chapter 2 provides an overview of lipid analysis, focusing on the analytical relevance, regulatory framework, and methodological challenges associated with the determination of fatty acid methyl esters (FAMEs) and sterols in food matrices. This chapter establishes the chemical and analytical background necessary to contextualize the experimental work presented in the subsequent chapters, highlighting the limitations of conventional sample preparation methods and the opportunities for improvement through microwave-assisted approaches.

The experimental investigation begins in Chapter 3, which is devoted to the microwave-assisted methodologies for FAMEs analysis. This chapter presents the experimental workflows developed during the PhD, including the integration of sample preparation steps and the evaluation of analytical performance, throughput, and sustainability using established assessment tools.

Chapter 4 focuses on sterol analysis, addressing a more complex and labor-intensive analytical problem. In this chapter, microwave-assisted saponification and extraction (MASE) are systematically optimized, and traditional purification strategies are replaced by solid-phase extraction.

Finally, Chapter 5 explores a forward-looking application of microwave technology through the development of a laboratory-scale prototype combining microwave irradiation with supercritical CO_2 extraction. This chapter is presented as proof of concept, demonstrating the feasibility of integrating these two advanced technologies and laying the groundwork for future research and technological development. A struction of the thesis is reported in Figure 1.

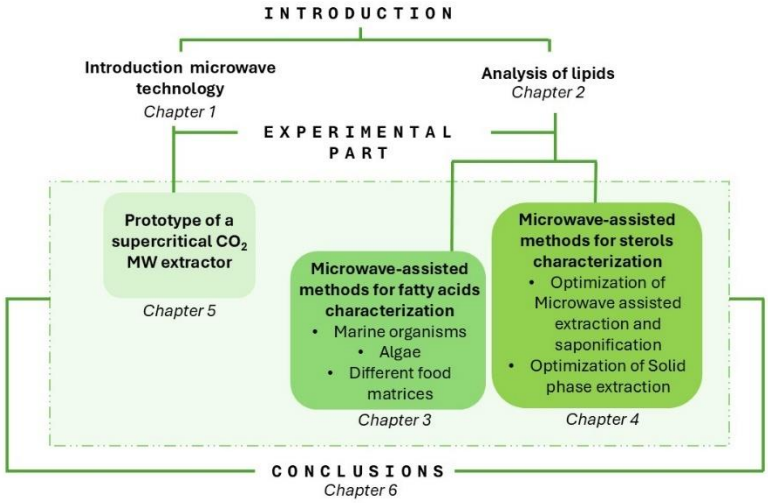

Figure 1. Thesis overview

Overall, the thesis presents a coherent progression from theoretical foundations to applied method development and technological innovation, illustrating the potential of microwave-assisted sample preparation to enhance efficiency, sustainability, and analytical performance in food lipid analysis.

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INTRODUCTION

Chapter 1

Introduction to microwave technology

This chapter reproduces material published in:

Ferrara D, Beccaria M, Cordero CE, Purcaro G. Microwave-assisted extraction in closed vessel in food analysis. J Sep Sci. 2023; 46:2300390. https://doi.org/10.1002/jssc.202300390

Chapter "Microwave-assisted extraction" for the Comprehensive Sampling and Sample Preparation, 2e . Authors Ferrara D, Purcaro

1. History of microwave

The discovery of microwave (MW) radiation dates back to 1945, during World War II, and was entirely serendipitous. Percy Spencer, an engineer at Raytheon, was testing a magnetron intended for combat radar systems when he noticed that the chocolate bar in his pocket had melted. The potential of this phenomenon was quickly recognized, and a patent was filed for its application in cooking. The first commercial microwave oven came out in 1947, but it didn't become popular right away because it was very expensive and required a lot of power. It was also huge: it weighed about 340 kg and was almost 1.8 meters tall.

After the patent expired, Japanese researchers redesigned the MW generator in the 1970s, making domestic MW ovens more affordable and enabling convenient food heating and large-scale processing [1]. The use of MWs in chemical laboratories began in 1975, when Samra et al. [2] harnessed the heat produced by a MW oven to analyze metals in biological samples, marking the first instance of MW-assisted digestion, which later became one of the main MW applications. In 1986, the first MW-assisted organic synthesis was reported, demonstrating faster reaction rates, improved kinetics, cleaner products, and higher yields [4,5]. That same year, Ganzler et al. [3] introduced the first extraction of organic compounds from soils, seeds, foods, and feed using a domestic MW oven, a technique that was subsequently named microwave-assisted extraction (MAE).

Since then, several laboratories have investigated the analytical capabilities of MW in the sample preparation step, mainly using domestic MW ovens. Initially, MAE was mainly proposed for the extraction of organic contaminants, such as polycyclic aromatic hydrocarbons (PAHs), polychlorobiphenyls, pesticides, herbicides, phenols, neutral and basic priority pollutants from various matrices such as sediments, soils, or atmospheric particles [6]. In 1993, Streinheimer [7] described a method for the quantitative estimation of atrazine and its principal degradation products in agricultural soils and groundwater using a laboratory MAE system, and in 1994 Lopez et al. [8] used a laboratory MW oven dedicated to digestion for extracting organic contaminants using a closed-vessel MAE approach for PAHs extraction from soils and sediments, indicating MAE as a valuable alternative to the traditional Soxhlet and sonication methods.

Although these early works showed the potential of the technology in chemical laboratories, many limitations were evident. Using a domestic MW oven, there was no control of temperature and pressure, and the use of flammable solvent was not possible unless to assume the risk for the operators. It appeared clear, though, that a specific system for laboratory applications was necessary to fully exploit the technique's potential avoiding any risk. Initially, systems designed for digestion started to be applied for extraction but without fine control of the temperature (a time-

power program was settable, and no temperature control was possible). In the '90s, thermocouples and optic fibers started to appear to record process temperature, but active control of the temperature was still not implemented. Starting from the mid-90s the back regulation of the power based on the actual temperature was introduced, thus allowing for a fine regulation. Despite being longly investigated, the use of MW for analytes extraction has never spread as much as for mineralization/digestion and synthesis. However, more recently, MAE has gained more attention among greener and more sustainable analytical sample preparation techniques [9-11]. Several reviews have been published focusing on the use of MAE for the large-scale extraction of active compounds [12,13], while much less is available on the use of this technology at the lab scale along with analytical workflows. Most of the reviews are very specific [14,15] and deal with pharmaceutical and personal care products applications [16], and environmental and biological samples [17,18]. Only a few reviews have been found on the use of MAE in food-related analytical determinations. Of those available, the focus is only on contaminants [19,20]. In this doctoral thesis, the use of microwave-assisted extraction (MAE) is instead explored in the field of food analysis with a specific focus on applications beyond the determination of contaminants. In particular, MAE is investigated as a versatile sample preparation strategy for the extraction of other classes of target compounds of analytical and nutritional relevance, thereby extending its application beyond the scenarios predominantly addressed in the existing literature

2. Theoretical considerations on microwave technology

MW radiation refers to the electromagnetic radiation spanning the 300 MHz–300 GHz frequency range. The energy provided by this radiation causes dielectric polarization, which is composed of four components: i) electronic polarization; ii) atomic polarization; iii) dipolar polarization; and iv) interfacial polarization. However, electronic and atomic polarization are neglectable at the frequency applied for MW, while interfacial polarization is strongly dependent on the frequency, thus, dipolar polarization is the most relevant component generating heat in the microwave. Typically, molecules in a sample with permanent or induced dipole moments are randomly oriented. When microwave energy is applied, the electric field causes oscillation of the electric dipoles to orient themselves in phase with the electric field that oscillates at a very high frequency. This phenomenon causes heat production through frictional force. Similarly, the ions in the samples move following the electric field oscillation (*i.e.*, ionic conduction) [21,22]. For both mechanisms producing heat, that is, ionic conduction and dipole rotation, the extent strongly depends on the nature of the extraction solvent and the sample. The ability of a material to transform microwave energy into heat is given by the dissipation factor (or loss tangent, tan $\delta = \varepsilon''/\varepsilon'$); where, ε'' is the dielectric loss factor, which represents the ability of the material to dissipate the absorbed electromagnetic energy, converting it into heat; and ε' is the dielectric constant, which represents a measure of the ability of a material to be polarized by an external electric field. Table 1 [23] reports the tan δ values of a series of common solvents.

Table1. Physical constants and dissipation factors for some solvents commonly used in MAE. Table adapted from [23].

Solvents	Boiling Point (°C)	Dielectric Constant[a], ε′	Dissipation factor[b], tanδ	Closed-vessel temperature [c](°C)
Acetone	56	20.7	0.054	164
Acetonitrile	82	37.5	0.062	194
Ethanol	78	24.3	0.941	164
Hexane	69	1.89	0.020	-
Methanol	65	32.6	0.659	151
Water	100	78.3	0.123	-
Hexane-acetone (1:1)	*52*	-	-	156
2-Propanol	82	19.9	0.799	145

[a]Determined at 20°C,
[b]Determined at 20°C and 2.45 GHz,
[c] Determined at 1207 kPa

A solvent with a high tan δ value is highly adsorptive to MW, while those with lower tan δ value tend to be transparent to MW (meaning that they are less/not affected by the energy transmitted) [5]. The tan δ of most solvents decreases at higher temperatures, making it more challenging to heat hot solvents than cold solvents. When exploiting MWs for chemical applications, the behavior of the extraction solvent can be tuned using a mixture of solvents.

At a fundamental level, the main difference between MW and conduction heating is related to efficiency. In conductive heating (Figure 1A), the heat is generated by an isomantle or hotplate, transmitted to the vessel before being transferred to the solution, where a temperature gradient is established through convection current.

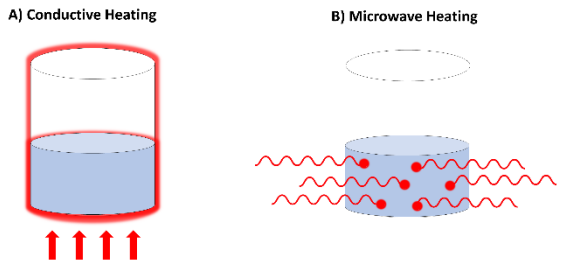

Figure 1**.** Scheme of the heating principle by conduction (A) and by microwave irradiation (B).

Therefore, the internal temperature is lower than the external one, and the thermic equilibrium may require a long time to be reached. Stirring may improve the process, but the overall control is weak, with risks of overheating the external surfaces before reaching the required temperature.
Contrarily, in microwave heating (Figure 1B), the reaction vessels are transparent to MWs (they are usually made of Teflon, tan $\delta = 1.5 \times 10^{-4}$), so the heating occurs directly within the sample while the vessels remain cold. Therefore, the targeted temperature is reached more efficiently and in a significantly shorter time [24].

3. Microwave instruments for laboratory applications

A MW instrument for laboratory use has the same mechanical part as a MW system used in the kitchen. Both systems have a MW generator (a "magnetron"), the waveguide, the applicator or the resonance cavity, and the circulator.
Among different MW sources, the magnetron is the one that has found the most significant applicability in industrial, defense, and space applications due to its efficient and compact size. The magnetron uses the interaction of electron flow, driven by a magnetic field, with cavities to produce MW radiation. It consists of an anode with a series of cavities that open into a central vacuum chamber containing a metal cathode. A permanent magnet provides a magnetic field that runs parallel to the cylinder axis. The cathode is heated by a high voltage direct current, producing electrons that flow toward the cylinder wall at 90° angles to the magnetic field. The electrons are deflected by the field in curved paths, causing the creation of circular currents within the cavities. These currents produce MW radiation at frequencies related to the size of the cavities. Therefore, the MWs must be directed to where they are needed. This is achieved by a metal structure known as a waveguide, along which the MWs travel. The propagation unit is a rectangular or, less often, circular channel of reflective material. To focus MW radiation on the sample, the applicator, which is the device used to insert the sample, can either be a multimode cavity (Figure 2A), or the waveguide itself (single mode) (Figure 2B). If the cavity is a multimode type, the MW radiation that exits the waveguide is randomly spread in the chamber, so to achieve a uniform distribution of MW radiation is necessary to use some tricks. For example, the use of stirrer mode, which is a fan-shaped blade used to reflect and mix the energy entering the microwave cavity from the waveguide. And, the fact that the samples can turn inside the chamber, the sample heating will be more position-independent, and multiple samples can be treated at the same time. The single-mode instrument guarantees stronger and homogenous irradiation, but generally, it processes a sample at the time, and it is mainly used for atmospheric pressure systems, called focused MAE or atmospheric pressure MAE. In this kind of system, the sample vessel is positioned directly in the waveguide at a maximum of MW radiation. Is sure that all the MW that are generated are directed on the sample, but only sample at the time can be processed, which can be interesting in terms of throughput.

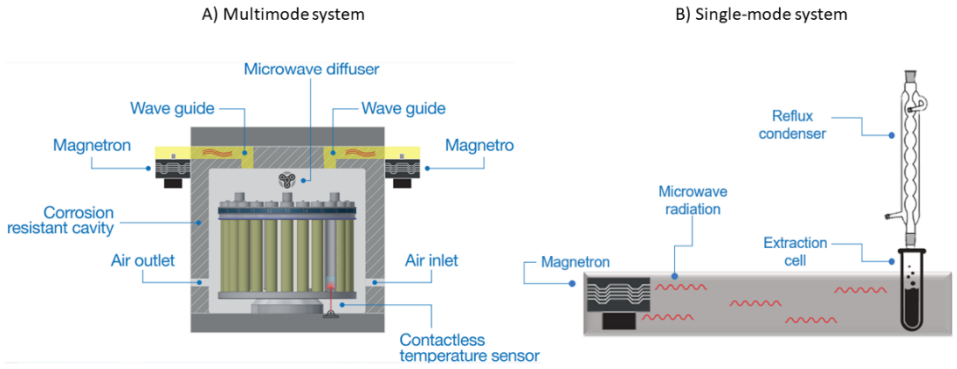

Figure 2. Scheme of the different MW designs, namely A) multi-mode cavity or B) focused or single-mode.

An important distinction among commercially available instruments is between open and closed vessels, with the latter being the most suitable from an analytical viewpoint.

In a closed vessel system, temperature and pressure are the two essential parameters to control. The most used temperature sensor is the thermocouple; however, its use has been debated as the metallic nature of the thermocouple can disturb the MW or even cause the concentration of heat at its tip. Metallic components are indeed generally not recommended in MW. An alternative is using optical fibers, which can also be coated with polytetrafluoroethylene to operate in the most severe environments. Optical fibers provide more accurate measurements than metal thermocouples, which can self-heat in MW and thus give inaccurate readings. The downside of the optical fiber is that it is expensive. These two contact temperature sensors have been generally replaced by non-contact methods, such as optical pyrometers and infrared sensors [22-25].

When operating in a closed vessel, control of the operative pressure is crucial. The generation of overpressure within the vessel is beneficial as it increases the solvent's boiling point, but, if out of control, it can be dangerous for the equipment and the operator. The pressure measurements are performed with classical pressure sensors (such as piezoelectric, membranes, and load cells), but some devices have been devised specifically for use in the presence of high-strength electromagnetic fields. An example of this is the use of a glass ring that changes the color of the transmitted polarized light according to the pressure at which it is exposed in the reaction chamber [22]. Fiber optics can also be used to measure pressure [22]. In modern systems, the

closure of the vessel is studied with a calibrated spring that opens to release the overpressure and then quickly reseals the vessel.

4. MAE: optimization of the analytical conditions

As for all the extraction techniques, the optimization of microwave-assisted extraction (MAE) conditions is strongly dependent on the characteristics of the sample matrix and on the physicochemical properties of the target analytes. To ensure method repeatability, samples should be as homogeneous as possible. In the case of solid matrices, fine grinding is therefore recommended, as it not only improves sample homogeneity but also increases the surface-to-volume ratio, thereby enhancing mass transfer and, ultimately, extraction efficiency.

Among the parameters requiring careful optimization, the water content of the sample plays a pivotal role. Water is characterized by a high dielectric constant and, consequently, absorbs microwave energy very efficiently. On the one hand, this property enables rapid and uniform heating of the sample–solvent system; on the other hand, the evaporation of matrix-associated water leads to the expansion of internal pores and cell structures, facilitating solvent penetration and promoting analyte–matrix interactions, thus favoring the release of target compounds into the extraction solvent.

However, excessive water content may negatively affect the extraction process by forming a protective layer around the analytes and/or causing solubility issues, particularly when apolar solvents are employed for the extraction of non-polar compounds. For example, an increase in the extraction yield of polycyclic aromatic hydrocarbons (PAHs) in dichloromethane has been reported when the water content was raised from 0% to 30%, whereas further increases resulted in decreased recoveries [26]. Consequently, sample drying or lyophilization may be advantageous, and water content should be regarded as a critical parameter during method development.

As a general principle, the sample should be fully immersed in the extraction solvent. Nevertheless, owing to the enhanced solubility of analytes under microwave irradiation, the total solvent volume required is typically substantially lower than that used in conventional extraction techniques [19]. With respect to solvent selection, in addition to classical considerations such as analyte solubility, matrix interactions, and compatibility with subsequent analytical steps, the ability of the solvent to absorb microwave energy must also be taken into account. Different strategies may be adopted depending on the analytical objective. Polar solvents, which strongly absorb microwave radiation, are generally suitable for the extraction of polar compounds. Conversely, when thermolabile analytes are targeted—such as essential oils extracted from water-rich plant matrices (e.g., leaves or peels)—an alternative approach can be employed [17]. In this case, the matrix itself efficiently absorbs microwave energy, leading to tissue disruption and analyte release, while a low-dielectric-constant solvent can be used to limit thermal degradation of the extract [17].

Solvent mixtures with different dielectric properties may also be employed; however, this often results in reduced selectivity, necessitating additional purification steps, as

reported for the extraction of PAHs from smoked meat using hexane–acetone mixtures [27]. Alternatively, microwave-transparent solvents (e.g., hexane) can be used in combination with highly microwave-absorbing inserts, typically composed of carbon-based materials, so that heating occurs predominantly through conduction rather than direct microwave irradiation [27].

Finally, extraction temperature and time must be carefully optimized, as these parameters are closely interrelated: higher temperatures generally allow for shorter extraction times.

Obviously, attention must be paid to the thermal stability of the analytes and the matrix to set the optimal conditions. As aforementioned, when using closed-vessel MW systems, the solvent may increase its boiling point due to the overpressure created by the vapors in the closed-vessel (as in a pressure cooker). Although to a limited extent, this reflects one of the benefits of the pressurized liquid extraction (PLE) system. Under these conditions, analyte solubility and diffusion rates are enhanced, while solvent viscosity and surface tension are reduced, facilitating solvent penetration into the matrix and the disruption of analyte–matrix interactions. Overall, these effects contribute to improved mass transfer and higher extraction yields. The careful optimization of these parameters not only enhances analytical performance but also enables a reduction in solvent consumption, energy input, and extraction time, thereby establishing a clear conceptual link between microwave-assisted extraction and the fundamental principles of green analytical chemistry, which are discussed in the following section [23-28].

5. Applications of MW technology for extraction procedures

5.1 Environmental

The application of MAE in environmental analysis is probably the most developed field of application, among which there are several official methods approved. The first official method approved, besides the ones for elemental analysis, was the US Environmental Protection Agency (EPA) Method 3546 for the analysis of water-insoluble or slightly water-soluble organic compounds from several environmental compartments (i.e., soil, clays, sediments, sludge, and other solid waste) in 1997 [29]. It provided performance similar to the Soxhlet but used 10 times less solvent and reduced the preparation time from hours to minutes. The method was not developed to be optimized for the extraction of specific analytes from specific samples or in terms of throughput but rather to be a robust and versatile procedure for a wide variety of environmental contaminants and in a large variety of matrices. It was what today we would call a robust, untargeted universal method. This versatility and high efficiency, along with the rapidity of the process, are one of the main characteristics that make MAE such an exciting tool in analytical sample preparation. Various multi-target methods have been developed over the years and are nicely summarized in some reviews to which the readers are directed [17,30]. Methods have been optimized by exploiting either the extraction solvent or the sample matrix's heating properties, especially when contaminants were extracted from samples with high water content,

such as plants. Both strategies proved to be efficient and allowed for sufficient selectivity. Particularly interesting is the observation reported in the first application of MAE for the extraction of contaminants from mint plants [31]. The degree of degradation of the plant was examined with an electron micrograph, showing that the extent of disruption of the biological structure after 20s under microwave conditions was comparable to 2 hours of steam distillation and 6 hours of Soxhlet. Moreover, the MAE extract was much less pigmented than the steam distillate. This could be explained by the lower penetration of the extraction fluid used and the different mass diffusion rates of the analytes and the pigments. Thus, during the short extraction period applied (2-3 minutes), pigments were not extracted [30].

5.2 Foods

MAE has found a broad application in the field of food analysis; besides the more traditional use for total crude fat extraction, many targeted applications (e.g., contaminants, active compounds, etc) have been proposed. A general review has been recently published on the topic [32] and a few more specific reviews [17,20,29-32]. Here, the goal is not to be comprehensive but rather to provide a general overview.

A major application of MAE in food analysis is the determination of active compounds (referring to natural components proven to have an impact in the prevention of various chronic diseases, including cancer, cardiovascular disease, inflammation, and other oxidative stress-related pathologies [33]), among which phenolic compounds have been the most studied ones using MAE. Indeed, besides reducing time and solvent consumption, MAE plays an important role in minimizing the detrimental impact of the high reactivity of phenolic compounds to thermal and oxidative degradation, allowing for shorter and milder extraction conditions with comparable, if not superior, performance. For instance, MAE provided recoveries similar to traditional maceration for eight phenolic compounds extracted from grape skin and reduced the extraction time from 5h to 5 min while extracting three additional compounds [34]. No further increase in the yield was observed after 5 min. On the contrary, extending the extraction above 15 min negatively impacted the recoveries [34], as also reported for (poly)phenols, anthocyanins, flavonoids, phytosterols, carotenoids, and saponins [35]; and for carotenoids due to oxidation and isomerization [36]. Optimal extraction conditions for carotenoids were defined as short as 120 s at 60°C from paprika powder [37]. Diterpenes were also successfully extracted from coffee by exploiting microwave-assisted methanolysis, thus improving the yield from 25.9% with conventional heating to 99% with MAE [38].

For lipid extraction MAE has been proven superior to traditional methods such as Soxhlet, Folch, and Bligh and Dyer methods [39,40]. The benefit of the MAE, besides the significant reduction in time and solvent consumption, is that it can efficiently disrupt the matrix (e.g., tissue, cell wall), favoring the extraction of the lipids without using dedicated procedures [41,42].

The possibility of merging different steps during a single MAE treatment has also been explored. For instance, the simultaneous extraction and derivatization of fats in

fatty acid methyl esters have been successfully proposed [43], proving the equivalence with the official method, which involves a tedious extraction followed by a derivatization method based on the use of BF_3 and MeOH [44].

Microwave-assisted saponification (MAS) has also been proposed for several applications, such as the analysis of sterols and dialkyl ketones (DAK) in fats [45], cholesterol and cholesterol oxides (COPs) in shrimps (raw, salted, and dried-salted) [46].

MAS was similarly used for the simultaneous enrichment and extraction of contaminants, such as polycyclic aromatic hydrocarbons (PAHs) and mineral oils (MOH) from different samples [47-49]. Indeed, PAHs and MOH are notably lipophilic compounds, but at the same time, they are stable and have low reactivity. Therefore, saponification allows for easy removal of the bulk of the triglycerides, possibly co-extracted, significantly improving sensitivity [20, 25, 29-32]. Nevertheless, it was also shown that the use of more harsh conditions for food samples allowed for more quantitative extraction of the MOH from the matrix, reflecting not only the superficial contamination due to migration from food contact materials but also the pre-existing contamination [50].

MAE has also a wide application for the analysis of pesticides, where it allows for a broad extraction in terms of pesticide classes and exploitation of the interaction of the microwave with the matrix [51]. Indeed, the extraction from vegetable products benefits from the high-water content of the matrix (over 80%), which ensures efficient heating while disrupting the interaction of pesticides with the matrix thanks to the local expansion of water [52]. The choice of the solvent or the solvent mixture is critical to tuning the selectivity of the extraction. For instance, acetonitrile/acetone (1/1 v/v) succeeded in the efficient extraction of 72 pesticides from 35 different matrices [52]. Acetonitrile was used for the combined extraction of pesticides and mycotoxins (total of 46 analytes) from apples in a single step, providing comparable performances with the QuEChERS procedure but in a significantly simplified workflow [53].

5.3 Clinical

While many studies can be found where microwaves are used for the extraction of analytes from environmental and food matrices, as described above, the same technique is rarely employed for biological matrices in clinical/toxicological fields, although it can provide significant benefits.

Rapid and clean-up-free MAE methods were successfully used to extract drugs from blood, serum, urine, human hair, and vitreous humor samples. Recoveries ranging between 87-99.3% were reported, compared to recoveries in the 52.1-99.1% range reported for solid-phase extraction [54-57].

In particular, the procedure for the analysis of hairs is significantly simplified using MAE. Indeed, the preparation of hair samples is complex, time-consuming (from 1h to several hours), and requires many steps of clean-up before instrumental analysis. Wietecha-Posłuszny *et al.* optimized a 10 minute extraction method (at 75°C) for the

analysis of six benzodiazepines from human hair by UHPLC-TOF-MS, achieving limits of detections in the 0.003-0.025 ng/mg range without the need of additional purification [58].

Microwaves can also play an important role in lipidomic studies, although careful optimization is needed according to the matrix and the class of lipids targeted [59-60]. For instance, Morais *et al.* reported the use of MAE for the extraction of 24 fatty acids from human plasma, showing a slightly lower recovery compared to the traditional Folch method (97.22 % vs 102.21%) [61]. On the contrary, Gonzalez-Illan *et al*. [62] and Khoomrung *et al*. [63] described MAE as an efficient and reproducible method for the extraction and quantification of fatty-acid ethyl esters in skin and yeast cells, respectively.

Moreover, the lysis of more than 95% of mammalian cells was observed when exposed to microwave energy, providing 50% more mass peak than the traditional Blingh and Dyer method for lipidomic studies [64]. The ability of cell lysis, thanks to the mechanical movement generated by microwave energy, makes application in the field particularly interesting to avoid the use of harsher conditions and more aggressive and less eco-friendly solvents. Nevertheless, the clinical setting is generally characterized by limited sample availability and small volume, therefore, the miniaturization of the sample preparation step is an important requirement that commercial systems cannot yet fully fulfill.

6. Microwave and Green Analytical Chemistry: the use of greener solvents

MAE plays also an important role in the perspective of green analytical chemistry. Per se, it already leads to a reduction of hazardous reagents and waste, lower energy consumption, and higher safety of the analytical procedure. Indeed, energy consumption is reduced thanks to the higher efficiency and lower heating time, along with the possibility of treating multiple samples simultaneously. Besides, the procedure in a closed vessel minimizes the risk for the operators, and permits a reduction of the amount of solvents, reagents, and sample needed. Nevertheless, the nature of the solvent used remains generally not green (e.g., hexane, acetone, etc.). However, more recently, attention has been paid to the use of alternative greener solvents in the analytical chemistry process [18], which has also started to enter the field of MAE. Obviously, the ideal green situation involves no use of solvents. Solvent-free MAE has been proposed for the extraction of essential oils, mixing an absorbent, such as carbon powder or ionic liquid, with the dry matrices and exploiting the swelling and the disruption effect of the internal water on the cell wall, with the consequence release of the essential oil trapped in the sorbent. Nevertheless, the applicability of this kind of approach is very limited, and it is at the border between laboratory analytical chemistry and an industrial chemical process for essential oil extraction [65,66].

The use of water as an extraction solvent is also a highly green choice. In particular, water above 100°C has a lower dielectric constant and polarity, similar to organic solvents such as ethanol, acetonitrile, and acetone. Therefore, subcritical water (which

means water in the 100-274°C temperature range) may allow the extraction of less polar compounds, which would not be extractable in water at lower temperatures. Exploiting this water property in combination with a microwave is named microwave-assisted water extraction (MWE) or microwave-assisted aqueous extraction (MAAE). This technique has been proven efficient for the extraction of bioactive compounds, such as polyphenols, reducing the extraction time by about one-third [67,68].

The use of supramolecular solvents (i.e., surfactants of different types, such as anionic, cationic, nonionic, or a mixture) has also been proposed. Surfactants are considered greener alternatives thanks to their low toxicity, low volatility, and biodegradability. The use of surfactant in MAE is named microwave-assisted micellar extraction (MAME) or cloud point extraction. It has been mainly used for the analysis of metals from different matrices [16] but has also found some applications in the analysis of alkaloids, hydrocarbons, PAHs, sulphonamide, and phenols.

The use of ionic liquids (ILs) as an extraction solvent has also been investigated. ILs have gained attention over the years for their peculiar characteristics and their low volatility, which make them a greener choice over traditional organic solvents [69], although the extent of their green aspects is, at present, under discussion [70]. ILs are perfect solvents for microwave heating as they are salts with a melting point below 100°C, which means that they are directly excited by the microwave, showing a characteristic heating behavior [71]. Nevertheless, usually, they are added in low amounts to increase the heating ability of the medium but are not used as a unique extraction solvent.

Finally, of interest is the recent introduction of deep eutectic solvents (DESs) as extraction solvents in the sample preparation step. Deep eutectic solvents (DESs) are a class of solvents formed by the combination of two or more components capable of interacting through hydrogen bonding, resulting in a eutectic mixture with a melting point significantly lower than that of the individual constituents. While early DESs were typically based on quaternary ammonium salts (e.g., choline chloride) combined with hydrogen-bond donors (e.g., sugars, alcohols, or carboxylic acids), more recent developments have expanded this definition to include a broader range of systems, such as natural deep eutectic solvents (NADES) and non-ionic DESs. DESs are characterized by tunable physicochemical properties and are often considered environmentally friendly due to their low toxicity, low cost, and potential biodegradability. [72-74]. That makes this solvent category of high interest. DESs are very viscous at room temperature, and therefore, to maximize their penetration and diffusivity capacity into the samples, they benefit from the use of high extraction temperature, which, on the contrary, can be detrimental to the degradation of the targeted analytes; therefore, these aspects must carefully be optimized [75].

7. Conclusion

MAE is a powerful technique which has proven its benefits in many fields of applications. The main advantage is the increased extraction yield thanks to the matrix disruption effect due to the mechanical oscillations of the dipolar molecules.

Moreover, the more efficient heating process improves the sample throughput, reducing energy consumption, waste production, and using less solvent volume. The attention towards the green analytical aspects has opened a new field of research related to the use of more eco-friendly solvents, which will lead in the near future to an even more acceptability of MAE as a greener technique. It is the authors' opinion that miniaturization of the system will push the technique towards even wider applicability, allowing incorporation into robotic automation and into critical fields, such as clinical, where the sample availability and dimension represent a critical issue.

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Chapter 2

Analysis of lipids

1. Lipids definition

Lipids constitute a broad and structurally heterogeneous class of biomolecules that play a fundamental role in biological systems and food matrices. Despite the absence of a universally accepted definition, lipids are generally described as naturally occurring compounds that are insoluble in water and soluble in non-polar organic solvents, primarily composed of carbon, hydrogen, and oxygen, and frequently containing heteroatoms such as phosphorus, nitrogen, or sulfur [1]. From a nutritional and physiological perspective, lipids serve as a major source of metabolic energy, provide essential fatty acids that cannot be synthesized endogenously, and act as key structural components of cell membranes. In addition, they play a crucial role in the transport and bioavailability of fat-soluble vitamins and in the regulation of several metabolic and signaling pathways. In the context of food science, lipids are equally relevant, as they strongly influence sensory attributes such as flavor, texture, juiciness, and overall palatability, while also affecting technological properties during food processing, including emulsification, rheology, oxidative stability, and shelf life.

Given their structural complexity and functional relevance, lipids are commonly classified according to their chemical structure, which represents the most informative and robust classification approach. Based on this criterion, lipids can be grouped into simple, complex, and derived lipids. Simple lipids consist of esters formed by fatty acids and alcohols, whereas complex lipids additionally contain other functional groups such as phosphate, carbohydrate, or nitrogen-containing moieties. Derived lipids include fatty acids and other compounds obtained from the hydrolysis of simple and complex lipids, representing key building blocks within lipid metabolism and food systems [1].

2. Fatty acids

Among lipid constituents, fatty acids represent the fundamental structural units of most lipid classes and are therefore of primary analytical interest [2]. Chemically, fatty acids are composed of a linear aliphatic hydrocarbon chain containing four or more carbon atoms, terminated by a carboxylic acid functional group (–COOH) at one end and a methyl group ($–CH_3$) at the other. This amphiphilic nature confers limited solubility in aqueous media while favoring solubility in non-polar organic solvents.

The physicochemical properties of fatty acids, including melting point, solubility, and chemical reactivity, are strongly influenced by chain length and degree of unsaturation. Based on the number of carbon atoms, fatty acids are commonly classified into short-chain (C4–C10), medium-chain (C12–C14), long-chain (C16–C18), and very-long-chain fatty acids (>C20). This classification generally excludes acetic (C2) and propionic (C3) acids, as they are not typical constituents of natural fats and oils. Most naturally occurring fatty acids possess an even number of carbon atoms, reflecting their biosynthetic origin, whereas odd-chain fatty acids are less abundant and are mainly found in specific animal and marine lipid sources [2].

Fatty acids can be further classified according to their degree of unsaturation into saturated fatty acids (SFAs), which contain no carbon–carbon double bonds, and unsaturated fatty acids, which contain one or more double bonds along the hydrocarbon chain. Unsaturated fatty acids are subdivided into monounsaturated fatty acids (MUFAs), characterized by the presence of a single double bond, and polyunsaturated fatty acids (PUFAs), which contain two or more double bonds. The position of the first double bond relative to the terminal methyl group allows further classification into omega families, most notably omega-3 and omega-6 fatty acids, which differ in their chemical behavior, natural distribution, and biological functions.

In addition to chain length and unsaturation degree, the geometric configuration of double bonds represents a crucial structural feature. Double bonds may occur in either cis (Z) or trans (E) configuration, with the cis configuration being predominant in naturally occurring unsaturated fatty acids. Trans fatty acids may be formed during industrial processing or high-temperature treatments, resulting in a more linear molecular structure, higher melting point, and increased thermodynamic stability compared to their cis counterparts.

From an analytical standpoint, the structural diversity of fatty acids has important implications for their determination. Variations in volatility, polarity, and thermal stability influence both sample preparation and chromatographic behavior, making fatty acid analysis highly dependent on appropriate derivatization and extraction strategies. These aspects are particularly relevant in food analysis, where complex matrices and a wide range of fatty acid classes coexist, necessitating robust and selective analytical approaches [2,3].

2.1 Fatty acids and correlations with health

The relationship between dietary fatty acids and human health is complex and cannot be adequately described through a purely quantitative assessment of total fat intake. Rather, the health impact of lipids is strongly influenced by the qualitative composition of fatty acids, their structural characteristics, and the food matrix in which they are consumed. For several decades, research in this field has primarily focused on the potential adverse effects associated with saturated fatty acids (SFAs) and trans fatty acids (TFAs), as well as on the beneficial roles attributed to specific classes of unsaturated fatty acids.

Saturated fatty acids play essential physiological roles, including participation in membrane structure, protein modification, regulation of gene expression, and energy storage. The human body is capable of synthesizing SFAs de novo, and their circulating levels are therefore influenced by both dietary intake and endogenous metabolism. Historically, high consumption of SFAs—particularly from animal-derived fats—has been associated with increased plasma cholesterol levels and elevated risk of cardiovascular diseases. However, more recent evidence suggests that the relationship between SFA intake and health outcomes is modulated by factors such as fatty acid chain length, food matrix effects, and overall dietary patterns, challenging the oversimplified classification of SFAs as uniformly detrimental.

Within the group of unsaturated fatty acids, monounsaturated fatty acids (MUFAs) have been consistently associated with positive health outcomes. Diets rich in MUFAs have been linked to improved lipid profiles and reduced cardiovascular risk, leading international health organizations to recommend their inclusion as a substantial fraction of total dietary fat intake. Polyunsaturated fatty acids (PUFAs), particularly those belonging to the omega-3 and omega-6 families, are of special nutritional relevance, as they include essential fatty acids that must be supplied through the diet. PUFAs are involved in a wide range of biological processes, including inflammation modulation, immune response, neuronal function, and cardiovascular protection.

Trans fatty acids represent a distinct category of unsaturated fatty acids characterized by at least one double bond in the trans configuration. Their physicochemical properties resemble those of saturated fatty acids, resulting in increased melting points and enhanced structural rigidity. Epidemiological and clinical studies have consistently demonstrated that industrially produced TFAs adversely affect lipid metabolism by increasing low-density lipoprotein (LDL) cholesterol and decreasing high-density lipoprotein (HDL) cholesterol, thereby significantly increasing the risk of cardiovascular diseases. As a consequence, international health authorities have promoted strategies aimed at minimizing TFA intake, leading to strict regulatory measures in many countries.

Overall, these findings highlight the importance of accurately characterizing fatty acid profiles rather than relying solely on total fat content as a nutritional indicator. From an analytical perspective, this reinforces the need for robust, precise, and comprehensive methods for fatty acid determination, capable of distinguishing among different fatty acid classes and isomers. In this context, fatty acid methyl ester (FAME) analysis represents a cornerstone technique, providing the detailed compositional information required to support nutritional assessment, food quality evaluation, and regulatory compliance [4].

3. Sterols

3.1 Chemistry of Sterols

Chemically, phytosterols are characterized by a tetracyclic perhydro-cyclopentano-phenanthrene core with a flexible aliphatic side chain at carbon C-17 and a hydroxyl group in the 3β-position [5]. Most phytosterols contain 28 or 29 carbon atoms and possess one or two carbon–carbon double bonds, typically located within the sterol nucleus and occasionally in the side chain [6].

According to IUPAC nomenclature (1989), sterols consist of four fused rings—A, B, C, and D—with standardized carbon numbering (Figure 1) [7]. Rings A, B, and C contain six carbon atoms each and are fused in a non-linear arrangement, whereas ring D contains five carbon atoms. Structural diversity among phytosterols arises from variations in the length and substitution of the side chain attached to C-17 and from the number and position of double bonds present in both the sterol nucleus and the

aliphatic chain. Specific structural modifications determine their physiological functions.

Plant sterols (phytosterols) constitute the majority of the unsaponifiable fraction in vegetable oils and are classified according to the presence or absence of methyl substituents at the C4 position of the A ring:

- 4-desmethylsterols (no methyl groups on ring A)
- 4-monomethylsterols (one methyl group)
- 4,4-dimethylsterols (two methyl groups; triterpenic alcohols)
- In addition to these classes, the unsaponifiable fraction of olive oil also contains triterpene dialcohols, a group of compounds that co-elute chromatographically with 4-desmethylsterols [8]. Among these, erythrodiol and uvaol are the predominant triterpene dialcohols found in olive oil.

Figure 1 illustrates the typical structures of the most common plant sterols.

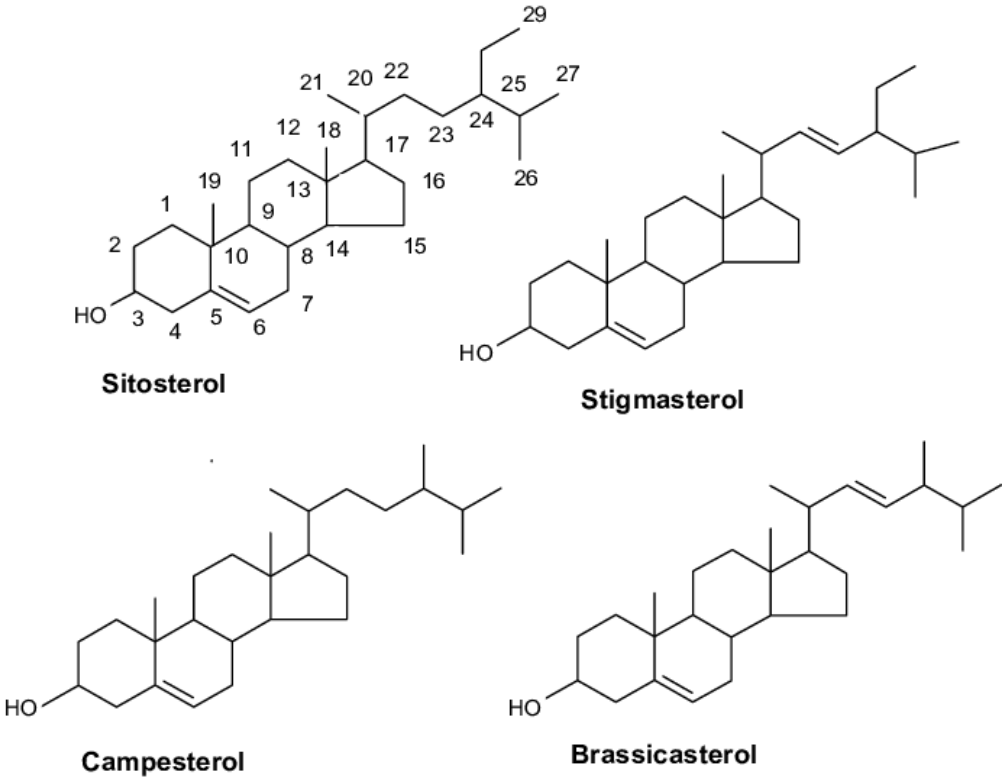

Figure 1. Chemical structure of selected plant sterols (sitosterol, campesterol, stigmasterol, and brassicasterol)

Due to the hydroxyl group at carbon 3 of ring A, phytosterols can occur in both free and esterified forms. In 4-monomethylsterols we can cite Citrostadienol, and finally

in the class of 4,4-dimethylsterols we can cite Cyclartenol and 24-Methylenecycloartanol.

The reader is encouraged to keep in mind the nomenclature and structural attributes of the sterols presented in this section, as they will play an essential role in the methodological and analytical considerations developed in the experimental chapter.

Moreover, based on the number and position of double bonds in the B ring, 4-desmethylsterols are divided into three groups: Δ^5-sterols, Δ^7-sterols, and $\Delta^{5,7}$-sterols [9]. This class includes the most common phytosterols with 28- or 29-carbon skeletons, as well as cholesterol, which possesses a 27-carbon structure [9]. Although cholesterol is the predominant sterol in animal cells, it represents only a minor component in plant tissues [10]. Structurally, cholesterol is closely related to phytosterols, differing primarily in the composition of its side chain.

3.2 Sterols in EVOO

Virgin olive oil is obtained exclusively from the fruit of the olive tree (*Olea europaea* L.) through mechanical or physically based extraction processes that prevent any thermal or chemical alteration of the product. Traditional techniques such as pressing, centrifugation, and percolation enable the separation of oil from the olive paste while preserving the physicochemical and sensory properties naturally present in the fruit. In general, the industrial processing of olives yields two main products: virgin olive oil (VOO) and olive pomace oil. According to both European legislation and the International Olive Council (IOC), virgin olive oils must be obtained solely through mechanical means that do not alter the oil composition. Within this category, oils are further classified as extra virgin olive oil (EVOO), virgin olive oil (VOO), and lampante olive oil (LOO). EVOO represents the highest-quality grade, distinguished by stringent chemical and sensory criteria, whereas LOO—due to its high acidity and sensory defects—cannot be directly marketed and must undergo refining before consumption. Differentiating EVOO from VOO is often challenging because the two share similar compositional attributes; however, the significant price difference between these categories makes EVOO particularly susceptible to fraud. Ensuring the authenticity of virgin olive oil is therefore crucial from both a public-health and economic perspective. EVOO is valued not only for its superior organoleptic characteristics but also for its well-documented health benefits, including antioxidant activity, anti-inflammatory effects, and cardioprotective properties. These attributes contribute to its higher market price compared with refined or blended oils, creating incentives for fraudulent practices. Common frauds involve mislabeling lower-grade oils as EVOO or diluting authentic EVOO with cheaper vegetable oils. It is estimated that olive oil accounts for nearly 24% of all food fraud cases [11].

In olive oil specifically, the lipid fraction determines its nutritional quality, sensory attributes, and technological behavior. The oil consists predominantly of triacylglycerols (95–99%), while the remaining 1–5% comprises minor lipid components—including sterols, tocopherols, hydrocarbons (notably squalene), aliphatic and triterpenic alcohols, phenolic compounds, and pigments. The qualitative

and quantitative composition of these minor constituents depends on several factors, such as olive cultivar, geographic origin, climate, degree of ripening, and extraction method. Because these components form a unique "chemical signature," the minor lipid fraction plays a pivotal role in authenticity assessment [12]. Unlike TAGs, which can sometimes be manipulated to mimic genuine profiles, minor lipids serve as intrinsic markers that are far more challenging to alter. Their patterns can even provide insight into the geographical origin of the oil—an increasingly important factor as olive cultivation expands beyond the Mediterranean Basin. To ensure fair production and trading practices, olive oils are regulated by stringent international standards. The primary regulatory bodies are the European Commission (EU), the International Olive Council (IOC), and the Codex Alimentarius. EU legislation—most recently defined by Commission Delegated Regulation (EU) 2022/2104—establishes mandatory requirements for olive oil marketing standards within Member States. IOC standards, while not legally binding, serve as widely adopted international guidelines. Both frameworks define the legal chemical and sensory parameters for olive oil categories and specify analytical procedures for compliance testing.

Table 1 reports the sterol composition allowed in different oil samples according to Regulation (EU) 2022/2104 [13].

Analytical methods recommended by both regulatory bodies are often the result of extensive research by Member States and have been updated over time to improve analytical performance. Recent advancements focus on:

- advanced analytical instrumentation
- safer and more sustainable solvents
- faster and more efficient procedures

Table 1. Sterol composition allowed in different oil samples according to Regulation (EU) 2022/2104 [3].

Category	Sterols composition						Total Sterols (mg/kg)
	Cholesterol (%)	Brassicasterol	Campesterol (2)(%)	Stigmasterol (%)	App β-sitosterol (3)(%)	Delta-7-stigmasterol (2) (%)	
1. Extra virgin olive oil	≤ 0.5	≤ 0.1	≤ 4.0	< camp	≥ 93.0	≤ 0.5	≥ 1000
2. Virgin olive oil	≤ 0.5	≤ 0.1	≤ 4.0	< camp	≥ 93.0	≤ 0.5	≥ 1000
3. Lampante olive oil	≤ 0.5	≤ 0.1	≤ 4.0		≥ 93.0	≤ 0.5	≥ 1000
4. Refined olive oil	≤ 0.5	≤ 0.1	≤ 4.0	< camp	≥ 93.0	≤ 0.5	≥ 1000
5. Olive oil composed of refined and virgin olive oils	≤ 0.5	≤ 0.1	≤ 4.0	< camp	≥ 93.0	≤ 0.5	≥ 1000
6. Crude olive- pomace oil	≤ 0.5	≤ 0.2	≤ 4.0		≥ 93.0	≤ 0.5	≥ 2500
7. Refined olive- pomace oil	≤ 0.5	≤ 0.2	≤ 4.0	< camp	≥ 93.0	≤ 0.5	≥ 1800

3.3 Sterols, an important tool in Detecting Extra Virgin Olive Oil Fraud

The predominant sterol in olive oil is β-sitosterol, which typically accounts for more than 80% of the total sterol content. Other major sterols include campesterol,

stigmasterol, and Δ^5-avenasterol, each contributing to the characteristic sterolic fingerprint of extra virgin olive oil. Because sterol composition is strongly influenced by botanical origin, cultivar, environmental conditions, and extraction practices, each vegetable oil exhibits a unique sterol profile [14]. This makes sterol analysis an exceptionally robust and widely accepted tool for detecting adulteration and verifying authenticity.

The addition of lower-cost vegetable oils—such as soybean, sunflower, or palm oil—alters the intrinsic sterol composition of EVOO, often resulting in elevated campesterol levels or the appearance of marker sterols not naturally present in olive oil. One of the most diagnostic markers is brassicasterol, a sterol characteristic of rapeseed (canola) oil and absent, or present only in trace amounts, in authentic EVOO. Even minor additions of rapeseed oil can be detected by tracing brassicasterol levels.

For this reason, sterol composition is strictly regulated. According to EU Regulation 2022/2104, the proportion of campesterol in EVOO must not exceed 4.0% of total sterols, while brassicasterol levels above 0.1% are indicative of adulteration [13]. Additional parameters, such as the ratio between β-sitosterol and Δ^5-avenasterol, or the presence of sterols typical of seed oils (e.g., Δ^7-stigmastenol, $\Delta^{5,22}$-stigmastadiene), can further support authenticity assessment.

4. Analytical determination of lipids

4.1 Fatty acid analysis

Fatt acids are most usually analyzed in the form of Fatty Acid Methyl Esters (FAMEs). They are obtained through esterification or transesterification reactions in which free fatty acids or acylglycerols react with a short-chain alcohol, most commonly methanol, in the presence of an acid or base catalyst. This process leads to the formation of fatty acid esters and glycerol as a by-product. The transesterification reaction is reversible, and its efficiency depends on reaction conditions, including catalyst type, temperature, and reactant ratios.

FAMEs analysis represents a cornerstone technique for fatty acid profiling, and the resulting compositional information provides insight into the relative abundance of individual fatty acids, enabling the assessment of nutritional quality, technological properties, and product authenticity, as well as compliance with labeling regulations (e.g., saturated fatty acids or ω-3 fatty acids).

Historically, early FAMEs analytical protocols relied on a two-step approach consisting of lipid extraction from the matrix followed by chemical derivatization. A wide range of extraction techniques has been proposed, and the choice of method and solvent system is strongly dependent on the nature of the sample matrix and the polarity of the lipid fraction. Since food lipids encompass compounds with different polarities, mixtures of two or more solvents are often employed to ensure exhaustive extraction.

Among traditional extraction methods, Soxhlet extraction has been extensively used. In this technique, the sample is placed in a porous thimble within an extraction chamber, while the solvent is heated, evaporated, condensed, and continuously recycled through the sample, allowing lipids to be solubilized and collected in the boiling flask. Despite its effectiveness, Soxhlet extraction is time-consuming and solvent intensive. Alternative conventional methods, such as the Folch and Bligh and Dyer [15,16] procedures, are based on chloroform–methanol mixtures and remain widely applied for total lipid extraction from biological and food samples.

In contrast, microwave-assisted extraction (MAE) has emerged as a faster, more efficient, and more robust alternative to conventional extraction techniques, as discussed in detail in Chapter 1. Other non-conventional extraction approaches, including supercritical fluid extraction (SFE) and ultrasound-assisted extraction (UAE), have also been investigated as promising strategies to reduce extraction time and solvent consumption.

Following lipid extraction, derivatization of fatty acids into their methyl ester form is required to improve volatility and chromatographic performance. Several derivatization strategies have been developed and can be broadly classified into basic, acidic, or combined approaches. In some studies, derivatization has also been performed under microwave irradiation after the extraction step. As early as 1992, Dasgupta et al. [17] reported the application of microwave energy to transesterify lipids using BF_3 in methanol, demonstrating the feasibility of microwave-assisted derivatization.

Basic-catalyzed derivatization methods commonly employ sodium methoxide or potassium hydroxide in anhydrous methanol. Typically, sodium methoxide (0.5 M) is added to the lipid extract, and the reaction is carried out at moderate temperatures for a few minutes, followed by neutralization with sodium bisulfate. These methods offer several advantages, including short reaction times, operational simplicity, and limited double-bond isomerization. However, they are not suitable for the esterification of free fatty acids, which limits their applicability in complex matrices.

Acid-catalyzed derivatization methods overcome this limitation and are effective for both esterified and free fatty acids. Methanolic hydrogen chloride is widely used due to its relatively mild reactivity, high esterification yield, and operational simplicity. Other acidic reagents, such as acetyl chloride, sulfuric acid, and boron trifluoride (BF_3) in methanol, have also been extensively applied. Among these, BF_3 has been one of the most commonly used catalysts owing to its high derivatization efficiency and relatively short reaction times. Nevertheless, its limited stability and the potential formation of analytical artifacts require careful handling and have raised concerns regarding its routine use.

In some protocols, a combined approach involving acid catalysis following an initial alkali-catalyzed transesterification has been proposed. In these cases, a preliminary basic treatment promotes rapid transesterification, while a subsequent acid-catalyzed step ensures the esterification of free fatty acids. Although sulfuric acid may be employed, BF_3 is often preferred due to its stronger esterifying power. Such multi-

step strategies aim to maximize derivatization completeness, albeit at the expense of increased procedural complexity.

Table 2 provides an overview of the extraction and derivatization techniques most commonly employed in literature for the analysis of food samples.

Table 2. Extraction and derivatization techniques according to the matrix processed.

FOOD MATRIX	EXTRACTION METHOD	DERIVATIZATION METHOD	REFERENCE
MEAT	Soxhlet Method (PE/EE) Bligh and Dyer Method Folch Method Rapid microwave solvent extraction SFE	Base catalyst sodium methoxide method BF_3-MeOH method	AOAC Official Method 960.39, 2012 AOAC, Official Method 991.36, 2012 AOAC, Official Method 985.15, 2012 AOAC, Official Method 969.33, 2012 Pérez-Palacios et al. (2008), Fiori et al. (2013) O'fallon, et al. (2007)
NUTS AND SEEDS	Soxhlet Method (EE/Hex) UAE	BF_3-MeOH method	Al Juhaimi et al. (2018), Aremu and Akinwumi (2014), AOAC Official Method 948.22 (2012), Luque and De Castro (2004), Mello et al. (2017)
BUTTER	Soxhlet method (Hex) Bligh and Dyer method Folch method	Acid catalyst conc. H_2SO_4 (95%) method BF_3 -MeOH method	Abdul-Mumeen et al. (2019), Rutkowska and Adamska (2011), Ozcan et al. (2016), AOAC Official Method 938.06, 2012, Nazari et al. (2012)
FAST FOOD	Non-meat foods: Solvent extraction (PE/Hex) Meat foods: Folch method MAE SFE	Base catalyst sodium methoxide method BF_3 -MeOH method	Musaiger et al. (2008), Ferna´ndez and Juan (2000), Nazari et al. (2012), Virot et al. (2008), Devineni et al. (1997).
DAIRY PRODUCTS	Modified Folch method Chloroform:Methanol (2:1, v/v) Modified Bligh and Dyer Chloroform:Methanol: Water (1:4:0.8, v/v)	BF_3 -MeOH method Base catalyst (sodium methoxide/ KOH method) Acid catalyst (5% HCl/MeOH)	Nazari et al. (2012), Castanha et al. (2013), Cruz-Hernandez et al (2004), Amores and Virto (2019)
FLOUR PRODUCTS AND CHOCOLATE	Solvent extraction (PE/Hex) Folch method Soxhlet extraction (Hex) MAE Bligh and Dyer	Base catalyst sodium methoxide method BF_3 -MeOH method	Fernandez and Juan (2000), Nazari et al. (2012), Virot et al. (2008), Patrignani et al. (2015), Bahrami et al. (2014)

Over time, increasing attention has been devoted to the development of one-step extraction–derivatization strategies, with the aim of simplifying the analytical workflow for FAMEs determination and reducing sample handling, solvent consumption, and analysis time. In these approaches, lipid extraction and fatty acid derivatization are combined into a single operation, typically exploiting strong acidic or basic catalysts. Several reagents commonly used for conventional derivatization, including methanolic hydrogen chloride, acetyl chloride, sulfuric acid, and boron trifluoride (BF_3), have been successfully applied in one-step protocols.

In this context, Jariyasopit et al. [18] proposed a direct derivatization procedure for dairy samples, in which small volumes of milk (200 µL) were reacted with BF_3 (14% in methanol) in the presence of hexane, without a prior lipid extraction step. Similarly, Meier et al. [19] validated a one-step extraction and methylation method using

methanolic hydrogen chloride for the determination of fatty acids in marine tissues, demonstrating higher recoveries of total fatty acids compared to conventional multi-step procedures.

The feasibility of direct methylation has also been recognized in official analytical protocols. Notably, the AOCS Official Method Ce 2b-11 is based on direct methylation of lipids in food matrices via alkali hydrolysis followed by acid catalysis, corresponding to the combined derivatization strategy previously described, but performed without an initial extraction step.

More recently, the application of microwave-assisted techniques has further expanded the potential of one-step extraction–derivatization methods. Microwave irradiation enables rapid and homogeneous heating of the reaction medium, enhancing mass transfer and accelerating reaction kinetics. In this regard, Rui-Lin Liu et al. [20] investigated a microwave-assisted one-step extraction–derivatization approach in which samples were treated with hexane, solid potassium hydroxide, and methanol, and subsequently irradiated at controlled microwave power and temperature, demonstrating the effectiveness of microwave energy in integrating extraction and derivatization into a single step.

Among the microwave-assisted one-step strategies proposed to date, the method developed by Fina et al. [21] represents one of the most comprehensive and robust approaches for FAMEs analysis in food matrices. This protocol combines microwave-assisted extraction and acid-catalyzed derivatization into a single closed-vessel step, allowing efficient conversion of both free and esterified fatty acids while significantly reducing extraction time, solvent consumption, and manual handling. Importantly, the method was systematically validated against the AOCS official procedure, demonstrating comparable fatty acid profiles and quantitative results across different food matrices. Owing to its analytical reliability, operational simplicity, and favorable sustainability profile, the MAED approach proposed by Fina et al. [21] can be considered a particularly effective alternative to conventional official methods and constitutes the methodological reference framework adopted and further investigated in the present thesis.

4.2 Analytical determination of FAMEs by gas chromatography

Gas chromatography (GC) represents the reference analytical technique for the determination of fatty acid methyl esters due to the volatility, thermal stability, and favorable chromatographic behavior of FAMEs. Among the available detection systems, flame ionization detection (FID) is the most widely employed in routine fatty acid analysis, owing to its robustness, wide linear dynamic range, high sensitivity toward organic compounds, and straightforward quantitative response. For these reasons, GC-FID is routinely adopted in official and standardized methods for FAMEs determination in food matrices and remains the benchmark technique for fatty acid profiling.

Despite its widespread application, conventional one-dimensional GC-FID may present limitations when complex food matrices are analyzed, particularly in the presence of co-eluting compounds, minor fatty acids, or structurally similar isomers. These challenges become more pronounced when fast and simplified sample preparation strategies, such as one-step extraction–derivatization approaches, are employed, as the resulting extracts may contain a broader range of co-extracted matrix components. For this reason, comprehensive two-dimensional gas chromatography (GC×GC) coupled with FID detection is proposed as an advanced analytical approach for FAMEs analysis [21].

4.3 GC×GC

As one-dimensional (1D) chromatography proved insufficient for the complete separation of highly complex samples, increasing attention has been directed toward the development and application of multidimensional chromatographic techniques. The use of comprehensive two-dimensional gas chromatography (GC×GC) is particularly advantageous for the analysis of FAMEs, which are characterized by high chemical similarity, limited selectivity in one-dimensional separations, and frequent coelutions. By combining two separation dimensions governed by different interaction mechanisms, GC×GC provides a substantial increase in peak capacity and enables structured chromatographic patterns based on carbon number, degree of unsaturation, and positional isomerism. This enhanced resolving power allows reliable identification and accurate profiling of FAMEs in complex lipid matrices, making GC×GC a highly effective technique for comprehensive fatty acid analysis. The fundamental principles underlying such systems were formally defined by Giddings and co-workers, who established two essential requirements for true multidimensional separations [22]. First, the separation processes in each dimension must be governed by distinct physicochemical mechanisms. Second, analytes resolved in the preceding separation step must remain separated throughout the subsequent separation process. According to these definitions, multidimensional chromatographic systems are most effective when the individual separation dimensions are based on different interaction mechanisms. Under these conditions, the separation is described as orthogonal, as later discussed by Venkatramani et al. [23]. In comprehensive gas chromatography (GC×GC), the entire sample is subjected to separation across two chromatographic dimensions. Specifically, all solutes eluting from the first-dimension column are periodically transferred and subsequently separated in the second-dimension column, ensuring that no portion of the sample bypasses the second separation step. The interface between the two separation dimensions is achieved through a modulation process, whereby the effluent from the first-dimension column is continuously collected, refocused, and reinjected into the second-dimension column at regular intervals. This operation has to be very fast, generally a pulse-time is in the range of 100-500 ms, and is a critical step for preserving chromatographic resolution and for maintaining the comprehensive nature

of the technique. Modulators employed in GC×GC can be broadly classified into two main categories, namely thermal modulators and flow-based modulators, each characterized by distinct operating principles and performance features [24]. With regard to modulation, this section focuses specifically on flow-based modulators, as this type of modulation is employed in the FAMEs studies presented in the following chapters. Flow modulators operate by periodically collecting fractions of the first-dimension effluent and transferring them into the second dimension using an auxiliary carrier-gas flow. This approach relies on pressure and flow control rather than thermal trapping and offers a simpler and more cost-effective alternative to cryogenic modulation. Because the 1D effluent continuously enters the modulation interface and the columns remain permanently connected, the carrier gas flow in the 2D column must be higher than that in the 1D column. This pressure imbalance is necessary to counteract the incoming 1D flow and prevent breakthrough into the second dimension [ref]. Depending on the specific design and configuration, flow-based modulators may operate in either forward fill/flush (FFF) or reverse fill/flush (RFF) mode. In FFF operation, the filling and flushing steps occur in the same flow direction, whereas in RFF mode they proceed in opposite directions. While both modes exhibit comparable performance at low analyte concentrations, RFF operation generally provides superior performance at higher concentrations, yielding reduced peak broadening, enhanced sensitivity, and increased peak capacity [24,25]. For these reasons, the reverse fill/flush (RFF) flow modulation mode was selected and is the configuration employed in the studies presented in Chapter 3.

4.4 Sterols Analysis

Several methods have been proposed for the recovery of phytosterols from sterol-containing materials. In raw matrices, sterols occur both in free form and, to a significant extent, as esterified compounds. Consequently, an initial pretreatment step is often required to hydrolyze steryl esters into free sterols, which belong to the unsaponifiable fraction. This hydrolysis can be achieved through different technological approaches. One method involves contacting the sterol ester–containing material with water under high temperature (200–260 °C) and elevated pressure (1.5–50 MPa), promoting ester cleavage. Alternatively, hydrolysis may be carried out by alkaline treatment through saponification using sodium or potassium hydroxide at temperatures between 90 and 120 °C, optionally under pressure and continuous stirring. This latter approach is particularly advantageous, as it allows the simultaneous hydrolysis of steryl esters and saponification of the lipid matrix.

Following hydrolysis, current analytical processes aimed at separating and concentrating the unsaponifiable fraction from residues and by-products of animal and vegetable origin rely primarily on physicochemical differences between sterols and the saponified matrix. These processes are generally based either on differences in solubility between unsaponifiable compounds and soap matrices in selected solvents or solvent mixtures, or on differences in volatility between volatile unsaponifiable substances and non-volatile salts or soaps, with separation typically achieved by high-

vacuum distillation or evaporation techniques [26]. These differences in solubility and volatility between sterols and the saponified matrix form the basis of analytical methodologies developed for sterol determination in complex lipid matrices, including olive oil.

In the specific case of olive oil, the analysis of sterols reported in the majority of scientific studies is based on the standardized method developed by the International Olive Council (IOC) [27]. This method has been adopted as the official analytical procedure by Commission Regulation (EEC) No. 2568/91 and subsequent amendments [28] and is employed for the official control of olive oils placed on the European Union market. In addition to its regulatory use, the method is widely applied in the industrial sector, particularly in quality control laboratories of companies involved in the production, packaging, and commercialization of olive oil.

Comparable analytical steps are also described in the American Oil Chemists' Society (AOCS) Official Method Ch 6-91 [29]. Owing to its regulatory relevance, robustness, and extensive validation, the IOC method has become the most widely applied approach in scientific research focused on sterols and triterpene diols in olive oil.

The official method consists of a multi-step analytical procedure. Initially, olive oil samples undergo saponification, followed by liquid–liquid extraction of the unsaponifiable fraction using diethyl ether, including subsequent dealkalization and dehydration of the extract. Sterols and triterpene diols are then separated from other components of the unsaponifiable fraction—such as aliphatic and triterpenic alcohols, tocopherols, and polyphenols—by thin-layer chromatography (TLC) on silica gel plates. The corresponding silica bands are eluted with an organic solvent, derivatized into trimethylsilyl ethers, and analyzed by gas chromatography (GC).

Detection is performed using a flame ionization detector (FID), while identification is achieved by comparison with a reference chromatogram and by evaluation of retention times relative to an internal standard. Quantification is based on an internal standard, typically α-cholestanol or betulin, added at the beginning of the analytical procedure, assuming a response factor equal to unity. The results are expressed as total sterol content (mg/kg) and as relative percentages of individual sterols with respect to the total sterol fraction. The method allows the determination of fourteen major sterols, together with the triterpene diols erythrodiol and uvaol, and also enables the identification of non-characteristic sterols such as brassicasterol and ergosterol.

Despite its robustness, the official method is time-consuming and requires extensive sample preparation, the use of hazardous solvents, specialized laboratory equipment, and highly trained operators. As a result, it is considered analytically complex and costly, generating significant amounts of chemical waste. For these reasons, numerous efforts have been made to simplify the procedure and reduce solvent consumption. Several studies have proposed bypassing the TLC separation step by directly derivatizing the unsaponifiable fraction prior to GC analysis; however, such approaches generally focus on major sterols only.

Advanced gas chromatographic techniques coupled with mass spectrometric detection, including GC–MS and comprehensive two-dimensional gas

chromatography (GC×GC–MS), have also been developed for the simultaneous determination of sterols, triterpene diols, and other unsaponifiable compounds [30].

Liquid chromatography (LC) has been employed for the separation of olive oil unsaponifiable compounds for more than two decades. In the most recent revision of the IOC method, preparative LC using silica columns with ultraviolet or refractive index detection has been introduced as an alternative to TLC. Several authors have applied high-performance liquid chromatography coupled with mass spectrometry (HPLC–MS or UPLC–MS) for sterol analysis, typically following the same saponification and extraction steps as the official method but omitting derivatization. While these methods enable selective and sensitive detection, they generally allow the determination of a smaller number of sterols and may suffer from incomplete chromatographic resolution [31-33].

More recently, metabolomics-based approaches employing high-resolution mass spectrometry have enabled the identification and relative quantification of a substantially larger number of sterol-related compounds in olive oil. Using simplified extraction protocols and ultra-high-performance liquid chromatography coupled with electrospray quadrupole time-of-flight mass spectrometry (UHPLC–ESI/QTOF–MS), several hundred sterols have been tentatively annotated, representing a promising direction for future research in this field [34,35].

Hybrid LC–GC methodologies have also been proposed, offering reduced analysis time, lower solvent consumption, and enhanced sensitivity, albeit requiring highly specialized instrumentation [36]. Fully automated versions of these methods, involving saponification and extraction followed by LC–GC analysis without derivatization, have demonstrated quantitative results comparable to those obtained using the ISO reference method. Additional analytical techniques, including nuclear magnetic resonance (NMR) spectroscopy and Fourier-transform near-infrared (FT-NIR) spectroscopy, have further proven effective for the determination of total sterol content or specific sterol groups.

Table 3 summarizes the principal techniques employed to isolate individual sterol classes.

Table 3. Common methods used for separating sterols in vegetables oils.

Method	Stationary phase	Solvent system	Reference
Total sterols			
TLC	Silica	Hexane: diethyl ether: acetic acid (85:15:1)	Morales & León-Camacho, 2000
HPLC	Silica	Hexane: diethyl ether	Amelio *et al.*, 1992
SPE	C_{18}	Methanol: chloroform	Toivo *et al.*, 1998
SPE	C_{18}	Acetonitrile: toluene	Ham *et al.*, 2000
Sterol classes			
TLC	Silica	Hexane: diethyl ether: acetic acid (70:30:1)	Kornfeldt & Croon., 1981
HPLC	Silica	Petroleum ether: ethyl acetate	Li *et al.*, 2001

The use of SPE enables the simultaneous determination of sterols and the triterpene diols erythrodiol and uvaol, in full compliance with current regulatory requirements, while ensuring adherence to the precision limits established by the COI/T.20/Doc. No. 30/Rev. 2 standard [27] for the most critical sterolic compounds, namely Δ7-stigmastenol, and Δ7-avenasterol. In contrast, liquid chromatography (LC)-based approaches typically require more complex instrumentation, and advanced operator expertise, which limits their suitability for routine quality control applications. Contrarily, SPE is a more affordable technique, which can be more promptly implemented in routine laboratories to allow a significant reduction in analytical time and operational complexity compared to the official method, thereby facilitating the adoption of an abbreviated methodology capable of providing results fully comparable to those obtained using the official reference method.

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Chapter 3

Microwave-assisted methods for fatty acids characterization

1. Introduction

On the basis of the theoretical background presented in the previous sections, a part of this doctoral thesis is dedicated to the evaluation of microwave-assisted strategies for the determination of fatty acid methyl esters (FAMEs) in food and food-related matrices. The experimental design is structured around a systematic comparison with established reference methodologies in order to assess the analytical reliability and applicability of the proposed approaches. In this context, the official methods issued by the American Oil Chemists' Society (AOCS) [1] were adopted as reference procedures for FAMEs analysis. The microwave-assisted extraction and derivatization (MAED) approach, originally proposed by Fina et al. [2], is investigated as an alternative analytical strategy, to determine its suitability as a replacement for conventional multi-step workflows. Particular attention is devoted to evaluating the ability of the MAED method to reproduce fatty acid profiles obtained with different references methods, while offering advantages in terms of reduced analysis time, solvent consumption, and operational complexity.

The experimental work is articulated in two main parts. In the first part, the MAED protocol is applied to a broad range of food and food-related matrices to assess its robustness, repeatability, and degree of matrix-independence applicability. In the second part, different microwave-assisted workflows, including one-step and two-step extraction and derivatization strategies, are comparatively evaluated to elucidate the impact of methodological choices on analytical performance and selectivity. FAMEs determination is carried out using gas chromatographic techniques, with GC×GC-FID used as an advanced analytical tool. In addition to analytical performance, the proposed methodologies are systematically evaluated in terms of environmental impact and overall sustainability. Greenness and whiteness assessments are therefore performed using established metrics, allowing the MAED approach to be critically compared with AOCS or other reference methods not only on analytical grounds, but also within the broader framework of Green and White Analytical Chemistry [3,4]. The chapter is structured to first present two case studies focused on different matrices, namely marine organisms and algae, followed by a section in which MW approaches are systematically compared with one-step and two-step methods.

2. Case Studies on the Application of MAED to Different Food Matrices

2.1 Composition and nutritional values of fatty acids in marine organisms by one-step microwave-assisted extraction/derivatization and comprehensive two-dimensional gas chromatography -flame ionization detector [5]

This section is adapted from: Ferrara D., Beccaria M., Cordero C., Purcaro G., Comprehensive comparison of fatty acid methyl ester profile in different food matrices using microwave-assisted extraction and derivatization methods and comprehensive two-dimensional gas chromatography coupled with flame ionization detection, Advances in Sample Preparation,Volume 11,2024,100124,ISSN 2772-5820.

2.1.1 Introduction

The most scientific evidence of the beneficial effects on human health of marine organism lipids in the diet was proved by studies on the low incidence of heart disease and the complete absence of diabetes mellitus in the Greenlandic Eskimos population [6]. The predominant FAs in fish and marine organisms are PUFA, followed by MUFAs and saturated (S) FAs. However, the most significant contribution to the beneficial health effect of marine organisms' intake is given by the presence of the long- chain semi-essential omega-3 FAs, i.e. eicosapentaenoic acid (EPA, C20:5n3) and docosahexaenoic acid (DHA, C22:6n3). EPA and DHA are indispensable for many metabolic processes, protecting the circulatory system, preventing the onset of many inflammatory and cardiovascular diseases, regulating the triacylglycerols level in the blood, and promoting the development of memory and cognitive skills [7]. However, literature data report a wide variability among the ratio of PUFA/ MUFA/SFA [8]. Moreover, this highly variable composition of the marine fat depends not only on the animal species, but also on the geographical site, season, and animal's organs [9].

This work reports the characterization of the lipidic fraction of seven species of marine organisms gathered along the shoreline of the Po Delta Park of Emilia-Romagna Region (Italy) and of the north Adriatic Sea. Two species of oysters (*Crassostrea gigas* and *Ostrea edulis*), two species of clams (*Chamelea gallina* and *Ruditapes philippinarum*), one species of mussel (*Mytilus galloprovincialis*), one species of macroalgae (*Ulva rigida*), and one species of spiny dogfish (*Squalus acanthias*) were analyzed to characterize their fatty acids profile and related nutritional value. Moreover, to determine the food nutritional value of the samples investigated, the most common nutritional indices (index of atherogenicity, index thrombogenicity, hypocholesterolemic/hypercholesterolemic ratio, health-promoting index, unsaturation index, and the fish lipid quality index) were calculated from FAME profiles. The goal of the proposed research is to verify the suitability of the MAED-GC×GC-FID methodology for the analysis of samples containing high percentages of omega-3 PUFA. In this context, the higher separation power obtained using GC × GC compared to monodimensional GC allowed us to report highly accurate nutritional indices based on their FA distribution in the investigated matrices.

2.1.2 Materials and methods

Samples and sampling area ◦ Pacific oysters (*Crassostrea gigas*) and European oysters (*Ostrea edulis*) were collected in a farming area located in Valli di Comacchio (44.55–44.65 N; 12.10–12.25 ◦ E) which are a wide inner complex of shallow water brackish lagoons. Clams (*Chamelea gallina* and *Ruditapes philippinarum*), mussels (*Mytilus galloprovincialis*), and macroalgae (*Ulva rigida*) were collected in the Sacca di Goro, which is the southernmost lagoon of the actual Po River Delta (44.78–44.83 ◦ N; 12.25–12.33 ◦ E). Both these lagoons belong entirely to the Po Delta Park of the Emilia- Romagna Region and are considered Sites of European Community Interest (S.I.C.) under the "Habitat" Directive n ◦ 43/92. Spiny dogfish (*Squalus acanthias*) was collected in the north Adriatic Sea (45.04 12.33 ◦ ◦ N; E) Geographical Sub-Area GSA 17, according to the FAO General Fisheries Commission for the Mediterranean (GFCM) [10]. All samples were collected in spring 2022, selected and taxonomically identified by experts in the field. Information relative to full sample names, size, number of individuals, and moisture are reported in Table 1.

Table 1. Information relative to full sample names, size, number of individuals, and moisture.

ID Samples	Binomial nomenclature	edible part (g)	n. of individuals	Moisture (%)
Pacific oyster	*Crassostrea gigas*	391.05	23	83.31 ± 0.51
European oyster	*Ostrea edulis*	156.48	49	82.37 ± 0.64
Small saltwater clam	*Chamelea gallina*	310.84	427	89.17 ± 1.21
Clam	*Ruditapes philippinarum*	480.28	52	84.59 ± 1.74
Mussel	*Mytilus galloprovincialis*	476.81	68	78.56 ± 1.23
Spiny dogfish	*Squalus acanthias*	560.22	10	72.82 ± 0.68
Macroalgae	*Ulva rigida*	1015.6	ND	85.14 ± 1.64

2.1.3 Sample treatment

The entire soft tissues of the studied bivalves (*C. gigas, O.edulis, C. gallina, R. philippinarum and M. galloprovincialis*) were removed from the shell (by carefully cutting the adductor muscles with a scalpel) and then washed with distilled water to remove any remains of the shell or sand. The tissue was dried with filter paper and

then weighed and homogenized with a blender, frozen in the fridge at 20 ◦ C and subsequently freeze-dried with a freeze dryer (M. Christ G. GmbH - Alpha 1–2 LDplus). From the spiny dogfish (*Squalus acanthias*), fillets were taken and lyophilized, samples were individually weighed. Finally, macroalgae (*Ulva rigida*) were washed twice with artificial salt water, made at the same concentration present at the lagoon site (25 PSU), to remove sediment residues; two further thorough washes with distillate water were applied to remove salt residues; then dried at room temperature for 30 min on laboratory filter paper, weighed in freeze-dryer flasks, and stored at 20 ◦ C until the freeze-drying step. Freeze-dried macroalgae were homogenized with a blender. All freeze-dried samples were individually weighed again to evaluate their moisture content and ground into a homogeneous powder with a ceramic mortar and pestle.

2.1.4 Chemicals and reagents

Solvents (hexane, cyclohexane, methanol, chloroform, and methyl acetate), standards (Supelco C37 FAME Mix, pure standards solution of n-alkanes in a range from n-C7 to n-C30), and reactives (sodium hy droxide, sodium sulfate, 14 % BF3/CH 3 OH solution, acidic-methanolic solution) were acquired from Merck KGaA (Darmstadt, Germany).

2.1.5 One-step microwave-assisted extraction and derivatization (MAED)

The MAED procedure was recently described [2]. An ETHOS X MW system with an SR-12 eT TFM rotor (Milestone Srl, Bergamo, Italy) was used. Briefly, 500 mg of each sample was weighed into the MW vessel. Ten mL of acidic-methanolic solution and 25 mL of cyclohexane were added, then, the vessel was sealed and placed inside the MW oven under continuous stirring [11]. The MW conditions were as follows: temperature ramp reaching 120 ◦ C in 2 min and hold for 15 min. After cooling, an aliquot of the supernatant was collected for the following GC × GC analysis. All samples were analyzed in triplicate.

2.1.6 Official method Ce 2b-11 by AOCS

The AOCS Official (Ce 2b-11 by AOCS) was applied for direct methylation of lipids in foods [11]. Briefly, samples were weighted according to the reference method [11], and 5 mL of NaOH/methanol (20 g/L) was added. The solution was heated under reflux for 15 min. Then, 5 mL of a solution of boron trifluoride-methanol was added, keeping it under reflux for 2 additional minutes before adding 5 mL of hexane and removing it from the heat source. All samples analyzed following this procedure were analyzed in triplicate.

2.1.7 Comprehensive two-dimensional gas chromatography (GC × GC) instrumentation

All the samples were analyzed in a GC × GC-FID system equipped with a medium-polar stationary phase as a first dimension (1 D) and a non-polar stationary phase as a

second dimension (through a flow modulator. Briefly, the 1 2 D) connected D column was a SepSolve FAMEs (stationary phase not disclosed) 20 m × 0.18 mm × 0.1 highly polar fused silica capillary column and the 2 1 D- µ m D column was a SepSolve 0.1 µ 2 D- FAMEs (stationary phase not disclosed) 5 m × 0.25 mm × m non-polar fused silica capillary column (SepSolve Analytical Ltd, UK). Details regarding parameters, columns, instrumentation, and software are reported in Table 2.

Table 2. GC×GC-FID parameters

GC×GC-FID parameters	
Carrier gas	helium
^{1}D flow-rate	0.5 mL/min
^{2}D flow-rate	20 mL/min
Modulation period	3s
Reinjection time	100 ms
Oven temperature program	3 min at 40°C; from 40 to 260 °C at 9 °C/min and hold it for 2 min
Injection mode	split mode (1:10)
Injection volume	0.5 µL
Injector temperature	250°C
FID temperature	270°C
FID data acquisition frequency	100 Hz
FID parameters	air flow: 350 mL/min, H2: 35 mL/min; make-up gas: 20 mL/min
Autosampler	AOC -30i Autoinjector
GC	Nexis GC-2030
Detector	FID
^{1}D column	^{1}D-FAMEs 20 m × 0.18 mm × 0.1 µm polar fused silica capillary column
^{2}D column	^{2}D-FAMEs 5 m × 0.25 mm × 0.1 µm non-polar fused silica capillary column
Bleedline	4.20 m × 0.1 mm uncoated capillary segment
Modulator	reversed fill/flush INSIGHT flow modulator
Data acquisition	LabSolution Version 5.111
Data processing	ChromSpace Version 1.5.1

FAMEs were tentatively identified based on the retention time of the standards (Supelco C37 FAMEs Mix), their elution order on a polar column [12], their position on the 2D plot [2,13,14], and relevant literature data [2,15,16]. Linear retention indices (LRIs) were only used for aligning FAMEs.

2.1.8 Statistical analysis

FAME significance between MAED and AOCS methods was tested using a t-test with Holm-Bonferroni correction [17]. A significance level of $p<0.05$ was selected.

2.1.9 Nutritional evaluation

The most common nutritional indices such as the index of atherogenicity (IA) and thrombogenicity (IT) [18], hypocholesterolemic/hypercholesterolemic (HH) ratio [19,20], health-promoting index (HPI) [21], unsaturation index (UI) [22], and the fish lipid quality (FLQ) index [27,28] were calculated from identified FAs according to the equations reported in the references and below (Equations (1) to (6)):

$$IA = [C12{:}0 + (4 \times C14{:}0) + C16]/\sum UFA \quad (1)$$

$$IT = (C14{:}0 + C16{:}0 + C18{:}0){:}\,[(0.5 \times \sum MUFA) + (0.5 \times \sum n6 - PUFA) + (3 \times \sum n3 - PUFA) + (n3/n6)] \quad (2)$$

$$HH = (cis18{:}1 + \sum PUFA)/(C12{:}0 + C14{:}0 + C16{:}0) \quad (3)$$

$$HPI = \sum UFA/[C12{:}0 + (4 \times C14{:}0) + C16{:}0] \quad (4)$$

$$UI = 1 \times (monoenoics) + 2 \times (dienoics) + 3(trienoics) + 4 \times (tetraenoics) + 5(pentanoics) + 6 \times (hexanoics) \quad (5)$$

$$FLQ = 100 \times (C22{:}6n3 + C20{:}5n3)/\sum FA \quad (6)$$

2.1.10 Results and discussion

2.1.11 Method evaluation

As a first step, the applicability of the MAED method on the different fish and marine organisms herein investigated was verified. The sample of *Mylitus galloprovincialis*, selected as the representative for the mollusks group was used for a detailed comparison with the reference method [1]. Given their different nature to mollusks, *Ulva rigida* and *Squalus acanthias* were also analysed by comparing MAED and AOCS methods. Figure 1 displays the GC ×GC-FID chromatogram of the total FA fraction of *Mylitus galloprovincialis*.

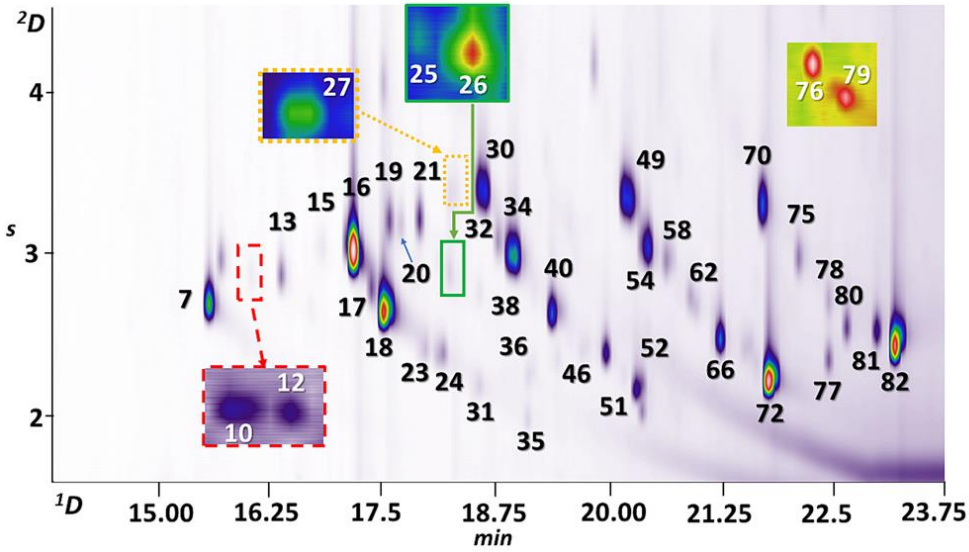


Figure 1. GC × GC-FID chromatogram of the total FA fraction of Mylitus galloprovincialis. Each number in Figure 1 corresponds to a different FAME, also listed in Table 4. Except for iso/anteiso and trans/cis isomers, FAMEs < 0.1% are not reported.

The GC×GC-FID chromatograms of the other marine organisms are reported in Figure 2.

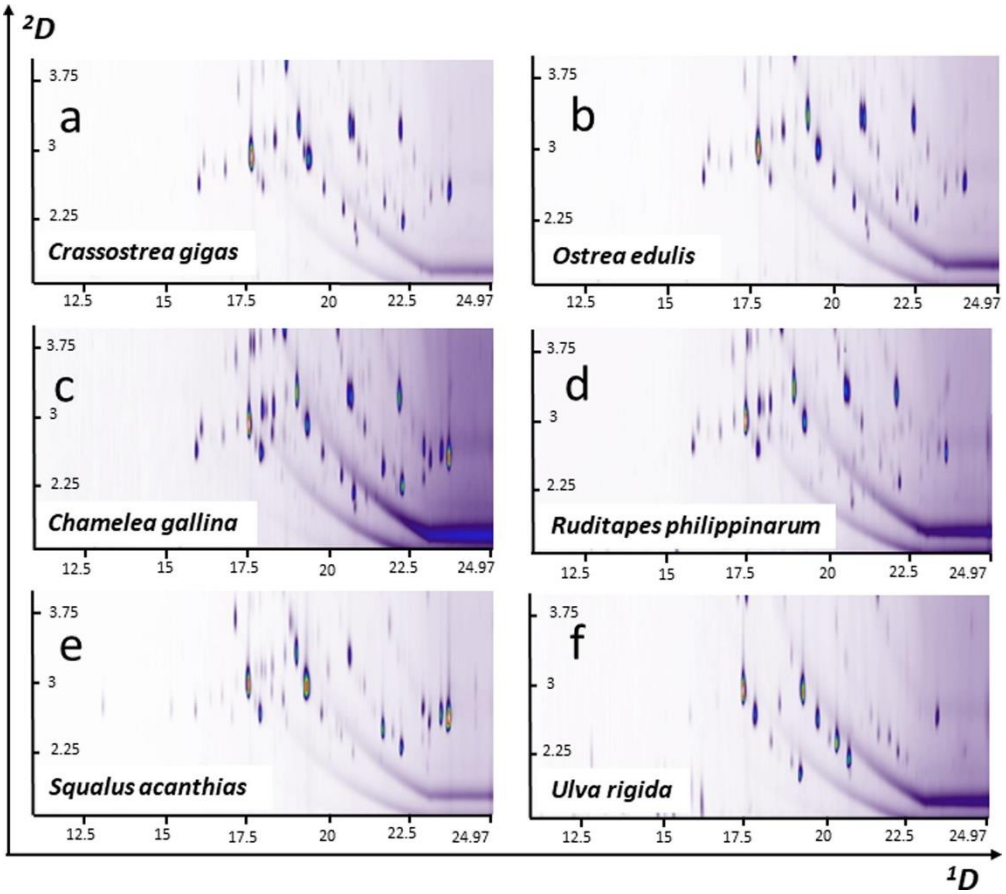


Figure 2. GC×GC-FID chromatograms of: *a) Crassostrea gigas; b) Ostrea edulis; c) Chamelea gallina; d) Ruditapes Philippinarum; e) Squalus acanthias; f) Ulva rigida.*

Except for iso/anteiso and trans/cis isomers, FAMEs <0.1 % are not reported in the figure for simplicity. Triplicate analyses of each sample were conducted using both methods. The comparison of the profiles of *Mylitus galloprovincialis* is reported in Figure 3, with error bars corresponding to the standard deviation (n =3).

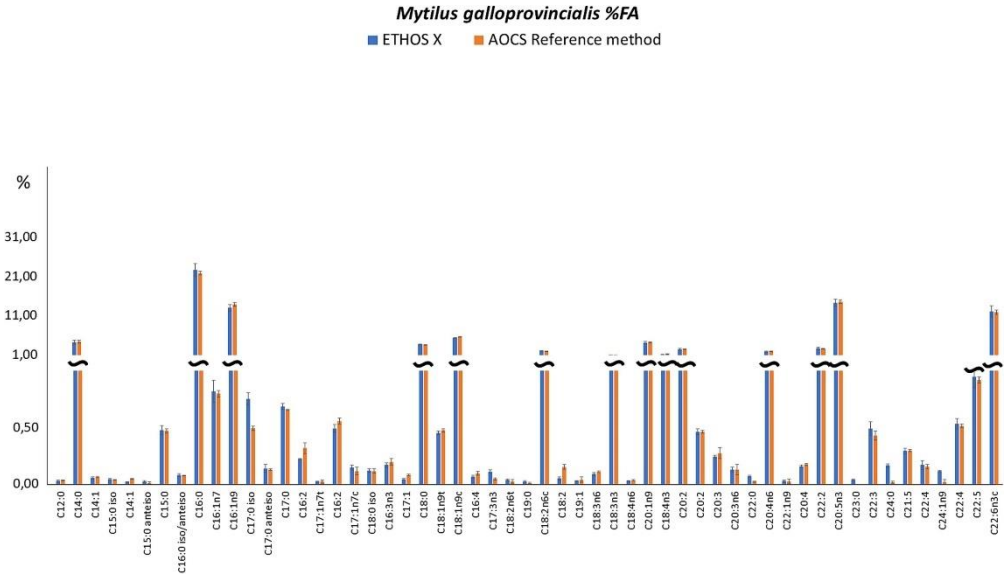


Figure 3. Comparison of the FAMEs percentage profile of *Mylitus galloprovinvialis* using the reference method (orange) [11] and the MAED method (blue). Error bars indicate the standard deviation of three replicates.

The comparison between the two methods for *Ulva rigida* and *Squalus acanthias* are reported in Figure 4 and 5, respectively.

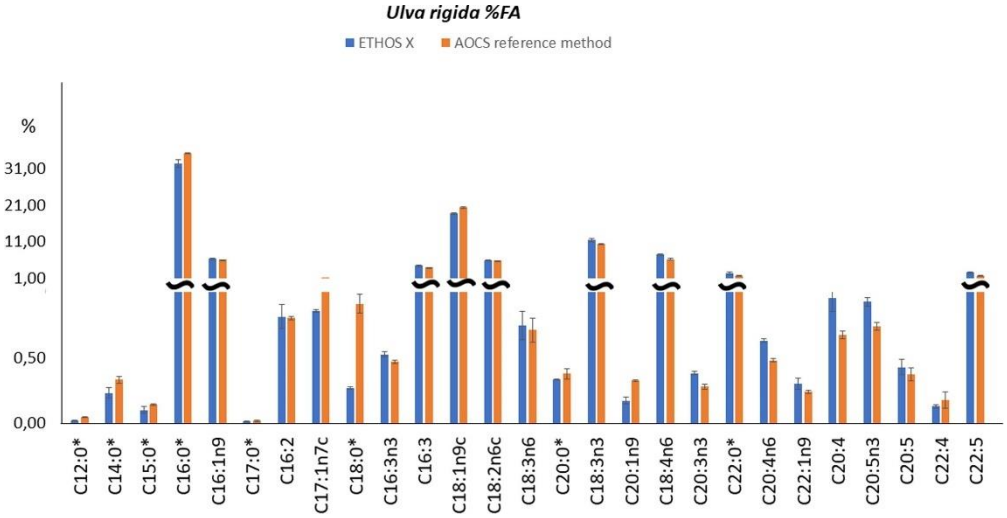


Figure 4. Comparison of the FAMEs percentage profile of *Ulva rigida* using the reference method (orange) [1] and the MAED method (blue). Error bars indicate the standard deviation of three replicates.

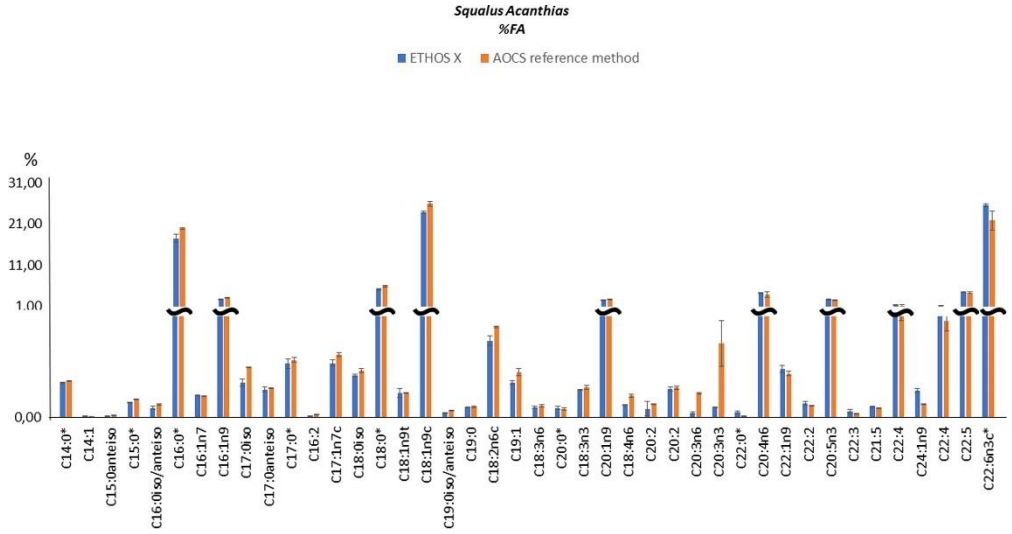


Figure 5. Comparison of the FAMEs percentage profile of *Squalus acanthias* using the reference method (orange) [1] and the MAED method (blue). Error bars indicate the standard deviation of three replicates.

Statistical analysis using a t-test with Holm-Bonferroni correction [17] revealed no significant differences (p-value >0.05) in most cases, except for a few components present in low quantities. The MAED method has shown to be suitable also for the analysis of marine organisms, significantly simplifying the overall sample preparation procedure compared to the official AOCS method, supporting the previous finding of Fina et al., pointing towards a broad application of the method [2]. Moreover, the use of GC ×GC-FID allowed for a detailed and sensitive characterization of the FAMEs profile without the need for long chromatographic runs and supporting the identification of the FAMEs thanks to the well-structured chromatographic separation.

2.1.12 Fatty acid compositions

The FA profile of the seven marine organisms investigated is pre sented in Table 3. SFA ranged from 25.05 % (*Squalus acanthias*) to 59.78 % (*Ruditapes philippinarum*) of total FAMEs. Amongst SFAs, palmitic acid (C16:0) was found as the most abundant, ranging between 17.46 % (*Squalus acanthias*) and 39.45 % (*Crassostrea gigas*), followed by octa decanoic acid (C18:0) ranging between 3.70 % (*Ulva rigida*) and 14.87 % (*Ruditapes philippinarum*). Low percentages of tetradecanoic (C14:0), pentadecanoic (C15:0) and heptadecanoic (C17:0) acids were present, ranging from 0.23 % (*Ulva rigida*) to 4.23 % (*Mytilus galloprovincial*), 0.10 % (*Ulva rigida*) to 0.95 % (*Crassostrea gigas*), and 0.02 % (*Ulva rigida*) to 2.66 % (*Chamelea gallina*), respectively. *Crassostrea gigas* was the only sample presenting traces of short-chain SFAs in a range of C6- C13. With regards to MUFA, the highest concentration was found in *Squalus acanthias* (33.19 %), while the lowest was in *Chamelea gallina*

(19.87 %). Oleic (C18:1n9) and palmitoleic (C16:1n9) were the most representative MUFAs, ranging between 5.34 % (*Mytilus galloprovincialis*) and 23.86 % (*Squalus acanthias*), and 1.45 % (*Crassostrea gigas*) and 13.03 % (*Mytillus galloprovincialis*), respectively. Gadoleic acids (C20:1n9) and an isomeric eicosaenoic acid (C20:1) were found in a range of 0.18 % (*Ulva rigida*) and 6.71 % (*Squalus acanthias*), and 0.35 % (*Ulva rigida*) and 7.97 % (*Ostrea edulis*) but not detected in *Mytilus galloprovincialis*, respectively. Trace/low concentrations (<0.01–1.36 %) of the other MUFAs were found. Furthermore, trans-ω9-octadecanoid acid (C18:1n9t) was found in 5 out of 7 samples investigated, in a range from 0.24 % (*Squalus acanthias*) to 1.75 % (*Crassostrea gigas*). The content of PUFAs ranged between 15.23 % (*Ruditapes philippinarum*) and 42.38 % (*Mytilus galloprovincialis*). DHA (C22:6n3), present in 6 out of 7 samples (not detected in Ulva rigida), ranged from 3.29 % (*Ruditapes philippinarum*) to 25.56 % (*Squalus acanthias*). Other ω 3 PUFA such as ω α-linolenic acid (ALA, C18:3n3), EPA (C20:5n3), and docosapentaenoic acid (DPA, C22:5) were present in the sample inves tigated in different concentrations. ALA ranged between 0.09 % (*Ruditapes philippinarum*) and 11.47 % (*Ulva rigida*), EPA was between 0.93 % (*Ulva rigida*) and 14.28 % (*Mytilus galloprovincialis*), while DPA was between 0.32 % (*Crassostrea gigas*) and 4.45 % (*Squalus acanthias*). Traces of 3 eptatridecenoic (C17:3n3) and eicosatrienoic acids (C20:3n3) were present, respectively, in 5 out of 7 samples investigated. Stear idonic acid (SDA, C18:4n3) was only detected in *Mytilus galloprovicialis* (1.11 %). Among the ω 6 PUFA, the most abundant were linoleic acid (L, C18:2n6c), followed by arachidonic acid (AA, C20:4n6c), ranging between 0.08 (*Ruditapes philippinarum*) and 5.90 % (*Ulva rigida*), and 0.48 (*Ruditapes philippinarum*) and 4.27 % (*Squalus acanthias*), respectively. Traces of γ-linolenic acid (GLA, C18:3n6) and homogammalinolenic acid (C20:3n6) were also present. Furthermore, traces of trans ω 6- octadecenoic acid (C18:2n6t) were detected in *Crassostrea gigas* (0.02 %) and *Mytilus galloprovincialis* (0.04 %). Other PUFAs were present in low concentrations in the sample investigated, but unfortunately, it was not always possible to determine the double bond position in the backbone of the FAs. Among them, a not well-defined docosadienoic acid (C22:2) was present in 6 out of 7 samples, ranging between 0.14 (*Squalus acanthias*) and 6.79 % (*Ruditapes philippinarum*). The composition of fats in marine organisms varies significantly in literature due to factors like animal species, geographical location, season, and specific organs. Indeed, reported ranges for SFAs, MUFAs, and PUFAs show considerable disparities [8,15,25-27].

2.1.13 Nutritional aspects

Dietary fats are generally FAs that may play positive or negative roles in the prevention and treatment of diseases. According to different food- based dietary guidelines for preventing cardiovascular diseases, an intake of FAs with ω 6/ ω 3 ratio of at least 5:1 with the diet is suggested for health promotion [28]. In Western diets, the ratio has been estimated between 15:1–16.7:1. This diet-habitude can promote the pathogenesis of many diseases, such as cardiovascular inflammatory and autoimmune diseases, whereas increased levels of ω 3 PUFAs (it means a low ω 6/ ω 3 ratio) exert

suppressive effects [29]. In all the samples investigated, a greater or equal content of values of ω 6/ ω ω 3 with respect to ω 6 FAs was reported, with ratios ranging from ~ 1:7 (*Mytilus galloprovincialis*) to ~ 1:1 (*Ulva rigida*). Regarding the nutritional indices, IT and IA assess the potential of a diet to promote the development of atherosclerosis and blood clot for mation. Higher values indicate a greater risk of cardiovascular disease, while lower values suggest a healthier diet in terms of reducing these risks [20]. Among the samples investigated, Crassostrea gigas had the highest values of IA (1.09) and *Ruditapes philippinarum* of IT (1.77), while *Squalus acanthias* was the sample with the lowest values in both indices (0.25 and 0.22). HH reflects the impact of a diet on cholesterol levels. A hypo cholesterolemic diet lowers cholesterol, while a hypercholesterolemic diet increases cholesterol levels. Higher HH values are desirable for cardiovascular health [19,20]. *Squalus acanthias* had the best HH score (3.69) while *Ruditapes philippinarum* was the lowest (0.68). HPI measures the overall health benefits of a diet based on multiple factors, such as nutrient composition, antioxidant content, and potential disease prevention. Higher HPI values indicate a diet that promotes better health and reduces the risk of chronic diseases [21]. HPI ranged between 3.99 (*Squalus acanthias*) and 0.92 (*Crassostrea gigas*). UI evaluates the degree of unsaturation or presence of unsaturated fats in a diet. Higher values indicate a greater proportion of unsaturated fats, such as MU and PU fats, which are considered healthier than saturated ones. A higher UI suggests a more heart-healthy diet [22]. The samples with the higher UI were *Squalus acanthias* (255.25) followed by *Mytilus galloprovincialis* (214.3), while the UI of *Ruditapes philippinarum* was the lowest (77.11). FLQ assesses the quality of lipids or fats derived from fish in the diet. It considers the balance of ω 3 FAs, particularly EPA and DHA (such as in the case of the samples investigated), which have numerous health benefits. A higher FLQ indicates a diet rich in beneficial ω 3 FAs, which contributes to lower cardiovascular risks and improved brain health [23,24]. FLQ ranged between 28.17 (*Squalus acanthias*) and 0.93 (*Ulva rigida*). The low value of *Ulva rigida* is due to the absence of DHA. These nutritional indices, reported in Table 4, provide valuable information about the potential health effects of different diets, guiding individuals toward making informed choices for their overall well- being. Indeed, FAs present in marine organisms play a crucial role in the human diet by providing essential/semi-essential ω 4 3-FA, which contributes to cardiovascular health and brain function [30].

Table 3. FAME profile (% ±standard deviation of three replicates) of the marine organisms investigated by MAED-GC ×GC-FID

N.	FAME	^{1}D-RT	^{2}D-RT	*Crassostrea gigas*	*Ostrea edulis*	*Chamelea gallina*	*Ruditapes philippinarum*	*Squalus acanthias*	*Ulva rigida*	*Mytilus galloprovincialis*
1	**C6:0***	7.60	1.80	0.04 ± 0.006						
2	**C8:0***	10.07	1.95	0.02 ± 0.005						
3	**C10:0***	12.24	2.15	tr						
4	**C11:0***	13.24	2.27	0.01 ± 0.001						
5	**C12:0***	14.16	2.38	0.02 ± 0.004	0.03 ± 0.003		0.03 ± 0.001		0.02 ± 0.001	0.03 ± 0.008
6	**C13:0***	15.06	2.52	0.02 ± 0.004	0.01 ± 0.00 3	tr	0.03 ± 0.003			tr
7	**C14:0***	15.89	2.63	1.98 ± 0.242	1.99 ± 0.111	1.98 ± 0.205	1.76 ± 0.011	0.34 ± 0.006	0.23 ± 0.038	4.23 ± 0.499
8	**C14:1**	16.14	2.46	0.02 ± 0.001	tr		tr	tr	tr	0.06 ± 0.011
9	**C14:1**	16.30	2.41	tr	tr	0.01 ± 0.002	0.03 ± 0.002	tr	0.02 ± 0.002	
10	**C15:0iso**	16.38	2.72	0.16 ± 0.023	0.18 ± 0.007	0.05 ± 0.007	0.17 ± 0.003		tr	0.04 ± 0.004
11	**C14:1**	16.41	2.37	0.01 ± 0.001	tr	0.01 ± 0.001	tr	0.01 ± 0.001		0.02 ± 0.003
12	**C15:0 anteiso**	16.49	2.78	0.01 ± 0.013	0.02 ± 0.000	0.02 ± 0.002	0.03 ± 0.001	0.01 ± 0.004		0.02 ± 0.006
13	**C15:0***	16.75	2.78	0.95 ± 0.107	0.67 ± 0.033	0.55 ± 0.050	0.82 ± 0.010	0.15 ± 0.004	0.10 ± 0.026	0.48 ± 0.041
14	**C15:1**	17.00	2.61					0.13 ± 0.017		
15	**C16:0 iso/anteiso**	17.16	2.92	0.12 ± 0.015	0.15 ± 0.025	0.52 ± 0.042	0.76 ± 0.010	0.09 ± 0.017	tr	0.08 ± 0.016
16	**C16:0***	17.47	2.96	39.45 ± 3.606	35.37 ± 1.417	23.94 ± 2.826	32.97 ± 0.489	17.46 ± 0.935	32.46 ± 1.050	22.68 ± 1.546

N.	FAME	^{1}D-RT	^{2}D-RT	*Crassostrea gigas*	*Ostrea edulis*	*Chamelea gallina*	*Ruditapes philippinarum*	*Squalus acanthias*	*Ulva rigida*	*Mytilus galloprovincialis*
17	**C16:1n7**	17.71	2.71	0.57 ± 0.069	0.56 ± 0.046	0.54 ± 0.092	0.45 ± 0.013	0.22 ± 0.001		0.82 ± 0.097
18	**C16:1n9**	17.84	2.62	1.45 ± 0.161	1.70 ± 0.246	3.90 ± 0.681	3.89 ± 0.223	2.70 ± 0.133	6.34 ± 0.254	13.03 ± 0.828
19	**C17:0iso**	17.90	3.09	0.59 ± 0.080	0.60 ± 0.010	2.91 ± 0.169	3.39 ± 0.404	0.34 ± 0.037		0.76 ± 0.055
20	**C17:0anteiso**	18.05	3.12	0.03 ± 0.043	0.14 ± 0.012	0.92 ± 0.047	0.62 ± 0.057	0.28 ± 0.022		0.14 ± 0.033
21	**C17:0***	18.23	3.12	2.37 ± 0.212	2.20 ± 0.210	2.66 ± 0.118	2.49 ± 0.095	0.53 ± 0.050	0.02 ± 0.005	0.69 ± 0.028
22	**C17:1**	18.32	2.83		0.01 ± 0.001	0.73 ± 0.031	0.62 ± 0.074	0.29 ± 0.009		0.04 ± 0.009
23	**C16:2**	18.32	2.42	tr		0.05 ± 0.004	tr	0.02 ± 0.002	0.82 ± 0.090	0.22 ± 0.005
24	**C16:2**	18.48	2.39	0.01 ± 0.001		0.04 ± 0.006	0.02 ± 0.004	0.02 ± 0.003		0.49 ± 0.032
25	**C17:1n7t**	18.50	2.99							0.02 ± 0.006
26	**C17:1n7c**	18.56	2.81	0.46 ± 0.049	0.14 ± 0.017	0.32 ± 0.059	0.23 ± 0.005	0.54 ± 0.032	0.86 ± 0.012	0.15 ± 0.021
27	**C18:0iso**	18.58	3.33	0.19 ± 0.025	0.17 ± 0.024	0.45 ± 0.022	0.03 ± 0.010	0.42 ± 0.012		0.12 ± 0.012
28	**C18:0anteiso**	18.74	3.23			0.03 ± 0.004	0.01 ± 0.000	0.01 ± 0.003		tr
29	**C17:2**	18.91	2.69	0.07 ± 0.017	0.11 ± 0.001	0.08 ± 0.002	0.12 ± 0.007			
30	**C18:0***	18.93	3.29	8.07 ± 0.597	12.06 ± 0.767	10.37 ± 0.141	14.87 ± 0.990	5.11 ± 0.217	0.27 ± 0.010	3.75 ± 0.042
31	**C16:3n3**	18.94	2.19	0.01 ± 0.001	0.01 ± 0.001	0.02 ± 0.003		0.01 ± 0.001	0.53 ± 0.020	0.17 ± 0.019
32	**C18:1n9t**	19.12	2.99	1.75 ± 0.175	0.27 ± 0.001	0.34 ± 0.046		0.24 ± 0.046		0.45 ± 0.016
33	**C16:3**	19.20	2.04	tr	0.01 ± 0.001	0.01 ± 0.004			4.40 ± 0.151	tr

N.	FAME	^{1}D-RT	^{2}D-RT	*Crassostrea gigas*	*Ostrea edulis*	*Chamelea gallina*	*Ruditapes philippinarum*	*Squalus acanthias*	*Ulva rigida*	*Mytilus galloprovincialis*
34	**C18:1n9c**	19.27	2.91	10.81 ± 0.911	9.27 ± 0.352	5.82 ± 0.908	8.36 ± 0.504	23.86 ± 0.310	18.73 ± 0.215	5.34 ± 0.044
35	**C16:4**	19.44	2.01		0.01 ± 0.001	0.01 ± 0.001				0.07 ± 0.012
36	**C17:3n3**	19.45	2.24	0.15 ± 0.085	0.06 ± 0.009	0.09 ± 0.011	0.30 ± 0.041			0.11 ± 0.013
37	**C19:0iso/anteiso**	19.44	3.42	0.04 ± 0.006	0.03 ± 0.002	0.05 ± 0.009	0.01 ± 0.001	0.05 ± 0.003		
38	**C18:2n6t**	19.49	2.69	0.02 ± 0.010		tr		0.05 ± 0.004		0.04 ± 0.006
39	**C19:0**	19.62	3.47	0.06 ± 0.006	0.12 ± 0.029	0.13 ± 0.024	0.14 ± 0.010	0.10 ± 0.003		0.02 ± 0.007
40	**C18:2n6c**	19.71	2.61	1.29 ± 0.077	1.61 ± 0.053	1.24 ± 0.009	0.08 ± 0.015	0.76 ± 0.045	5.90 ± 0.160	2.06 ± 0.016
41	**C20:0iso**	19.87	3.67	0.09 ± 0.019	0.04 ± 0.002					
42	**C19:1**	19.92	3.03	0.17 ± 0.046	0.21 ± 0.031			0.34 ± 0.020		0.03 ± 0.003
43	**C18:2**	19.49	2.76							0.05 ± 0.014
44	**C18:3n6**	20.05	2.40	0.02 ± 0.006		0.89 ± 0.077		0.10 ± 0.012	0.75 ± 0.1058	0.09 ± 0.015
45	**C20:0anteiso**	20.05	3.56	0.03 ± 0.004	0.07 ± 0.010					
46	**C20:0***	20.29	3.66	0.08 ± 0.002	0.15 ± 0.018	0.49 ± 0.071	0.75 ± 0.045	0.09 ± 0.015	0.34 ± 0.005	tr
47	**C18:3n3**	20.31	2.37	1.45 ± 0.068	1.94 ± 0.287	1.41 ± 0.264	0.09 ± 0.007	0.28 ± 0.001	11.47 ± 0.457	1.05 ± 0.021
48	**C18:4n3**	20.47	2.12							1.11 ± 0.073
49	**C20:1n9**	20.48	3.29	5.79 ± 0.364	4.22 ± 0.014	2.40 ± 0.047	6.71 ± 0.505	2.61 ± 0.006	0.18 ± 0.025	4.18 ± 0.243

N.	FAME	^{1}D-RT	^{2}D-RT	*Crassostrea gigas*	*Ostrea edulis*	*Chamelea gallina*	*Ruditapes philippinarum*	*Squalus acanthias*	*Ulva rigida*	*Mytilus galloprovincialis*
50	**C21:0iso/anteiso**	20.51	3.69				0.27 ± 0.036			
51	**C20:1**	20.59	3.22	2.76 ± 0.158	7.97 ± 1.118	5.30 ± 0.111	3.12 ± 0.180	1.50 ± 0.117	0.35 ± 0.005	
52	**C18:4n6**	20.66	2.19	0.75 ± 0.133	0.57 ± 0.053	2.37 ± 0.204	0.15 ± 0.012	0.13 ± 0.001	7.46 ± 0.314	0.03 ± 0.002
53	**C19:3**	20.76	2.50	0.25 ± 0.010	0.26 ± 0.034					
54	**C20:2**	20.78	2.98	0.31 ± 0.053	0.46 ± 0.070	0.61 ± 0.054	0.38 ± 0.043	0.09 ± 0.071		2.48 ± 0.153
55	**C21:1**	20.84	3.43			0.09 ± 0.017	0.05 ± 0.006			
56	**C21:0***	20.91	3.84	0.06 ± 0.008	0.06 ± 0.003	0.03 ± 0.001	0.04 ± 0.008			
57	**C21:1**	20.94	3.42			0.17 ± 0.001	0.03 ± 0.006			
58	**C20:2**	21.01	2.90	0.19 ± 0.153	0.53 ± 0.061	0.51 ± 0.054	0.48 ± 0.076	0.28 ± 0.020		0.46 ± 0.025
59	**C20:3**	21.09	2.80							0.25 ± 0.012
60	**C19:4**	21.11	2.33	0.27 ± 0.061	0.30 ± 0.043	0.33 ± 0.046				
61	**C21:1**	21.12	3.44		0.13 ± 0.011	0.15 ± 0.023	0.03 ± 0.006			tr
62	**C20:3n6**	21.25	2.68	0.09 ± 0.023	0.07 ± 0.013			0.05 ± 0.015		0.13 ± 0.021
63	**C20:3n3**	21.39	2.62	0.07 ± 0.008	0.10 ± 0.013	0.08 ± 0.012		0.10 ± 0.002	0.38 ± 0.013	
64	**C21:2**	21.48	3.12	0.03 ± 0.003	0.03 ± 0.004		0.03 ± 0.005			
65	**C22:0***	21.50	4.04	0.05 ± 0.013	0.07 ± 0.007	0.26 ± 0.032	0.26 ± 0.020	0.05 ± 0.010	2.42 ± 0.356	0.01 ± 0.001
66	**C20:4n6**	21.55	2.45	0.80 ± 0.032	1.09 ± 0.165	2.45 ± 0.243	0.48 ± 0.049	4.27 ± 0.094	0.63 ± 0.014	1.86 ± 0.115

N.	FAME	^{1}D-RT	^{2}D-RT	*Crassostrea gigas*	*Ostrea edulis*	*Chamelea gallina*	*Ruditapes philippinarum*	*Squalus acanthias*	*Ulva rigida*	*Mytilus galloprovincialis*
67	**C22:1n9**	21.73	3.62	0.19 ± 0.021	0.18 ± 0.030	0.08 ± 0.010	1.36 ± 0.032	0.48 ± 0.033	0.31 ± 0.042	0.01 ± 0.001
68	**C20:4**	21.87	2.42	0.15 ± 0.007	0.24 ± 0.017	0.27 ± 0.032	0.19 ± 0.033	0.30 ± 0.011	0.96 ± 0.097	0.16 ± 0.010
69	**C20:5**	21.96	2.24	0.17 ± 0.027	0.19 ± 0.030	0.20 ± 0.028	0.24 ± 0.005	tr		
70	**C22:2**	22.04	3.21	4.06 ± 0.276	5.25 ± 0.620	0.78 ± 0.064	6.79 ± 0.110	0.14 ± 0.020		2.69 ± 0.262
71	**C23:0***	22.09	4.21	4.31 ± 0.290	0.28 ± 0.072	0.14 ± 0.016	0.02 ± 0.002	tr		0.04 ± 0.005
72	**C20:5n3**	22.11	2.25	1.96 ± 0.141	2.42 ± 0.446	2.64 ± 0.330	1.33 ± 0.048	2.61 ± 0.078	0.93 ± 0.033	14.28 ± 0.849
73	**C21:3**	22.17	2.85	0.14 ± 0.014	0.12 ± 0.004					
74	**C20:5**	22.41	2.19	0.08 ± 0.005	0.17 ± 0.013				0.43 ± 0.059	
75	**C22:3**	22.47	2.92	0.29 ± 0.002	0.54 ± 0.073	0.29 ± 0.024	0.20 ± 0.041	0.06 ± 0.017	tr	0.49 ± 0.060
76	**C24:0***	22.69	4.34	0.17 ± 0.040	0.16 ± 0.029	0.23 ± 0.033	0.14 ± 0.002	tr	0.01 ± 0.001	0.17 ± 0.015
77	**C21:5**	22.77	2.34	0.08 ± 0.006	0.07 ± 0.013	0.50 ± 0.006	0.13 ± 0.018	0.10 ± 0.007		0.29 ± 0.024
78	**C22:4**	22.79	2.69	0.08 ± 0.013	0.16 ± 0.039	2.05 ± 0.133	0.16 ± 0.020	1.30 ± 0.008		0.17 ± 0.038
79	**C24:1n9**	22.94	3.87	tr	0.01 ± 0.001		0.02 ± 0.005	0.26 ± 0.022	tr	0.12 ± 0.003
80	**C22:4**	22.98	2.55	0.42 ± 0.011	0.69 ± 0.005	1.61 ± 0.121	0.19 ± 0.011	1.12 ± 0.022	0.13 ± 0.014	0.53 ± 0.044
81	**C22:5**	23.36	2.63	0.32 ± 0.004	0.40 ± 0.017	2.33 ± 0.243	0.58 ± 0.051	4.45 ± 0.019	2.53 ± 0.167	0.95 ± 0.093
82	**C22:6n3c***	23.54	2.58	3.47 ± 0.183	3.33 ± 0.325	14.05 ± 0.343	3.29 ± 0.030	25.56 ± 0.384		12.11 ± 1.413

Table 4. Nutritional indices of FAs present in marine organisms (IA: index of atherogenicity; IT: index of thrombogenicity; HH: hypocholesterolemic/hypercholesterolemic ratio; HPI: health-promoting index; FLQ: fish lipid quality index; UI: unsaturation index). SFA: saturated fatty acid; MUFA: monounsaturated fatty acid; PUFA: polyunsaturated fatty acid; UFA: unsaturated fatty acid. The values reported are the average of 3 replicates ±standard deviation.

Indices	*Crassostrea gigas*	*Ostrea edulis*	*Chamelea gallina*	*Ruditapes philippinarum*	*Squalus acanthias*	*Ulva rigida*	*Mytilus galloprovincialis*
Total SFA	52.39	53.07	43.29	54.08	24.71	36.83	33.25
Total MUFA	24.34	22.59	21.66	24.80	32.31	26.62	24.26
Total PUFA	22.05	22.67	30.36	15.36	41.71	35.99	42.38
TOTAL UFA	46.39	45.26	52.02	40.16	74.02	62.60	66.63
PUFA ω3	9.75	9.02	15.83	5.12	28.64	14.09	28.84
PUFA ω6	3.76	3.73	6.18	0.71	5.43	14.31	4.21
ω6/ω3	0.39	0.41	0.39	0.14	0.19	1.02	0.15
PUFA/SFA	0.42	0.43	0.70	0.28	1.69	0.98	1.27
AI	1.00	0.93	0.68	0.99	0.26	0.55	0.59
TI	1.10	1.20	0.63	1.75	0.23	0.52	0.14
HH	0.81	0.87	1.30	0.68	3.56	1.64	1.77
HPI	1.00	1.08	1.46	1.01	3.79	1.83	1.68
FLQ	7.85	6.40	14.48	4.88	28.31	0.91	26.42
UI	108.90	104.19	164.99	78.98	253.64	146.93	214.24

2.1.14 Conclusions

The lipid extraction/derivatization step by MAED occurred in ~ 15 min (up to 12 samples simultaneously), while the GC ×GC-FID time analysis was ~ 30 min for a single run. MAED-GC ×GC-FID methodology allowed a complete characterization of the FAs fraction present in marine organisms without the need for more powerful detectors such as mass spectrometry (MS) [31]. Indeed, the high separation capability of GC × GC alone, due to the formation of group-type patterning in the 2D space, was adequate for the tentative identification of FAs, eliminating the necessity for MS coupling, and further streamlining the method, thereby promoting a more widespread adoption of this methodology. Moreover, the detailed characterization of the FA composition in marine organisms can be crucial to i) provide valuable insights into the nutritional value of marine organisms as a food source, ii) help assess the potential health benefits associated with consuming these organisms, particularly due to the presence of essential ω 3 FAs; iii) aid in evaluating the ecological roles of marine organisms and their impact on marine ecosystems, possibly supporting sustainable fisheries management and conservation efforts by providing information on the lipid profiles of marine species. Among the samples investigated, *Squalus acanthias* always presented the best nutritional score, while *Ruditapes philippinarum* had the worst scores in 3 out of 6 indices. However, even if no experiments were done regarding geographical, seasonal, and environmental factors, it is worth reporting that the specific composition of the surrounding water, which varies by location, can affect the availability of nutrients for marine organisms. Seasonal changes can impact factors like water temperature and food availability, influencing the growth rates and nutritional content of marine organisms. Environmental factors, including pollution and changes in habitat, can introduce contaminants and/or alter nutrient sources, further influencing the overall nutritional profile of marine organisms. Considering all these reasons, the nutritional index cannot be the only parameter to take into account to assess food quality. Therefore, further dedicated studies are needed to increase knowledge about the nutritional quality of a much larger set of marine organisms collected in different geographical areas, seasons, and environmental conditions.

2.2 Advancing fatty acid profiling in microalgae: a comparison of greener approaches and enhanced chromatographic separation vs conventional methods [accepted in European Journal of Lipid Science and Technology]

2.2.1 Introduction

Microalgae are photosynthetic eukaryotic microorganisms highly efficient in the fixation of CO_2 and using solar energy to produce biomass. They provide a wide range of high-value compounds that are commercially exploited, such as lipids, carotenoids, or proteins [32]. Among these compounds, omega-3 long-chain polyunsaturated fatty acids (n-3 LC-PUFA) are the most promising, as microalgae are the primary producers of these essential fatty acids in aquatic systems. For decades, fish, particularly oily fish, like salmon and sardines, have been the most common source of n-3 LC-PUFA for human consumption [33]. However, global fisheries are facing a significant overfishing risk, which has prompted substantial efforts to explore new alternatives. Given their immense potential as lipid biofactories, microalgae could hold the key to a viable and sustainable source of n-3 LC-PUFA.

The effective utilization of microalgal lipids and fatty acids in various applications, including food, nutraceuticals and feed, necessitates rapid, reliable, and accurate methodologies for quality assessment [34]. Depending on the specific application, these methodologies may involve either lipid extraction or direct analysis from the raw biomass. Extracting lipids from microalgal biomass is often necessary, especially in cases where the objective is to recover high-value lipids for dietary supplements or functional foods. However, conventional extraction techniques, such as the widely used Bligh and Dyer or Folch methods [35,36], use high volumes of organic solvents and energy requirements, leading to environmental concerns. Additionally, the presence of a dense and firm microalgae cell wall, particularly in microalgae species like *Microchloropsis gaditana* (formerly *Nannochloropsis gaditana*) where the cell wall has a bilayer structure protected by an external hydrophobic algaenan layer [37], makes lipid extraction from microalgae a challenging task. In a previous study, we explored alternative approaches, such as ultrasound and enzymatic strategies, which showed promising results in optimizing lipid recovery conditions from the lyophilized biomass of *M. gaditana*, with the enzymatic solution Saczyme® Yield demonstrating superior yields [38]. Building on this, in the current study, we pursued to assess the applicability of the previously developed method to other microalgae species, this time utilizing spray-dried biomass. It is worth noting that drying technologies play a crucial role in influencing the quality and quantity of high-value compounds, including lipids, in microalgal cultures [39]. On a commercial scale, spray drying is widely favored for its effectiveness in producing high-value-added products. Furthermore, spray-dried microalgae biomass holds versatility, finding use in pharmaceuticals and human food supplements [40,41].

On the other hand, for quality control or monitoring applications where the focus is on understanding the fatty acid profile, it may be sufficient to analyze lipids directly without an extraction step. In this case MAED method already riscussed in the

previous section could be very useful for the objective of routine analysis. While this method has been successfully applied to a wide variety of food matrices [42] and marine organisms [5], it has not yet been tested on microalgal biomass.

Therefore, in this study, we compare a pool of advanced and environmentally friendly approaches for microalgal lipid extraction and fatty acids characterization as alternatives to conventional methods. Initially, we evaluated ultrasound-assisted enzymatic strategies for applications requiring lipid extraction. Subsequently, we explored the potential of a one-step MAED procedure, followed by GC×GC analysis, for cases where lipid extraction is unnecessary, offering a faster alternative to traditional analysis and very detailed characterization. For comparison, the results were also evaluated in relation to those obtained using the commonly employed gas chromatography-mass spectrometry (GC-MS) technique. Additionally, we examined various photoautotrophic eukaryotic microalgae species of interest classified as *Eustigmatophyta (Microchloropsis gaditana)*, *Haptophyta (Tisochrysis lutea)*, *Rhodophyta (Porphyridium cruentum)* or *diatom (Phaeodactylum tricornutum)* with different compositions and fatty acid profiles to assess the adaptability of these alternative methods. The findings of this study have the potential to establish greener and simpler strategies for fatty acid profiling in microalgae, providing a broad range of applications.

2.2.2 Materials and methods

2.2.3 Materials

The spray-dried microalgal biomass of *Microchloropsis gaditana* (batch 02092021Ng), *Tisochrysis lutea* (batch 02092021TL), *Phaeodactylum tricornutum* (batch 02092021Pt) and *Porphyridium cruentum* (batch 02092021Pc) was purchased from Cianoalgae SI (Gipuzkoa, Spain).

The enzymatic solution Saczyme® Yield used for the extraction of lipids was kindly donated by Novozymes A/S (Bagsvaerd, Denmark). Chloroform, methanol, and ethanol were purchased from Fisher Scientific GmbH (Wien, Austria). Sodium hydrogen carbonate and potassium hydroxide were purchased from Carl Roth (Karlsruhe, Germany). Cyclohexane and acidic-methanolic solution were acquired from Merck KGaA (Darmstadt, Germany). Fatty acid methyl esters standard (Supelco 37 FAME Mix) was from Supelco (Bellefonte, PA, USA).

2.2.4 Extraction of microalgal lipids using the conventional Folch method

The Folch extraction method was done following the original procedure [36]. Briefly, 1 g of microalgal biomass was extracted with 20 mL of chloroform:methanol (2:1) and vortexing for 2 min. The mixture was centrifuged at 3000 rpm for 10 min, and the organic layer was collected. The extraction process was carried out 3 times on the same biomass. The collected organic layers were purified by washing with water, centrifuged at 4000 rpm for 10 min and the chloroform layer containing the extracted lipids was recovered. Samples were evaporated in a rotary evaporator (Heidolph Hei-Vap Value HB/G3, Schwabach, Germany) under reduced pressure. The lipid content

was determined gravimetrically and was calculated as a weight percentage of dry biomass. Lipid extracts obtained were stored in dark vessels with an argon atmosphere at 4 °C until their analysis.

2.2.5 Extraction of microalgal lipids using ultrasounds and enzymatic strategies

2.2.6 Ultrasound-assisted extraction

Ultrasound-assisted extraction (UAE) was carried out with an ultrasound bath (Elmasonic P 30H, Elma Schmidbauer GmbH, Singen, Germany) using an ultrasound frequency of 37 kHz and ultrasonic power of 100 W. Extractions were done at 50 °C for 30 min with ethanol as solvent (biomass-solvent ratio of 1:10 (w/v)). After the treatment, samples were filtrated, evaporated, and treated as previously described.

2.2.7 Enzymatic extraction

The enzyme-based extraction was carried out following the optimized conditions previously described [38]. Briefly, 1 g of microalgae biomass was dispersed in 10 mL of acetate buffer (pH 4.5) and the enzymatic solution Saczyme® Yield (15 mg of protein per gram of biomass). The mixture was incubated at 50 °C with constant shaking (500 rpm) for 1 h by using an Eppendorf Thermomixer C (Hamburg, Germany). At the end of the treatment, the enzymatic solution was removed, and the pretreated biomass was used for lipid extraction using UAE (see section 2.2.6). A control experiment without an enzymatic solution was also done. After the treatment, samples were filtrated, evaporated, and treated as previously described.

2.2.7 One-step microwave-assisted extraction and derivatization

The one-step MAED procedure was performed on an ETHOS X MW system with an SR-12 eT TFM rotor (Milestone Srl, Bergamo, Italy). The procedure was carried out as previously described [2]. Briefly, 500 mg of sample was added with 10 mL of acidic-methanolic solution and 25 mL of cyclohexane directly in the microwave vessel. A stir bar was added before sealing the vessel. The microwave oven was set to reach 120 °C in 2 min and held for 15 min. After cooling, the supernatant was directly collected and injected into the GC×GC system. All samples were analyzed in triplicate.

2.2.8 Fatty acid composition characterization

Two analytical approaches were employed: (i) the commonly used GC-MS (Sections 2.2.13, 2.2.16, and 2.2.17), and (ii) the more comprehensive GC×GC-FID technique (Sections 2.2.16 and 2.2.17). The complementary use of these methods allowed for a thorough analysis by enabling both the identification of major compounds and a detailed characterization of sample complexity, which would be difficult to achieve using only one technique.

2.2.9 GC-MS

Fatty acid composition of the extracted microalgal lipids was analyzed by GC–MS by using an Agilent 7890A connected to an Agilent 5975C Inert XL EI/CI MSD (Palo Alto, CA, USA). Before analysis, FAME were prepared by base-catalyzed methanolysis of the glycerides (KOH in methanol). FAME were separated by using an HP-5ms ultra-inert column (30 m × 250 µm × 0.25 µm) (Palo Alto, CA, USA). A total of 1 µL of the sample was injected in spitless mode and an injector temperature of 280 °C. The initial oven temperature was set at 150 °C for 1 min, and the temperature was gradually raised to 220 °C at 3 °C/min with a final increase to 300 °C for 3 min. Helium was used as carrier gas at a constant column flow rate of 2.52 mL/min. The GC to MS interface temperature was fixed at 280 °C, the electron ionization was performed at 70 eV. The MS was set in scan mode (mass range was 50–600 m/z), where the MS quad and source temperatures were maintained at 150 °C and 230 °C, respectively. Fatty acids were identified by comparing their retention times and mass spectrum profiles with known standards (FAME mix supelco) and the NIST mass spectral library (Version 2.2).

2.2.10 GC×GC-FID analysis

The GC×GC analyses were performed in a Nexis GC-2030 equipped with an AOC-30i Autosampler and an FID (Shimadzu, Germany). The GC was equipped with an Insight® reversed fill/flush flow modulator from SepSolve Analytical (SepSolve Analytical Ltd, UK). The columns were a proprietary SepSolve 1D-FAME 20 m × 0.18 mm × 0.1 µm polar fused silica capillary column and a SepSolve 2D-FAME 5 m × 0.25 mm × 0.1 µm non-polar fused silica capillary column (SepSolve Analytical Ltd, UK). The bleeding line was 4.20 m × 0.1 mm uncoated capillary segment. The 1D flow rate was 0.5 mL/min; the 2D-flow rate was 20 mL/min. Helium was used as carrier gas. The modulation period was set at 3 s, including 100 ms of reinjection time. The oven temperature program started at 40 °C (hold 3 min) and increased to 260 °C (hold 2 min) at 9 °C/min. The injection was performed in split mode (1:10 ratio), injecting 0.5 µL at 250 °C. Detection was performed using an FID set at 270 °C (airflow: 350 mL/min, H2: 35 mL/min; make-up gas: 20 mL/min). Data acquisition frequency was set at 100 Hz. Data acquisition was performed with LabSolution Ver 5.111 (Shimadzu), while data elaboration was done with Chromspace Version 1.5.1 (SepSolve Analytical).

2.2.11 Statistics

All extractions were performed at least in triplicate ($n \geq 3$). The effect of the extraction method on the fatty acid composition was analyzed by using one-way ANOVA followed by Tukey post-hoc test (differences were considered statistically significant at $p < 0.05$). Statistical differences of MAE compared to the conventional Folch method were calculated with a pair-sample t-test ($p < 0.05$, $p < 0.01$ and $p < 0.001$).

2.2.12 Results and discussion

2.2.13 Ultrasound and enzymatic strategies for the recovery of microalgal lipids

As alternatives to the traditional Folch method, which is widely used for lipid extraction in microalgae, we propose the use of various ultrasound and enzymatic approaches for applications requiring lipid extraction in order to develop greener strategies. The effect on extraction yield and the fatty acid profile is presented in the following sections.

2.2.14 Effect of the different methods on the extraction yield

Regarding extraction yield (Figure 6), the microalgae investigated showed values ranging from 7.5 to 23.5%, depending on the species and the approach used. It is important to note that, in this study, the extraction yield refers to crude extracts, which may also contain lipophilic pigments such as carotenoids, as reported in previous studies [43].

For *M. gaditana*, Saczyme® Yield again showed the most effective strategy within this species (23.5% ± 1.1, $p < 0.05$ compared to other methods employed), suggesting that the enzymatic blend is particularly effective in breaking down the cell wall of *M. gaditana*, independent of the drying technique used. However, in contrast with the previous study [38], in this case, Saczyme® Yield exceeded the yield produced by the conventional Folch method. Indeed, this confirms that the drying technique can impact both the extraction process and the extraction yield, as reported in previous studies [44,45]. Additionally, other authors have confirmed the successful use of enzymatic blends, although not the same one used in this study. For instance, various enzymes such as cellulase, laccase, carbohydrases, and mannanase have been applied for lipid extraction in *Nannochloropsis* and *Microchloropsis* strains, resulting in yields ranging from 22 to 29% [46-49]. However, another point to consider when comparing the results with the bibliography, apart from the drying conditions of the biomass, is the cultivation conditions. These can lead to different lipid production, as reported for these species [50], complicating the comparison with existing literature.

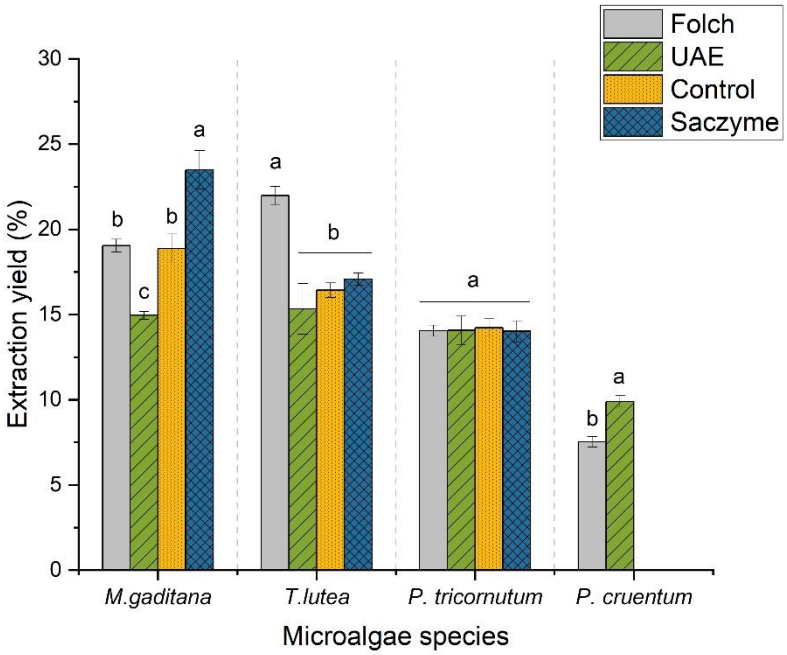


Figure 6. Extraction yield (%) of different microalgae species (*Microchloropsis gaditana, Tisochrysis lutea, Phaeodactylum tricornutum and Porphyridium cruentum*) using ultrasound-assisted enzymatic approaches and compared to the conventional Folch method. The ultrasound-assisted extraction (UAE) was carried out at 50 °C for 30 min using ethanol as solvent. The enzymatic approach was carried out using the enzymatic solution Saczyme® Yield (1 h, 50 °C), followed by a UAE extraction. A control experiment (control) without enzymatic solution was also conducted under the same conditions. Exceptionally, for P. cruentum, only the traditional Folch method and UAE were used, as enzymatic extraction was not feasible (indicated as "–" in Figure 1). Results are expressed as a percentage of dry weight. Error bars denote the standard deviation of at least three independent extractions ($n \geq 3$). Different letters indicate statistically significant differences in extraction method for each microalgae species at $p < 0.05$ (one-way ANOVA with post-hoc Tukey, a–c).

A different trend was found for T. lutea. Although neither ultrasound nor enzymatic strategies proved as effective as the conventional Folch method (22.0% ± 0.5, $p < 0.05$), it is noteworthy to highlight that the alternative approaches achieved an extraction efficiency of approximately 74%. The cell wall composition of T. lutea differs from that of *Microchloropsis* sp., primarily due to variations in monosaccharide content. While *M. gaditana* is composed of 75% glucose, *T. lutea* is

composed of a mixture of glucose, mannose, arabinose, and galactose [51], which may help explain the observed results. Other authors used a combination of pressurized liquids and higher temperatures than in this study to extract lipids from *T. lutea*, which may be necessary for this specific species [52,53].
A more positive outcome was found for *P. tricornutum*, as the methods used did not significantly influence the extraction yield ($p > 0.05$). All methods yielded approximately 14%, suggesting the efficiency of the alternative approaches to disrupt the cell wall and recover the lipids in a similar grade as the conventional Folch method but using a greener solvent. Using also enzymatic methods, specifically papain, other authors were able to release the triacylglycerols for biodiesel production [54]. Additionally, different methods have been used by other researchers to extract lipids from *P. tricornutum*, such as pressurized liquid extraction and microwave-assisted solvent extraction [55] or a simple 24h-string method with ethanol [56]. However, again, the growing and harvesting conditions affect lipid production [57,58], making it difficult to compare the results between studies.
For the lipid extraction of *P. cruentum*, only the traditional Folch method and UAE were used. The utilization of the enzymatic approach was not viable due to the presence of polysaccharides within the microalgae, with predominant sugars identified as xylose, glucose, and galactose [59]. Upon exposure to elevated temperatures, the interaction between the aqueous solution and the microalgae resulted in the formation of a viscous solution, rendering enzymatic extraction unfeasible. These challenges underscore the complexity of extracting lipids from microalgae using environmentally friendly technologies. Nonetheless, UAE combined with ethanol demonstrated greater efficiency, achieving a significantly higher yield compared to the Folch method ($9.9\% \pm 0.3$ and $7.5\% \pm 0.3$ for the UAE and the Folch method, respectively, $p < 0.05$).

2.2.15 Effect of the different methods on the fatty acid profile

Regarding the effect of the extraction strategy on the fatty acid profile (Figure 7), we studied the relative content of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), omega-3, and omega-6 fatty acids of the different produced lipid extracts using the widely employed GC-MS technique. Although differences were observed across species (see section 2.2.17), the extraction method had little influence on the relative content of fatty acid types, with only minor exceptions. For instance, in *P. tricornutum*, a higher PUFA content, particularly omega-3 fatty acids, was observed with the enzymatic approach using Saczyme® Yield. However, overall differences in fatty acid profiles across the various extraction methods were minimal, and the impact of the method was generally negligible. These findings are consistent with previous research, which also reported no major alterations in fatty acid composition regardless of the extraction technique [38,60,61]. These results highlight the effectiveness of the alternative extraction methods in terms of fatty acid profiling.

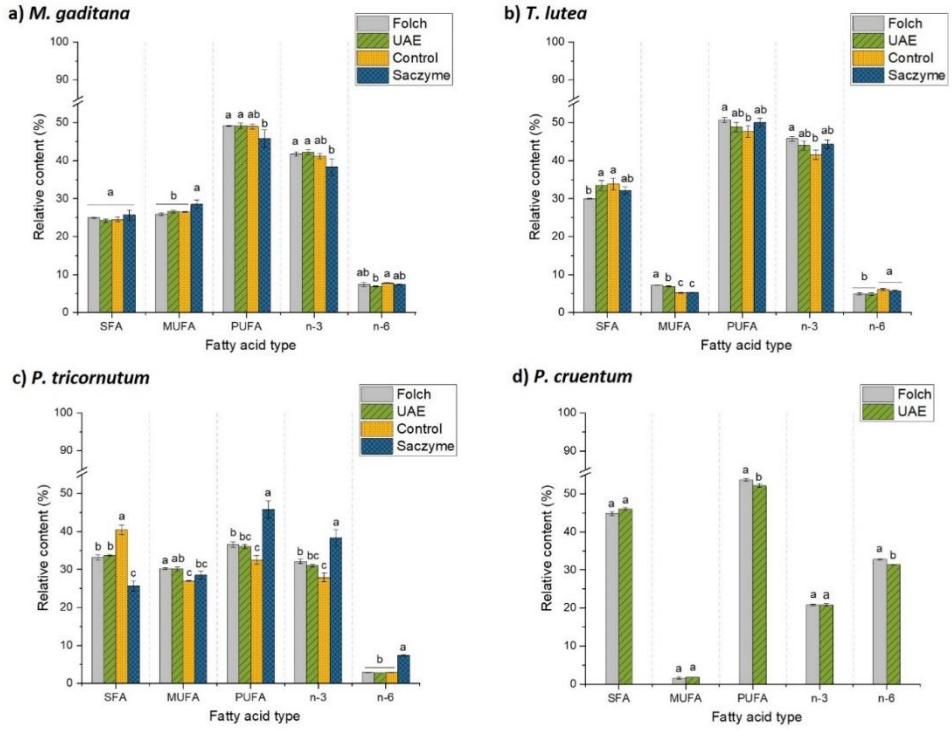


Figure 7. Effect of extraction method on the relative content of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), omega 3 (n-3) and omega-6 (n-6) fatty acids of different microalgae using GC-MS.

2.2.16 Single-step microwave-assisted extraction and derivatization method for microalgal lipids

Since the MAED method has already been proven valid for a wide variety of food commodities [42] and marine organisms [5], one of the goals of this work was to assess whether it is also effective with microalgae exhibiting different characteristics (e.g., different species, cell wall compositions, or fatty acid profiles). Moreover, it is important to highlight an additional challenge, as microalgae predominantly comprise long-chain PUFA with multiple double bonds. The MAED method may offer a suitable solution in cases where lipid extraction is not required, offering a faster and simpler alternative to conventional multi-step procedures. To the best of our knowledge, this is the first time that the MAED method has been used to analyze *M. gaditana, T. lutea, P. tricornutum, and P. cruentum.*

Figure 8 shows the fatty acid profile for the different microalgae species, categorized by type (SFA, MUFA, PUFA, omega-3, and omega-6), obtained using two approaches: (i) the novel MAED method and GC×GC-FID analysis and (ii) the traditional Folch method and GC-MS. The latter was included for comparison, as it

is the most commonly used method for analyzing microalgal fatty acids. In general, the MAED method produced a similar fatty acid profile to the traditional Folch method across all species, apart from *P. cruentum*. Once again, *P. cruentum* exhibited a different trend compared to the others, showing a slightly higher content of PUFA, particularly omega-3 fatty acids, when using the Folch method. These results also could be related to the different analytical techniques used. Nonetheless, apart from that exception, the results presented herein show that the MAED method is a promising solution for maximizing sample throughput in the analysis of fatty acids from microalgal species. Some adjustments on the MAED conditions might need to be made depending on the microalgal species, but this strategy could serve as a good starting point for a quick overview of the fatty acid profiles and potential applications of microalgae, even extending to unknown species.

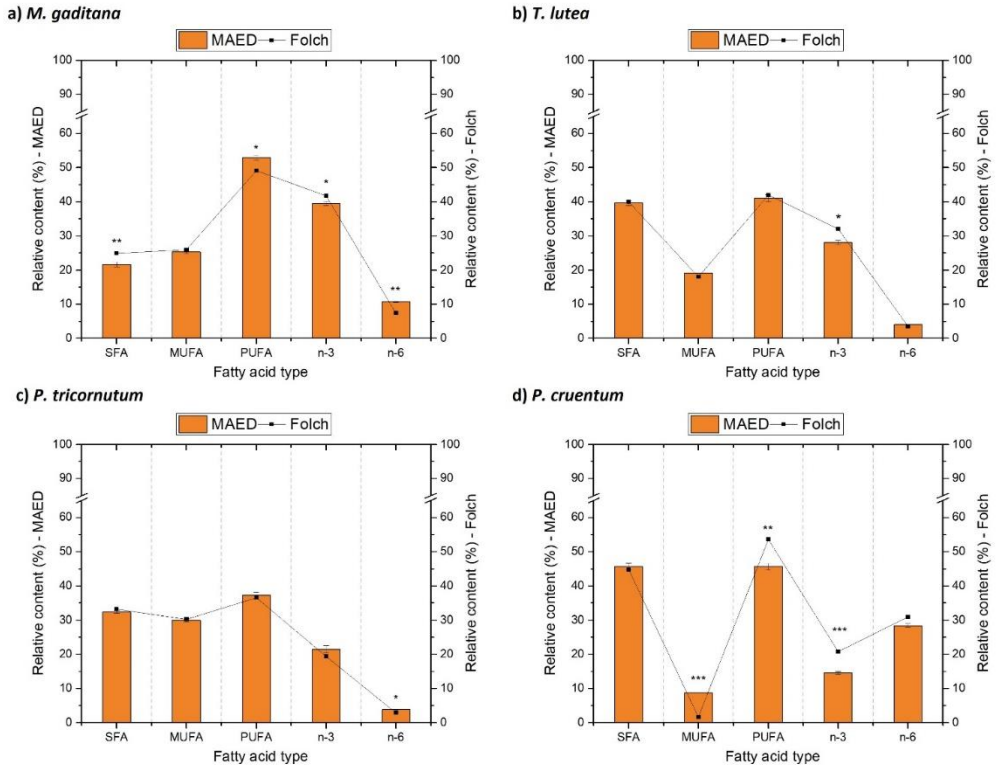


Figure 8. Relative content (percentage of the total fatty acids) of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), omega 3 (n-3), and omega-6 (n-6) fatty acids using the single-step microwave-assisted extraction and derivatization (MAED) approach followed by GC×GC-FID analysis compared to the conventional Folch method followed by GC-MS of different microalgae: (a) *Microchloropsis gaditana*, (b) *Tisochrysis lutea*, (c) *Phaeodactylum tricornutum*, and (d) *Porphyridium cruentum*. Results are expressed as a percentage of the total content (relative content). Values are the mean ± SD of three

determinations. Different letters indicate statistically significant differences in MAED compared to the conventional Folch method calculated with a pair-sample t-test (* p, ** p, *** p < 0.05, 0.01, 0.001).

2.2.17 Comprehensive fatty acid profiling across different microalgal species

Fatty acids are commonly analyzed using GC-FID or GC-MS, which are widely recognized as standard techniques in this field. These methods have been extensively employed to characterize fatty acid profiles in various matrices, providing reliable and reproducible results. However, when analyzing complex and less-explored samples, such as microalgae, more comprehensive techniques like GC×GC may be required.

The complete fatty acid profile of the microalgae species analyzed by GC×GC-FID and compared with GC-MS is presented in Table 5. A total of 44 FAMEs were tentatively identified across the different microalgal species studied. Figure 9 displays the GC×GC-FID chromatogram of *T. lutea* as an example, with the numbers corresponding to the FAME listed in Table 5. The GC×GC-FID chromatograms for the other microalgal species are shown in Figure 10, Figure 11, and Figure 12.

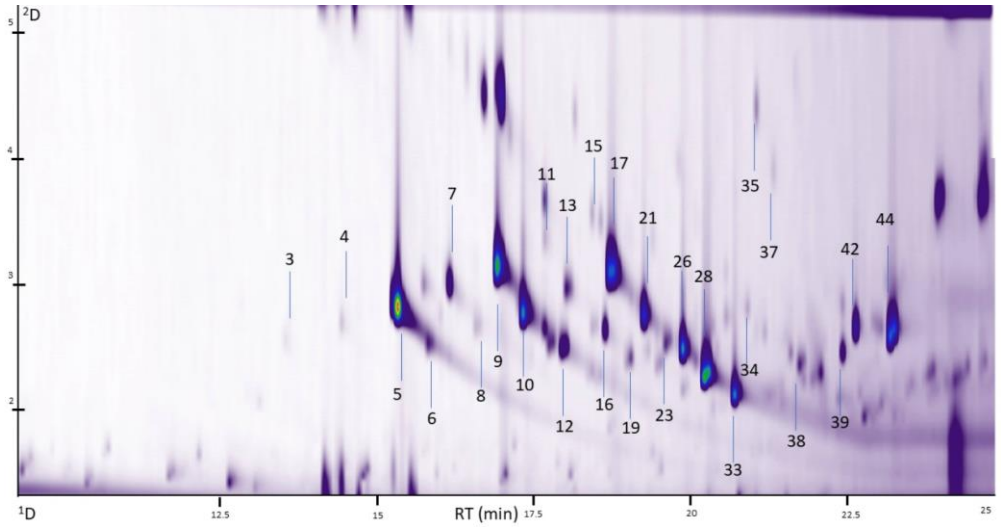


Figure 9. GC×GC-FID chromatogram of *T.Lutea*. Each number in the figure corresponds to a different fatty acid listed in Table 5

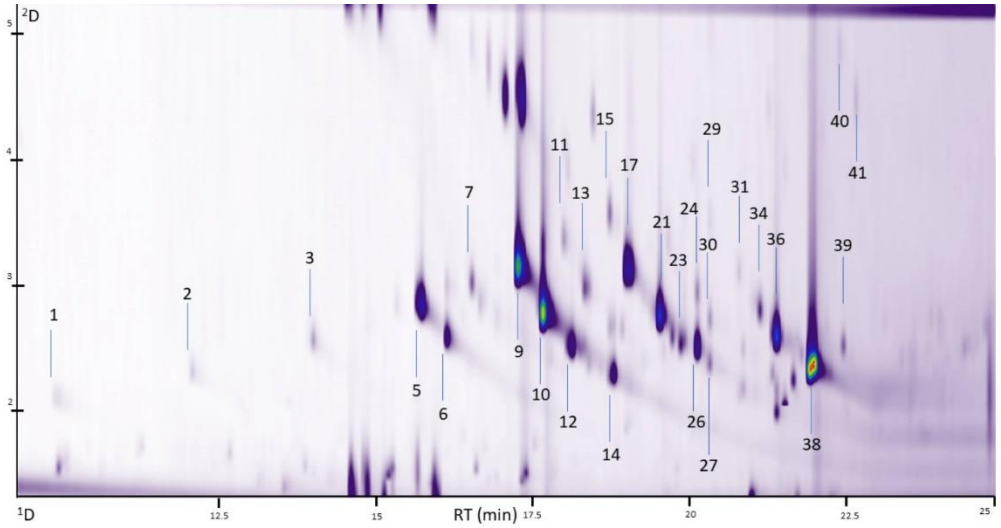


Figure 10. GC×GC-FID chromatogram of *Microchloropsis gaditana*. Each number in the figure corresponds to a different fatty acid listed in Table 5.

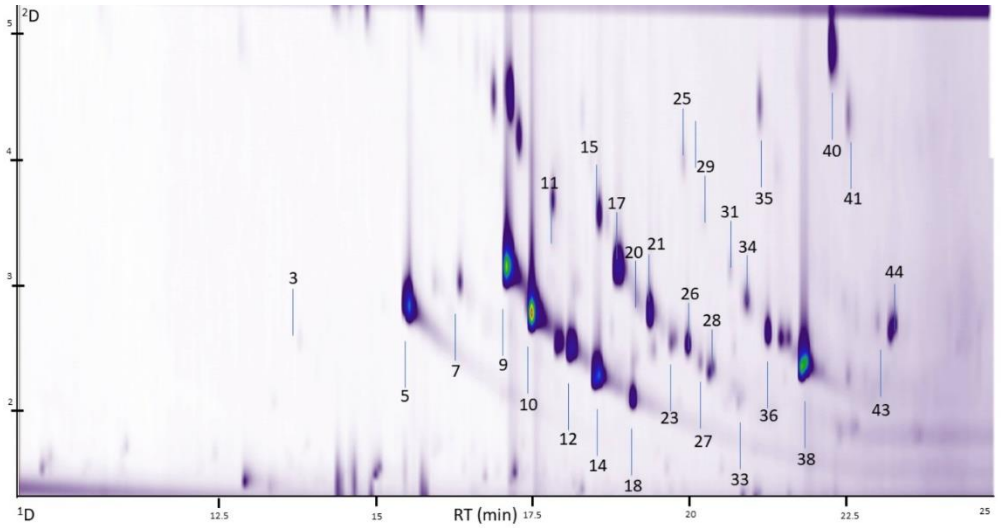


Figure 11. GC×GC-FID chromatogram of *Phaeodactylum tricornutum*. Each number in the figure corresponds to a different fatty acid listed in Table 5.

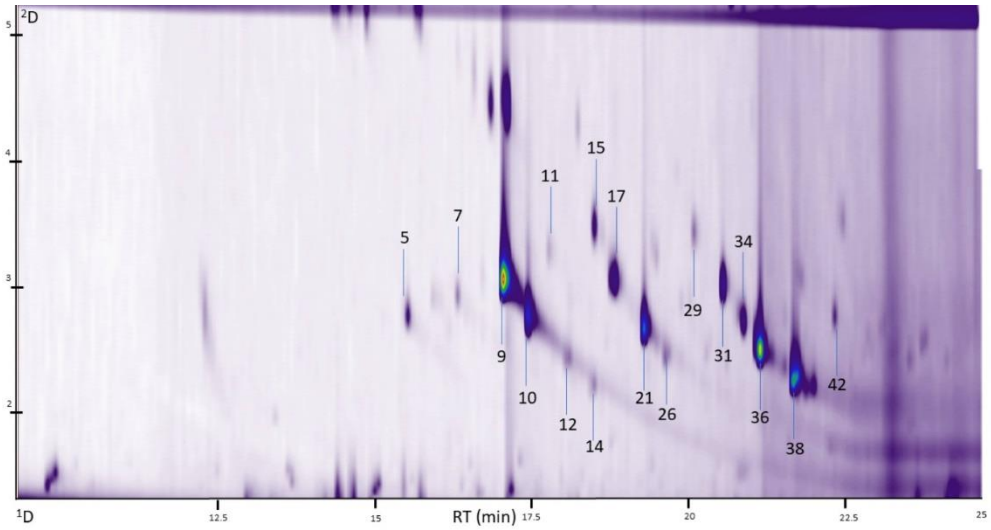


Figure 12. GC×GC-FID chromatogram of *Porphyridium cruentum*. Each number in the figure corresponds to a different fatty acid listed in Table 5.

In this study, GC-MS provided results similar to those fatty acid profiles reported in the literature. However, when samples were analyzed by GC×GC, a significantly more extensive fatty acid profile was obtained compared to both GC-MS and those typically documented in the literature. The enhanced resolution and sensitivity of GC×GC allowed for the detection of additional fatty acids, underscoring its capability to provide a deeper and more detailed characterization of the different fatty acid profiles, as further discussed for each microalgae species below.

Table 5. Fatty acid composition of the microalgal species processed using (i) one-step microwave-assisted extraction and derivatization (MAED) procedure and analyzed by GC×GC-FID and (ii) the Folch method followed by GC-MS analysis.

		Fatty acid percentage (%)							
		M. gaditana		*T. lutea*		*P. tricornutum*		*P. cruentum*	
Number	**Fatty acid**	**GC×GC**	**GC-MS**	**GC×GC**	**GC-MS**	**GC×GC**	**GC-MS**	**GC×GC**	**GC-MS**
1	**C8:0***	0.08 ± 0.01	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
2	**C10:0***	0.07 ± 0.01	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
3	**C12:0***	0.13 ± 0.01	n.d.	0.02 ± 0.01	n.d.	0.02 ± 0.00	n.d.	n.d.	n.d.
4	**C13:0***	0.01 ± 0.00	n.d.	0.06 ± 0.01	n.d.	n.d.	n.d.	n.d.	n.d.
5	**C14:0***	3.08 ± 0.10	4.2 ± 0.06	24.22 ± 0.68	27.55 ± 0.56	7.05 ± 0.45	8.04 ± 0.17	0.41 ± 0.07	n.d.
6	**C14:1***	0.74 ± 0.04	n.d.	0.49 ± 0.05	n.d.	n.d.	n.d.	n.d.	n.d.
7	**C15:0***	0.24 ± 0.01	n.d.	1.01 ± 0.05	0.80 ± 0.07	0.30 ± 0.02	n.d.	0.17 ± 0.01	n.d.
8	**C15:1***	n.d.	n.d.	0.11 ± 0.00	n.d.	n.d.	n.d.	n.d.	n.d.
9	**C16:0***	17.76 ± 0.93	20.75 ± 0.14	14.05 ± 0.11	11.64 ± 0.43	22.80 ± 0.47	24.91 ± 0.47	43.78 ± 0.56	44.34 ± 0.47
10	**C16:1***	20.46 ± 0.53	21.62 ± 0.25	7.20 ± 0.12	7.21 ± 0.08	26.15 ± 0.55	25.69 ± 0.22	6.61 ± 0.06	n.d.
11	**C17:0***	0.14 ± 0.01	n.d.	0.03 ± 0.01	n.d.	n.d.	n.d.	0.15 ± 0.07	n.d.
12	**C16:2**	1.47 ± 0.10	n.d.	1.15 ± 0.02	n.d.	3.19 ± 0.12	n.d.	0.36 ± 0.03	n.d.
13	**C17:1***	0.36 ± 0.00	n.d.	0.44 ± 0.03	n.d.	n.d.	n.d.	n.d.	n.d.
14	**C16:3**	0.87 ± 0.03	n.d.	0.13 ± 0.02	n.d.	7.06 ± 0.20	12.69 ± 0.75	0.21 ± 0.05	n.d.

15	**C18:0***	0.20 ± 0.01	n.d.	0.10 ± 0.02	n.d.	0.60 ± 0.03	n.d.	0.60 ± 0.06	0.46 ± 0.04
16	**C17:2**	n.d.	n.d.	0.57 ± 0.01	n.d.	n.d.	n.d.	n.d.	n.d.
17	**C18:1n9c***	3.75 ± 0.04	4.29 ± 0.53	10.87 ± 0.13	10.83 ± 1.16	3.55 ± 0.06	4.55 ± 0.03	1.94 ± 0.02	1.60 ± 0.34
18	**C16:4**	n.d.	n.d.	n.d.	n.d.	1.36 ± 0.08	1.53 ± 0.14	n.d.	n.d.
19	**C17:3**	n.d.	n.d.	0.22 ± 0.00	n.d.	n.d.	n.d.	n.d.	n.d.
20	**C18:2n6t***	n.d.	n.d.	n.d.	n.d.	0.23 ± 0.01	n.d.	n.d.	n.d.
21	**C18:2n6c***	4.86 ± 0.02	3.48 ± 0.33	3.52 ± 0.04	3.55 ± 0.13	2.00 ± 0.06	2.29 ± 0.04	6.98 ± 0.12	6.43 ± 0.15
22	**C19:1**	0.05 ± 0.00	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
23	**C18:3n6***	0.41 ± 0.04	n.d.	0.34 ± 0.01	n.d.	0.31 ± 0.00	n.d.	0.25 ± 0.07	n.d.
24	**C19:2**	0.13 ± 0.01	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
25	**C20:0**	n.d.	n.d.	n.d.	n.d.	0.08 ± 0.01	n.d.	n.d.	n.d.
26	**C18:3n3***	1.53 ± 0.04	n.d.	5.89 ± 0.04	1.96 ± 0.12	0.65 ± 0.03	n.d.	n.d.	n.d.
27	**C18:4n6**	0.19 ± 0.02	n.d.	0.13 ± 0.01		0.13 ± 0.01	n.d.	n.d.	n.d.
28	**C18:4n3**	n.d.	n.d.	13.90 ± 0.17	17.73 ± 1.27	0.41 ± 0.01	n.d.	n.d.	n.d.
29	**C20:1n9***	0.02 ± 0.00	n.d.	n.d.	n.d.	0.03 ± 0.00	n.d.	0.19 ± 0.03	n.d.
30	**C19:3**	0.11 ± 0.01	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
31	**C20:2***	0.04 ± 0.00	n.d.	0.01 ± 0.01	n.d.	0.06 ± 0.00	n.d.	1.89 ± 0.03	1.87 ± 0.07
32	**C19:4**	0.04 ± 0.01	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
33	**C18:5**		n.d.	4.96 ± 0.09	n.d.	0.10 ± 0.01	n.d.	n.d.	n.d.
34	**C20:3n6***	0.30 ± 0.03	n.d.	0.06 ± 0.00	n.d.	0.37 ± 0.04	n.d.	0.97 ± 0.02	0.86 ± 0.06
35	**C22:0**		n.d.	0.15 ± 0.02	n.d.	0.29 ± 0.03	0.23 ± 0.02	n.d.	n.d.
36	**C20:4n6***	4.95 ± 0.11	3.92 ± 0.13	0.04 ± 0.01	n.d.	0.96 ± 0.04	0.63 ± 0.05	20.20 ± 0.38	23.66 ± 0.29
37	**C22:1n9***			0.05 ± 0.00	n.d.	n.d.	n.d.	n.d.	n.d.

38	**C20:5n3***	37.95 ± 0.58	41.74 ± 0.60	0.19 ± 0.00	n.d.	19.66 ± 0.95	18.51 ± 0.82	15.54 ± 0.41	20.80 ± 0.27
39	**C21:5**	0.11 ± 0.01	n.d.	0.29 ± 0.09	n.d.	n.d.	n.d.	n.d.	n.d.
40	**C24:0***	0.00 ± 0.00	n.d.	n.d.	n.d.	1.87 ± 0.07	n.d.	n.d.	n.d.
41	**C24:1n9***	0.06 ± 0.00	n.d.	n.d.	n.d.	0.27 ± 0.05	n.d.	n.d.	n.d.
42	**C22:4**	n.d.	n.d.	1.43 ± 0.05	n.d.	n.d.	n.d.	0.26 ± 0.08	n.d.
43	**C22:5**	n.d.	n.d.	0.19 ± 0.00	6.36 ± 0.19	0.08 ± 0.01	n.d.	n.d.	n.d.
44	**C22:6n3***	n.d.	n.d.	8.13 ± 0.40	12.39 ± 0.38	0.70 ± 0.05	0.93 ± 0.06	n.d.	n.d.

Results are expressed as a percentage of the total content (relative content) and presented as mean ± SD (n = 3). Samples were treated using two approaches: (i) the novel MAED method followed by GC×GC-FID analysis, and (ii) the traditional Folch method followed by GC-MS, included for comparison as it is the most commonly used technique for analyzing microalgal fatty acids. *Fatty acids were identified using the Supelco 37 FAME Mix standard. Abbreviations: n.d., not detected.

For *M. gaditana*, almost all the fatty acids commonly reported in the literature were identified in GC-MS. The main fatty acids detected in *M. gaditana* are long-chain fatty acids, predominantly with an even number of carbon atoms, including C12:0, C14:0, C16:0, C18:0, C16:1, C18:1, C18:2, C20:4, and C20:5. Recent studies using GC-MS have also confirmed the presence of omega-6 fatty acids, such as 20:4, and omega-3 fatty acids, such as 20:5. Except for the C12:0 and C18:0, all the mentioned fatty acids were identified in GC-MS. On the other hand, GC×GC enabled the identification of a far greater number of fatty acids compared to those of GC-MS and the literature. Specifically, 30 fatty acids were identified with GC×GC, while 8 were detected using GC-MS and in previously published studies [62,63].

T. lutea was the species with the richest fatty acid profile among the species analyzed. In total, 31 fatty acids are detected in GC × GC and 10 in GC-MS. In the literature, C14:0, C18:4n-3, and C22:6n-3 are reported as the most abundant fatty acids [53], findings that align with the results of our study, both in GC×GC and GC-MS. However, we also reported an abundant level of C16:0. In some studies [64,65], C18:0, C18:3n6, C20:0 C22:0, C20:4n6, and C20:5n3 were also found, which were confirmed in our GC×GC analysis but not in the GC-MS due to the very low amount. Other fatty acids were identified in GC×GC, namely C18:4n6, C20:2, C18:5, C20:3n6, C22:1n9, and C21:5, which have never been reported before.

P. tricornutum consisted mainly of mid-chain SFA and MUFA, including C14:0, C16:0, C16:1, and C17:1, while C20:5n3 was the most abundant PUFA present. There are studies on *P. tricornutum* in which the species is subjected to various growth conditions, such as different salinity levels, nitrogen concentrations, light intensities, and temperatures [66,65]. These studies report a more extensive fatty acid profile, including fatty acids like C16:2, C16:3, C17:1, C18:0, C18:3n-3, and C18:4n-6. In our study, these particular fatty acids were not identified in GC-MS, but they were identified in GC×GC. Also, for this species, with GC×GC, a larger number of fatty acids were identified compared to GC-MS (28 fatty acids detected by GC×GC and 12 by GC-MS), and the literature, particularly long-chain saturated and polyunsaturated fatty acids like C20:0, C24:0, C18:2n6t, C18:4n3, C18:5, C20:3n6, and C24:1n9.

In general, the fatty acid profile of *P. cruentum* obtained using GC×GC aligns with what is typically found in the literature, with the exception of a few fatty acids such as C15:0, C16:1, and C18:3n-6, which were not detected either in our study with GC-MS. Even when compared to studies that evaluate the fatty acid composition of *P. cruentum* under different growth conditions [68,69,70], our results remain consistent. Moreover, when comparing the profiles obtained from the literature and GC-MS with those from GC×GC, 8 additional fatty acids were identified, in particular C14:0, C17:0, C16:2, C16:3, and C20:1n9.

In conclusion, the fatty acid profile obtained by GC-MS for the analyzed microalgae species showed results consistent with those previously reported in the literature, confirming the reliability of this method for routine analysis. However, using GC×GC-FID provided a significantly richer and more detailed fatty acid profile, demonstrating its superior analytical abilities. As aforementioned, the key advantage of GC×GC lies in its ability to generate 2D plots that clearly highlight chemical group patterns, supporting the identification without requiring an MS detector. Moreover, the significantly increased peak capacity and sensitivity obtained using GC×GC allows for the effective separation of compounds that might co-elute in one-dimensional GC and the detection of trace FAME in less than 30 minutes. To the best of our knowledge, this represents a superior identification compared to previously reported profiles. In general, no previous studies have reported the analysis of these microalgae species using GC×GC for fatty acid analysis. However, thanks to this technique, many more fatty acids of different chain lengths have been detected in these species, including those present in less than 0.1%, as shown in Table 5.

This enhanced detection capability provides a more detailed profile of the distribution of SFA, MUFA, and PUFA, offering deeper insights into the fatty acid composition of these microalgae. So, it is possible to say that all microalgae species were characterized by a high content of PUFA, ranging between 37.27% for *P. tricornutum* and 52.96 % for *M. gaditana*. For all the species, the SFA profile was predominantly characterized by C16:0, which is common in many microalgae. *P. tricornutum* exhibited a more balanced distribution between SFA and PUFA, with notable quantities of C16:0, C16:1, and C20:5n3. *T. lutea* showed a high amount of SFA among which the C14:0 was the most abundant (24.2%). Differently from the other species, *T. lutea* also showed a high amount of C18:1n9c, C 18:4n3, and C22:6n3, but it was the only one with low C20:5n3. Among the PUFA, the majority were omega-3 fatty acids, particularly in *M. gaditana* and *T. lutea* (39.5% and 28.1%, respectively), with lower amounts found in *P. cruentum* and *P. tricornutum* (14.5% and 21.4%).

2.2.18 Conclusions

The proposed strategies provide alternative methods for extracting and analyzing fatty acids in microalgae. On the one hand, for the applications needed to recover microalgal lipids, we successfully reported the use of ultrasound and enzymatic-assisted extractions, expanding our study to different microalgal species. Specifically, superior yields to the traditional Folch method were obtained for the microalga *M. gaditana* and the enzymatic solution Saczyme® Yield, while for *P. tricornutum*, the combination of ethanol and ultrasounds was demonstrated to be an effective strategy to recover the lipids for this microalga. However, we also faced some drawbacks; for instance, the presence of polysaccharides in *P. cruentum* made the enzymatic extraction unfeasible, while the green methods were less effective for *T. lutea*, likely due to the different cell wall composition, which might affect the complete lipid recovery. These drawbacks highlight the complexity of working with microalgal lipids and green technologies and the necessity to individually optimize the extraction

conditions for using eco-friendly methods. Nevertheless, the use of these greener techniques did not affect the relative content of SFA, MUFA, PUFA, omega-3, and omega-6; the results were comparable to those obtained using the traditional Folch method.

On the other hand, the single-step microwave-assisted extraction and derivatization method was successfully applied for the first time to different microalgal species. The MAED method produced a fatty acid profile similar to the traditional Folch method across all species. Thus, this method represents an excellent alternative for applications requiring a quick overview of the fatty acids in microalgae without requiring an additional extraction step. This is particularly beneficial in high-throughput scenarios or when time efficiency is crucial. Moreover, thanks to GC×GC-FID, we reported a complete characterization of the fatty acids for these four microalgal species, including the identification of a larger number of fatty acids in comparison with those reported by GC-MS or the literature. Furthermore, the enhanced resolution provided by GC×GC-FID ensures high-quality and reproducible results. Thus, the results presented herein mark a significant advancement for future analyses of microalgae, particularly in applications within the food and nutraceutical fields.

2.3 Comprehensive comparison of fatty acid methyl ester profile in different food matrices using microwave-assisted extraction and derivatization methods and comprehensive two-dimensional gas chromatography coupled with flame ionization detection [71]

This section is adapted from: Donatella Ferrara, Marco Beccaria, Chiara E. Cordero, Giorgia Purcaro,
Comprehensive comparison of fatty acid methyl ester profile in different food matrices using microwave-assisted extraction and derivatization methods and comprehensive two-dimensional gas chromatography coupled with flame ionization detection, Advances in Sample Preparation, Volume 11,2024, https://doi.org/10.1016/j.sampre.2024.100124.

2.3.1 Introduction

The one-step MAED method was previously validated in comparison with the AOCS Ce 2b-11 method for a variety of food commodities (n = 11) [2] and edible marine organisms (n = 7) as discussed in the first part of the chapter [5]. Nevertheless, from discussions with many routine laboratories, it emerged that an intermediate step is often needed to determine the total/crude fat of the sample for which the FAME profile is requested. Indeed, fat content is often a crucial parameter in food analysis. Determining total fat, along with SFA, MUFA, and trans-fat content, is essential for dosing ingredients accurately, complying with nutritional standards, producing high-quality, low-fat foods, and optimizing processing conditions to set product quality. So, in order to align methodological development with the practical requirements of routine food analysis, the following section expands the scope of the previously validated MAED approach by explicitly addressing the need for flexible workflows that can accommodate both total fat determination and detailed FAMEs profiling.
We decided to evaluate microwaves 'role in the different sample preparation steps, based on the most common procedures used, both for a one-step procedure and a two-step one (i.e., extraction + derivatization). The final results in terms of FAMEs profile were compared to evaluate possible bias due to the different procedures applied against the reference methods. It was decided to also extend the previous comparison of MAED against the AOCS Ce 2b-11 [1] with other matrices and compare it against the AOCS Ce 2c-11 [72]. The latter method is specific to some food categories, such as oat-based products, which require acid hydrolysis for the extraction of the total fat. Therefore, six food matrices complementary to the ones previously used [2,5] were selected, namely: pastry, cordon bleu, oat, cheese (gouda), spreadable cream, and infant formula. All of them were analyzed using both one-step (i.e., the two AOCS and the MAED), and the two-step procedures. In the latter cases, the extraction was energy-assisted by microwave. Once the lipid fraction was extracted, the total/crude fat underwent two acid-based derivatization methods: I) Conventional BF_3 derivatization [1]; II) HCl/MeOH procedure, with the support of the microwave. The complete scheme of the study is reported in Figure 13.

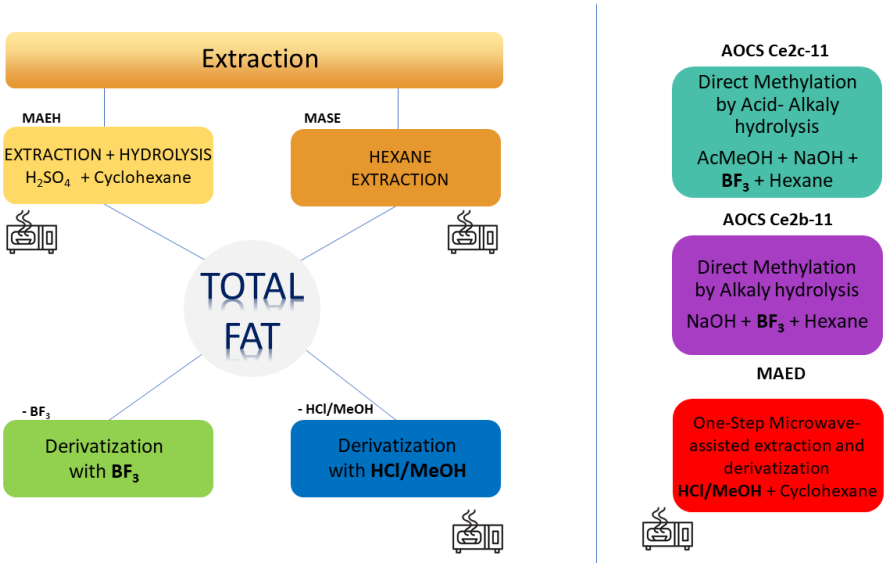


Figure 13. General scheme of the compared methods.

2.3.2 Material and methods

2.3.2.1 Samples and sample treatment

The samples targeted for the study were cordon blue, factory-made pastry (later called simply pastry), spreadable cream, oats, infant formula, and cheese (gouda). All products were bought from a local supermarket, except the cordon bleu (RCIL n◦ 2022-2023-0544) and pastry (RCIL n◦2022-2023-0446) that were samples from BIPEA (Paris, France), provider of proficiency testing programs and external reference materials, having certified value for the FAME profile (Table 6-7). 2.2. Chemicals and reagents All chemicals, including solvents (cyclohexane > 99 %, hexane and methanol in HPLC grade), reference standard (Supelco C37 FAME Mix), and derivatization agents, i.e., 14 % boron trifluoride (BF_3) / methanol solution and HCl/MeOH solution 1.25 M, were acquired from Merck KGaA (Darmstadt, Germany).

2.3.2.2 Direct extraction and derivatization methods

2.3.2.3 One-step microwave-assisted extraction and derivatization (MAED)

MAED procedure was previously described. Briefly, 500 mg of the sample was weighed in the SR-12 vessels of an ETHOS X system from Milestone srl (Sorisole, Bergamo, Italy). Subsequently, 10 mL of methanolic hydrogen chloride solution (HCl/MeOH, Merk KGaA) and 25 mL of cyclohexane were added. The vessels were sealed and placed inside the microwave oven, where the samples were heated under continuous stirring with the following temperature program: 120 ◦C in 2 min, 15 min holding. After cooling, an aliquot of the supernatant containing FAMEs was collected for the following chromatographic analysis. All samples were analyzed in duplicate.

2.3.2.4 Official method CE 2b-11 by AOCS

For the direct methylation of lipids in food by Alkali Hydrolysis, the AOCS Official Method 2b-11 was applied [1]. Briefly, the samples were weighted following the guidance of the table included in the reference method. Then, 5 mL of NaOH/MeOH (0.5 M) was added and the solution was heated under reflux for 15 min. Afterward, 5 mL of 14 % BF_3 in MeOH was added, maintaining the solution under reflux for an additional 2 min. Finally, 5 mL of hexane was added and then everything was removed from the heating source. After cooling, an aliquot of the supernatant containing FAMEs was collected for the following analysis. All samples were analyzed in duplicate.

2.3.2.5 Official method CE 2c-11 by AOCS

For the direct methylation of lipids in food by Acid–Alkali Hydrolysis, the AOCS Official Method 2c-11 was applied [2]. The samples were weighted following the guidance of the table included in the reference method. Then, 5 mL of acidic-methanolic solution (1.25 M) was added and heated under reflux for 15 min. Subsequently, 5 mL of NaOH/MeOH (2.3 M) was added and the solution was heated under reflux for an additional 15 min. Then, 10 mL of 14 % BF_3 in MeOH was added, maintaining the solution under reflux for another 2 min. Finally, 5 mL of hexane was added and then everything was removed from the heating source. After cooling, an aliquot of the supernatant containing FAMEs was collected for the following analysis. All samples were analyzed in duplicate.

2.3.2.6 Extraction and derivatization methods with intermediate total fat determination

2.3.2.7 Microwave-assisted hydrolysis extraction (MAEH)

Approximately 3 g of sample was weighed in the SR-12 extraction vessel of the ETHOS X microwave system; 10 mL of sulfuric acid 25 % and 25 mL of cyclohexane were added. The vessels were closed and heated under continuous stirring with the following temperature program: reaching 90 ◦C in 3 min, reaching 135 ◦C in 4 min, and holding 135 ◦C for 40 min. At the end of the program, the vessels were cooled and the organic phase was evaporated using the RAR 15 evaporation rotor from Milestone connected to a pump and heated for 20 min at 110 ◦C [73]. The total fat was determined before weighing 100 mg for the following derivatization step. The derivatization step was performed either following the procedure described in 2.3.6 using BF_3 (MAEH-BF_3) or the procedure described in 2.3.5 using HCl/MeOH (MAEH-HCl/MeOH).

2.3.2.8 Microwave-assisted solvent extraction (MASE)

Approximately 3 g of the sample was weighed in the SR-12 extraction vessel of the ETHOS X microwave system; 30 mL of hexane was added. The vessels were closed and heated under continuous stirring with the following temperature program: reaching 100 ◦C in 10 min and holding that temperature for 40 min. At the end of the program, the vessels were cooled and the organic phase was evaporated using the

RAR 15 evaporation rotor from Milestone connected to a pump and heated for 20 min at 110 ◦C. The crude fat was determined before the derivatization step that was performed either following the procedure described in 2.3.6 using BF_3 (MASE-BF_3) or the procedure described in 2.3.5 using HCl/ MeOH (MASE-HCl/MeOH).

2.3.2.9 GC × GC-FID instrumentation

All the samples were analyzed by GC × GC-FID. The system (Nexis GC-2030, Shimadzu) was equipped with an AOC-30i autoinjector (Shimadzu). The first dimension (1 D) column was a SepSolve 1D-FAMEs 20 m × 0.18 mm × 0.1 μm polar fused silica capillary column; the second dimension column (2 D) was SepSolve 2D-FAMEs 5 m × 0.25 mm × 0.1 μm non-polar fused silica capillary column. The two columns were connected through an INSIGHT RFF modulator (SepSolve Analytical Ltd, UK). The oven temperature program was: 40 ◦C (2 min), to 250 ◦C (2 min) at 11 ◦C/min. The temperature program was optimized using the standard mixture Supelco C37 FAMEs. The flow rates were as follows: the first dimension flow was set at 0.5 mL/min, and the second dimension flow at 20 mL/min through an auxiliary flow controller (APC). Helium was used as carrier gas. The modulation period was set at 4 s, including 100 ms of reinjection time. The injection was performed in split mode (1:50 ratio), injecting 1.0 μL at 250 ◦C. Detection was performed using an FID set at 250 ◦C (airflow: 300 mL/min, H2: 30 mL/min; make-up gas: 10 mL/min). Data acquisition frequency was set to 100 Hz. Data was acquired by LabSolution Verison 5.111 and processed by ChromSpace Version 1.5.1 by Markes International Limited (Bridgend, UK).

2.3.3 Results and discussion

FAMEs were tentatively identified by comparing their retention time with the Supelco C37 FAME Mix standards, examining their elution on a polar column, and, most importantly, using the position of the FAMEs in a 2D chromatogram [2,13,14]. In this study, the use of GC × GC enabled a detailed and sensitive characterization of FAMEs profile in less than 30 min, and the use of an RFF flow modulator provided good re-injection, resulting in satisfied 2D peak width and symmetry [2,5], and comparable separation to cryogenic modulators. All these advantages are also accompanied by a significant cost reduction since the flow modulator is consumable-free and does not require the use of cryogenic fluids like a cryogenic modulator. Surely, the consumption of carrier gas is higher than 1D analysis, but the cost can be further reduced by moving to hydrogen. All the GC × GC chromatograms were integrated, and the FAMEs profile was calculated to compare the different procedures. A quantitative comparison was performed on the FAMEs, which were ≥0.1 %. The 2D-GC chromatogram of the infant formula with the identified FAMEs is reported in Figure 14 as an example of the separation obtained.

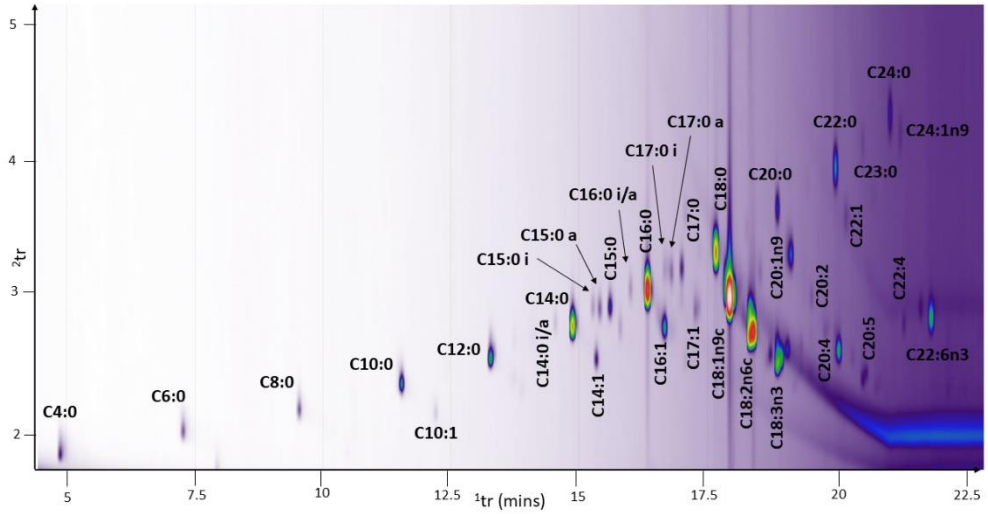


Figure 14. 2D-GC FAMEs profile of infant formula

2.3.3.1 One-step methods: comparison of MAED and official methods AOCS CE 2b-11 and CE 2c-11

A first comparison was performed considering only the one-step methods to further confirm the value of the MAED method previously optimized. Figure 15A shows the summary of the saturated (SFA), monounsaturated (MUFA), and polyunsaturated fatty acids (PUFA) present in the different samples (error bars correspond to the standard deviation (n = 2)) and Figure 15B shows the radar plot comparison of the different FAMEs.

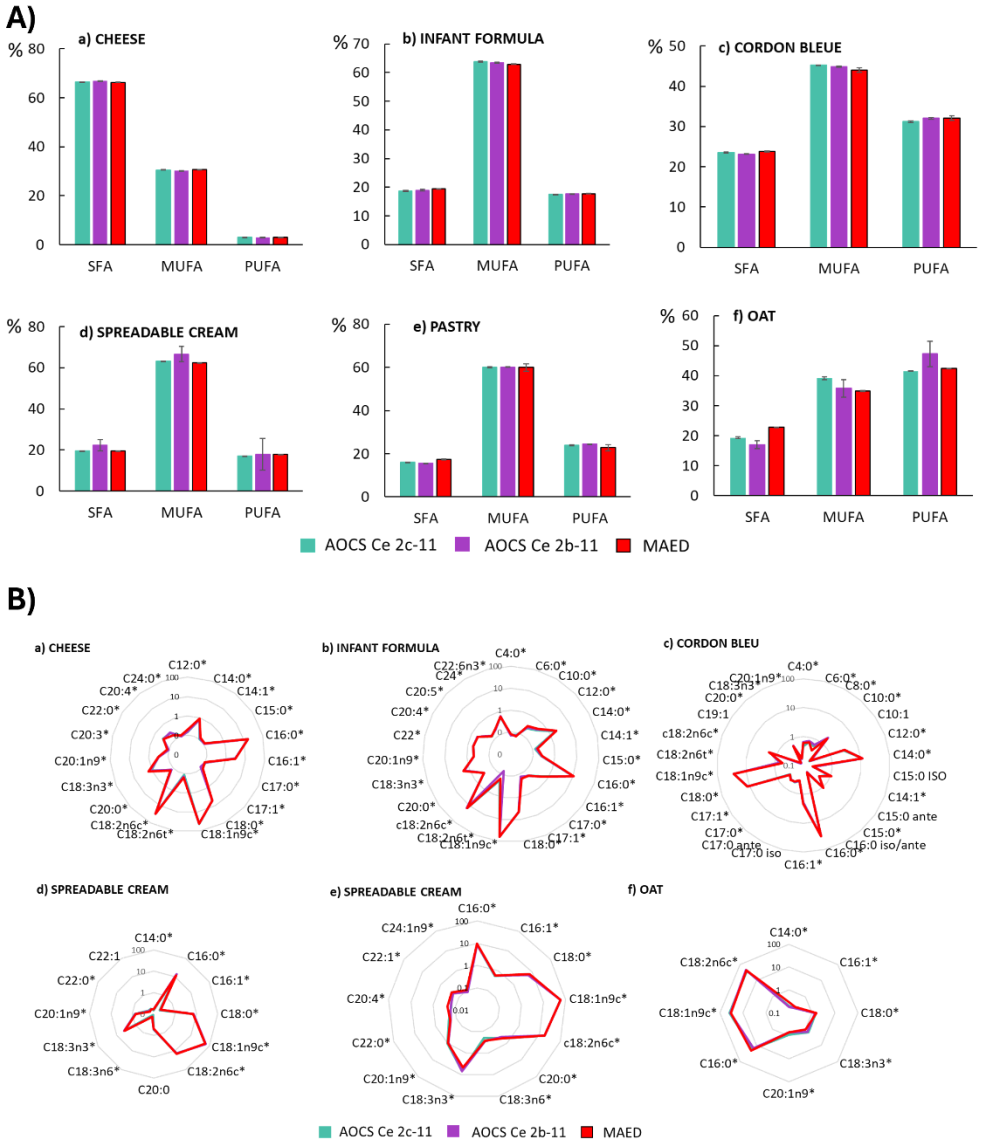


Figure 15. A)) Percentage profile of Saturated (SFA), Monounsaturated (MUFA), and Polyunsaturated fatty acids (PUFA) in a) cheese, b) infant formula, c) cordon bleu, d) spreadable cream, e) pastry, and f) oat performed with Official methods AOCS Ce 2b-11 (purple), Ce 2c-11 (green), and MAED (red). B) Percentage profile of all detected fatty acids in a) cheese, b) infant formula, c) cordon bleu, d) spreadable cream, e) pastry, and f) oat performed with Official methods AOCS Ce 2b-11 and 2c11 and MAED.

All the values for the FAMEs for all the matrices are reported in Table 6-11. The results obtained performing the MAED procedure aligned perfectly with the two official methods, both in terms of total SFA, MUFA, and PUFA (Figure 15A); as well as looking at the single FAMEs (Figure 15B) for all the matrices, except the oat. For the particular samples of pastry and cordon bleu, the results are also consistent with those reported in the BIPEA reports, whose comparison is shown in Table 6-7. These outcomes confirmed the results already obtained on previous studies on the broad and efficient applicability of the MAED method. At the same time, it is confirmed that the AOCS Ce 2b-11 method fails for oat-based samples, where acidic hydrolysis is needed. The special case of the oat is later discussed.

2.3.3.2 Two-step procedure: comparison of BF_3 and HCL/MeOH derivatizations and MASE and MAEH extractions.

From a survey among different laboratories, two main extraction procedures were indicated as the most common, namely, Soxhlet extraction using hexane and the extraction with cyclohexane after previous hydrolysis with H_2SO_4. Both extraction procedures can easily be performed with the support of microwave technology, thus simplifying the procedure, improving the throughput, and reducing the overall energy consumption. Therefore, two equivalent extraction methods, named microwave-assisted solvent extraction (MASE) to replace the Soxhlet and microwave-assisted extraction with hydrolysis (MAEH) were applied. The two extracts obtained were derivatized in two different ways: 1. Using the most used derivatization agents BF_3 (MASE-BF_3 and MAEH-BF_3), without the microwave-assisted derivatization. Using less hazardous derivatization agents, HCl/MeOH (MASE-HCl/ MeOH and MAEH-HCl/MeOH), and performing microwave-assisted derivatization.

2.3.3.3 Determination of total and crude fat

Various methods exist for analyzing fat in food samples [73]. The efficiency of lipid extraction depends on the polarity of both the lipids and the solvent. Polar lipids, such as glycolipids and phospholipids, are more soluble in polar organic solvents (e.g., alcohols). On the other hand, nonpolar lipids (e.g., triacylglycerols, and sterol esters) are more soluble in nonpolar solvents (e.g., hexane and heptane). The Soxhlet method considered the “gold standard” method for fat and oil extractions, is a traditional technique for extracting lipids from foods. It is widely used due to its simplicity and the ability to operate unattended [74]. However, it has several disadvantages, including the use of hazardous and flammable organic solvents, the need for more expensive and higher purity solvents, and being time- and solvent-consuming. Modifications of the methods developed in the 1950s, such as Folch, Bligh and Dyer, have become increasingly common in lipid extraction in food samples due to the use of solvents with different polarities during extraction (a combination of chloroform, MeOH, and water). In both methods, a monophasic solvent system of chloroform and methanol is used to extract and dissolve fats.

Table 6. FAME Percentage profile of cordon blue obtained using AOCS Ce 2-11, AOCS Ce 2c-11, MAEH-HCl/MeOH, MAEH-BF_3, MASE-HCl/MeOH, MASE-BF_3, MAED, compared also with results from BIPEA**.**

Group	1tr	2tr	BIPEA			AOCS Ce 2c-11			AOCS Ce 2b-11			MAEH-HCl/MeOH			MAEH-BF3			MASE-HCl//MeOH			MASE-BF3			MAED		
C12:0*	13.3	2.6	0.2	±	0	0.2	±	0.01	0.2	±	0.007	0.2	±	0.02	0.2	±	0.003	0.2	±	0.001	0.2	±	0.004	0.2	±	0.03
C14:0*	14.9	2.8	1	±	0	0.9	±	0.03	0.9	±	0.03	1.0	±	0.1	0.9	±	0.003	0.9	±	0.03	0.9	±	0.002	0.9	±	0.1
C14:1*	15.3	2.6	0.2	±	0	0.1	±	0.003	0.1	±	0.002	0.1	±	0.01	0.1	±	0.002	0.1	±	0.01	0.1	±	0.001	0.1	±	0.01
C15:0*	15.6	3.0	0.1	±	0	0.1	±	0.003	0.1	±	0.002	0.1	±	0.01	0.1	±	0.001	0.1	±	0.001	0.1	±	0.002	0.1	±	0.01
C16:0*	16.4	3.1	16.1	±	0.1	16.6	±	0.32	16.5	±	0.09	16.7	±	0.04	16	±	0.1	15.9	±	0.2	16.0	±	0.4	16.9	±	0.2
C16:1*	16.7	2.8	3.1	±	0	2.9	±	0.01	3.0	±	0.004	3.1	±	0.04	3.2	±	0.03	3.1	±	0.1	3.1	±	0.03	3.0	±	0.002
C17:0*	17.0	3.2	0.1	±	0	0.1	±	0.001	0.1	±	0.001	0.1	±	0.01	0.1	±	0.002	0.1	±	0.003	0.1	±	0.03	0.1	±	0.01
C17:1*	17.3	2.9	0.1	±	0	0.1	±	0.03	0.1	±	0.001	0.1	±	0.003	0.1	±	0.001	0.1	±	0.01	0.01	±	0.001	0.1	±	0.04
C18:0*	17.7	3.3	4.7	±	0	5.1	±	0.1	4.9	±	0.02	4.8	±	0.2	4.7	±	0.1	4.7	±	0.04	4.6	±	0.01	5.0	±	0.1
C18:1n9c*	18.0	3.0	40.6	±	0.1	41.8	±	0.02	41.4	±	0.2	40.6	±	0.2	41.2	±	0.1	41.8	±	0.5	41.7	±	0.5	40.5	±	0.8
C18:2n6t*	18.2	2.9				0.1	±	0.04	0.1	±	0.01	0.1	±	0.004	0.1	±	0.003	0.02	±	0.03	0.1	±	0.001	0.2	±	0.04
C18:2n6c*	18.4	2.7	30	±	0.1	29.6	±	0.1	30.2	±	0.1	30.3	±	0.4	30.7	±	0.2	30.3	±	0.1	30.5	±	0.1	30.1	±	0.8
C20:0*	18.9	3.7	0.2	±	0	0.2	±	0.002	0.1	±	0.01	0.1	±	0.01	0.1	±	0.003	0.2	±	0.003	0.1	±	0.003	0.2	±	0.004
C18:3n3*	19.1	2.6	1.6	±	0	1.1	±	0.4	1.3	±	0.2	1.6	±	0.01	1.6	±	0.001	1.5	±	0.03	1.5	±	0.02	1.6	±	0.1
C20:1n9*	19.1	3.3	0.3	±	0	0.3	±	0.004	0.3	±	0.004	0.3	±	0.01	0.3	±	0.002	0.3	±	0.002	0.3	±	0.002	0.3	±	0.01
C20:3*	19.8	2.8	0.1	±	0	0.1	±	0.002	0.1	±	0.001	0.1	±	0.01	0.1	±	0.004	0.1	±	0.01	0.1	±	0.002	0.1	±	0.02
C22:0*	20.1	0.0	0.3	±	0	0.3	±	0.01	0.3	±	0.001	0.3	±	0.003	0.3	±	0.01	0.3	±	0.01	0.3	±	0.01	0.3	±	0.03
C20:4*	20.1	2.6	0.2	±	0	0.3	±	0.02	0.3	±	0.02	0.3	±	0.01	0.3	±	0.01	0.2	±	0.02	0.2	±	0.001	0.2	±	0.004
C24:0*	21.1	0.4	0.1	±	0	0.1	±	0.01	0.1	±	0.001	0.1	±	0.01	0.1	±	0.002	0.1	±	0.003	0.1	±	0.001	0.1	±	0.01
SFA			23.6	±	0.2	23.5	±	0.2	23.2	±	0.1	23.5	±	0.2	22.5	±	0.1	22.5	±	0.2	22.5	±	0.3	23.8	±	0.1
MUFA			44.1	±	0.2	45.3	±	0.02	44.8	±	0.1	44.2	±	0.1	44.9	±	0.1	45.4	±	0.3	45.2	±	0.4	44.1	±	0.6
PUFA			32	±	0.1	31.2	±	0.2	32.0	±	0.2	32.3	±	0.3	32.7	±	0.2	32.1	±	0.1	32.3	±	0.1	32.1	±	0.6

Table 7. FAME Percentage profile of pastry obtained using AOCS Ce 2-11, AOCS Ce 2c-11, MAEH-HCl/MeOH, MAEH-BF_3, MASE-HCl/MeOH, MASE-BF_3, MAED, compared also with results from BIPEA.

Group	1tr	2tr	BIPEA			AOCS Ce 2c-11			AOCS Ce 2b-11			MAEH-HCl/MeOH			MAEH-BF3			MASE-HCl//MeOH			MASE-BF3			MAED		
C12:0*	13.3	2.6	0.2	±	0	0.2	±	0.01	0.2	±	0.007	0.2	±	0.02	0.2	±	0.003	0.2	±	0.001	0.2	±	0.004	0.2	±	0.03
C14:0*	14.9	2.8	1	±	0	0.9	±	0.03	0.9	±	0.03	1.0	±	0.1	0.9	±	0.003	0.9	±	0.03	0.9	±	0.002	0.9	±	0.1
C14:1*	15.3	2.6	0.2	±	0	0.1	±	0.003	0.1	±	0.002	0.1	±	0.01	0.1	±	0.002	0.1	±	0.01	0.1	±	0.001	0.1	±	0.01
C15:0*	15.6	3.0	0.1	±	0	0.1	±	0.003	0.1	±	0.002	0.1	±	0.01	0.1	±	0.001	0.1	±	0.001	0.1	±	0.002	0.1	±	0.01
C16:0*	16.4	3.1	16.1	±	0.1	16.6	±	0.32	16.5	±	0.09	16.7	±	0.04	16	±	0.1	15.9	±	0.2	16.0	±	0.4	16.9	±	0.2
C16:1*	16.7	2.8	3.1	±	0	2.9	±	0.01	3.0	±	0.004	3.1	±	0.04	3.2	±	0.03	3.1	±	0.1	3.1	±	0.03	3.0	±	0.002
C17:0*	17.0	3.2	0.1	±	0	0.1	±	0.001	0.1	±	0.001	0.1	±	0.01	0.1	±	0.002	0.1	±	0.003	0.1	±	0.03	0.1	±	0.01
C17:1*	17.3	2.9	0.1	±	0	0.1	±	0.03	0.1	±	0.001	0.1	±	0.003	0.1	±	0.001	0.1	±	0.01	0.01	±	0.001	0.1	±	0.04
C18:0*	17.7	3.3	4.7	±	0	5.1	±	0.1	4.9	±	0.02	4.8	±	0.2	4.7	±	0.1	4.7	±	0.04	4.6	±	0.01	5.0	±	0.1
C18:1n9c*	18.0	3.0	40.6	±	0.1	41.8	±	0.02	41.4	±	0.2	40.6	±	0.2	41.2	±	0.1	41.8	±	0.5	41.7	±	0.5	40.5	±	0.8
C18:2n6t*	18.2	2.9				0.1	±	0.04	0.1	±	0.01	0.1	±	0.004	0.1	±	0.003	0.02	±	0.03	0.1	±	0.001	0.2	±	0.04
C18:2n6c*	18.4	2.7	30	±	0.1	29.6	±	0.1	30.2	±	0.1	30.3	±	0.4	30.7	±	0.2	30.3	±	0.1	30.5	±	0.1	30.1	±	0.8
C20:0*	18.9	3.7	0.2	±	0	0.2	±	0.002	0.1	±	0.01	0.1	±	0.01	0.1	±	0.003	0.2	±	0.003	0.1	±	0.003	0.2	±	0.004
C18:3n3*	19.1	2.6	1.6	±	0	1.1	±	0.4	1.3	±	0.2	1.6	±	0.01	1.6	±	0.001	1.5	±	0.03	1.5	±	0.02	1.6	±	0.1
C20:1n9*	19.1	3.3	0.3	±	0	0.3	±	0.004	0.3	±	0.004	0.3	±	0.01	0.3	±	0.002	0.3	±	0.002	0.3	±	0.002	0.3	±	0.01
C20:3*	19.8	2.8	0.1	±	0	0.1	±	0.002	0.1	±	0.001	0.1	±	0.01	0.1	±	0.004	0.1	±	0.01	0.1	±	0.002	0.1	±	0.02
C22:0*	20.1	0.0	0.3	±	0	0.3	±	0.01	0.3	±	0.001	0.3	±	0.003	0.3	±	0.01	0.3	±	0.01	0.3	±	0.01	0.3	±	0.03
C20:4*	20.1	2.6	0.2	±	0	0.3	±	0.02	0.3	±	0.02	0.3	±	0.01	0.3	±	0.01	0.2	±	0.02	0.2	±	0.001	0.2	±	0.004
C24:0*	21.1	0.4	0.1	±	0	0.1	±	0.01	0.1	±	0.001	0.1	±	0.01	0.1	±	0.002	0.1	±	0.003	0.1	±	0.001	0.1	±	0.01
SFA			23.6	±	0.2	23.5	±	0.2	23.2	±	0.1	23.5	±	0.2	22.5	±	0.1	22.5	±	0.2	22.5	±	0.3	23.8	±	0.1
MUFA			44.1	±	0.2	45.3	±	0.02	44.8	±	0.1	44.2	±	0.1	44.9	±	0.1	45.4	±	0.3	45.2	±	0.4	44.1	±	0.6
PUFA			32	±	0.1	31.2	±	0.2	32.0	±	0.2	32.3	±	0.3	32.7	±	0.2	32.1	±	0.1	32.3	±	0.1	32.1	±	0.6

Table 8. FAME Percentage profile of cheese obtained using AOCS Ce 2-11, AOCS Ce 2c-11, MAEH-HCl/MeOH, MAEH-BF_3, MASE-HCl/MeOH, MASE-BF_3, MAED.

Group	1tr	2tr	BIPEA	AOCS Ce 2c-11	AOCS Ce 2b-11	MAEH-HCl/MeOH	MAEH-BF3	MASE-HCl/MeOH	MASE-BF3	MAED
C16:0*	16.4	3.1	8.2 ± 0.1	8.7 ± 0.1	8.8 ± 0.1	8.9 ± 0.1	8.7 ± 0.1	8.2 ± 0.1	7.8 ± 0.3	9.5 ± 0.1
C16:1*	16.7	2.8	0.5 ± 0	0.5 ± 0.002	0.6 ± 0.003	0.5 ± 0	0.5 ± 0.01	0.5 ± 0.01	0.5 ± 0.01	0.6 ± 0.01
C18:0*	17.8	3.3	5.8 ± 0.1	6.6 ± 0.03	6.0 ± 0.04	6.5 ± 0.1	6.2 ± 0.1	5.9 ± 0.1	5.5 ± 0.04	7.1 ± 0.1
C18:1n9c*	18.0	3.0	58.4 ± 0.2	58.1 ± 0.4	58.3 ± 0.1	57.6 ± 0.1	57.9 ± 0.1	59.0 ± 0.1	59.4 ± 0.6	58.0 ± 2.2
c18:2n6c*	18.4	2.7	17.5 ± 0.2	17.2 ± 0.1	17.4 ± 0.02	17.5 ± 0.2	17.6 ± 0.1	17.2 ± 0.1	17.4 ± 0.3	17.7 ± 0.7
C20:0*	18.9	3.7	0.5 ± 0	0.5 ± 0.04	0.4 ± 0.002	0.5 ± 0.01	0.5 ± 0.03	0.5 ± 0.001	0.5 ± 0.01	0.5 ± 0.03
C18:3n6*	18.7	2.6	±	0.2 ± 0.1	0.3 ± 0.01	0.3 ± 0.01	0.3 ± 0.02	0.3 ± 0.01	0.2 ± 0.03	0.3 ± 0.1
C18:3n3*	18.9	2.5	7.3 ± 0	6.4 ± 0.2	6.7 ± 0.02	6.7 ± 0.1	6.7 ± 0.04	6.8 ± 0.02	7.0 ± 0.10	4.6 ± 2.7
C20:1n9*	19.1	3.3	1.1 ± 0	1.0 ± 0.1	0.9 ± 0.01	1.0 ± 0.01	1.0 ± 0.04	1.0 ± 0.001	1 ± 0.02	1.0 ± 0.1
C22:0*	20.0	0.1	0.3 ± 0	0.2 ± 0.01	0.2 ± 0.002	0.2 ± 0.001	0.2 ± 0.01	0.2 ± 0.001	0.2 ± 0.01	0.2 ± 0.03
C20:4*	20.1	2.6	±	0.2 ± 0.01	0.2 ± 0.01	0.2 ± 0.003	0.1 ± 0.01	0.04 ± 0.01	0.03 ± 0.004	0.2 ± 0.03
C22:1*	20.2	3.6	0.3 ± 0	0.3 ± 0.01	0.2 ± 0.01	0.2 ± 0.001	0.2 ± 0.01	0.3 ± 0.004	0.3 ± 0.02	0.2 ± 0.01
C24:1n9*	21.3	0.3	0.1 ± 0	0.1 ± 0.01	0.1 ± 0.01	0.1 ± 0.001	0.1 ± 0.01	0.1 ± 0.002	0.1 ± 0.01	0.1 ± 0.01
SFA			15.1 ± 0.2	16.0 ± 0.02	15.4 ± 0.1	16.1 ± 0.1	15.6 ± 0.01	14.8 ± 0.1	14.1 ± 0.2	17.4 ± 0.2
MUFA			60.4 ± 0.4	60.0 ± 0.2	60.1 ± 0.1	59.4 ± 0.03	59.8 ± 0.04	60.9 ± 0.04	61.3 ± 0.5	59.9 ± 1.6
PUFA			24.4 ± 0.4	23.9 ± 0.2	24.5 ± 0.002	24.6 ± 0.2	24.7 ± 0.04	24.3 ± 0.1	24.7 ± 0.3	22.8 ± 1.5

Table 9. FAME Percentage profile of infant formula obtained using AOCS Ce 2-11, AOCS Ce 2c-11, MAEH-HCl/MeOH, MAEH-BF_3, MASE-HCl/MeOH, MASE-BF_3, MAED

Group	1tr	2tr	AOCS Ce 2c-11	AOCS Ce 2b-11	MAEH-HCl/MeOH	MAEH-BF3	MASE-HCl/MeOH	MASE-BF3	MAED
C4:0*	4.9	1.9	0.5 ± 0.08	0.7 ± 0.15	0.6 ± 0.1	0.3 ± 0.03	0.6 ± 0.2	0.5 ± 0.04	0.6 ± 0.04
C6:0*	7.3	2.0	0.7 ± 0.2	0.7 ± 0.00	0.7 ± 0.1	0.4 ± 0.01	0.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.01
C8:0*	9.5	2.2	0.6 ± 0.1	0.6 ± 0.002	0.4 ± 0.1	0.5 ± 0.000	0.4 ± 0.04	0.7 ± 0.1	0.4 ± 0.04
C10:0*	11.5	2.4	1.7 ± 0.2	1.9 ± 0.02	1.8 ± 0.3	1.8 ± 0.02	1.6 ± 0.04	2.1 ± 0.3	1.7 ± 0.1
C10:1	12.2	2.2	0.2 ± 0.02	0.2 ± 0.002	0.2 ± 0.04	0.2 ± 0.001	0.2 ± 0.01	0.2 ± 0.03	0.2 ± 0.02
C12:0*	13.3	2.6	3.1 ± 0.05	3.3 ± 0.1	3.3 ± 0.2	3.2 ± 0.03	3.1 ± 0.01	3.6 ± 0.2	3.3 ± 0.1
C14:0*	14.9	2.8	11.1 ± 0.04	11.4 ± 0.03	11.2 ± 0.3	11.1 ± 0.2	11.1 ± 0.1	11.9 ± 0.2	11.2 ± 0.2
C15:0 ISO	15.3	3.0	0.2 ± 0.01	0.2 ± 0.01	0.2 ± 0.002	0.2 ± 0.002	0.2 ± 0.002	0.2 ± 0.002	0.2 ± 0.01
C14:1*	15.3	2.6	0.9 ± 0.01	1.0 ± 0.01	1.0 ± 0.05	1.1 ± 0.02	1.0 ± 0.003	1.1 ± 0.04	1.1 ± 0.04
C15:0 ante	15.4	3.0	0.5 ± 0.01	0.5 ± 0.02	0.5 ± 0.004	0.5 ± 0.01	0.5 ± 0.003	0.5 ± 0.001	0.5 ± 0.01
C15:0*	15.6	3.0	1.0 ± 0.01	1.1 ± 0.02	1.1 ± 0.02	1.1 ± 0.01	1.1 ± 0.01	1.1 ± 0.01	1.1 ± 0.02
C16:0 iso/ante	16.0	3.1	0.2 ± 0.003	0.2 ± 0.001	0.2 ± 0.003	0.2 ± 0.000	0.2 ± 0.000	0.2 ± 0.001	0.2 ± 0.01
C16:0*	16.3	3.1	33.8 ± 0.3	33.5 ± 0.2	33.5 ± 0.3	33.0 ± 0.2	33.0 ± 0.4	33.5 ± 0.003	33.3 ± 0.3
C16:1*	16.7	2.8	1.9 ± 0.01	1.9 ± 0.03	2.0 ± 0.01	2.1 ± 0.1	2.0 ± 0.003	2.0 ± 0.01	2.1 ± 0.2

C17:0 iso	16.7	3.3	0.4	±	0.02	0.4	±	0.01	0.4	±	0.01	0.3	±	0.03	0.3	±	0.002	0.3	±	0.002	0.4	±	0.01
C17:0 ante	16.8	3.3	0.4	±	0.01	0.4	±	0.002	0.5	±	0.00	0.5	±	0.04	0.5	±	0.002	0.4	±	0.01	0.4	±	0.01
C17:0*	17.0	3.3	0.5	±	0.03	0.5	±	0.002	0.5	±	0.003	0.5	±	0.01	0.5	±	0.004	0.4	±	0.01	0.5	±	0.03
C17:1*	17.3	3.0	0.4	±	0.02	0.4	±	0.03	0.4	±	0.1	0.4	±	0.001	0.3	±	0.01	0.3	±	0.01	0.4	±	0.01
C18:0*	17.7	3.4	11.9	±	0.2	11.6	±	0.1	11.6	±	0.1	12.0	±	0.1	12.1	±	0.1	11.3	±	0.2	11.8	±	0.4
C18:1n9c*	17.9	3.1	26.8	±	0.2	26.4	±	0.1	26.5	±	0.8	27.4	±	0.1	27.2	±	0.1	26.2	±	0.7	26.6	±	0.004
C18:2n6t*	18.2	2.9	0.7	±	0.05	0.7	±	0.02	0.7	±	0.02	0.5	±	0.1	0.6	±	0.1	0.6	±	0.01	0.5	±	0.2
c18:2n6c*	18.4	2.8	1.7	±	0.05	1.7	±	0.02	1.8	±	0.04	2.0	±	0.04	1.8	±	0.1	1.7	±	0.02	1.9	±	0.2
C19:1	18.5	3.3	0.2	±	0.01	0.1	±	0.03	0.1	±	0.03	0.2	±	0.01	0.2	±	0.000	0.1	±	0.02	0.2	±	0.004
C20:0*	18.8	3.8	0.1	±	0.002	0.1	±	0.002	0.1	±	0.001	0.1	±	0.001	0.1	±	0.001	0.1	±	0.01	0.1	±	0.01
C18:3n3*	18.9	2.6	0.6	±	0.1	0.6	±	0.03	0.6	±	0.1	0.5	±	0.1	0.5	±	0.004	0.6	±	0.01	0.6	±	0.03
C20:1n9*	19.1	3.4	0.2	±	0.001	0.2	±	0.01	0.2	±	0.01	0.2	±	0.003	0.2	±	0.000	0.2	±	0.01	0.2	±	0.01
SFA			66.5	±	0.01	66.9	±	0.01	66.5	±	0.5	65.6	±	0.2	66.1	±	0.05	67.5	±	0.5	66.3	±	0.1
MUFA			30.5	±	0.1	30.2	±	0.04	30.4	±	0.5	31.5	±	0.2	31.0	±	0.1	30.1	±	0.5	30.7	±	0.1
PUFA			2.9	±	0.1	2.9	±	0.05	3.1	±	0.01	3.0	±	0.1	2.9	±	0.02	2.8	±	0.02	3.0	±	0.002

Table 10. FAME Percentage profile of spreadable cream obtained using AOCS Ce 2b-11, AOCS Ce 2c-11, MAEH-HCl/MeOH, MAEH-BF_3, MAEH-BF_3, MASE-HCl/MeOH, MASE-BF_3, MAED.

Group	1tr	2tr	AOCS Ce 2c-11	AOCS Ce 2b-11	MAEH-HCl/MeOH	MAEH-BF3	MASE-HCl/MeOH	MASE-BF3	MAED
C4:0*	4.9	1.9	0.1 ± 0.02	0.1 ± 0.02	0.1 ± 0.01	0.1 ± 0.002	0.1 ± 0.01	0.1 ± 0.01	0.1 ± 0.001
C6:0*	7.3	2.0	0.1 ± 0.003	0.1 ± 0.02	0.1 ± 0.01	0.1 ± 0.01	0.1 ± 0.01	0.1 ± 0.02	0.1 ± 0.01
C10:0*	11.5	2.4	0.2 ± 0.02	0.3 ± 0.02	0.3 ± 0.03	0.3 ± 0.01	0.2 ± 0.01	0.2 ± 0.02	0.3 ± 0.01
C12:0*	13.3	2.6	0.4 ± 0.03	0.4 ± 0.02	0.5 ± 0.02	0.5 ± 0.003	0.4 ± 0.02	0.4 ± 0.02	0.5 ± 0.01
C14:0*	14.9	2.8	1.9 ± 0.08	2.1 ± 0.04	2.1 ± 0.1	2.1 ± 0.01	1.8 ± 0.04	1.7 ± 0.01	2.1 ± 0.02
C14:1*	15.3	2.6	0.1 ± 0.01	0.2 ± 0.01	0.2 ± 0.01	0.2 ± 0.001	0.1 ± 0.003	0.1 ± 0.002	0.2 ± 0.004
C15:0*	15.6	3.0	0.3 ± 0.003	0.3 ± 0	0.3 ± 0.003	0.3 ± 0.002	0.2 ± 0.01	0.2 ± 0	0.3 ± 0.002
C16:0*	16.3	3.1	10.0 ± 0.2	10.1 ± 0.2	10.4 ± 0.1	10.0 ± 0.1	9.5 ± 0.2	9.3 ± 0.05	10.4 ± 0.1
C16:1*	16.6	2.8	0.5 ± 0.01	0.6 ± 0.01	0.6 ± 0.004	0.6 ± 0.01	0.5 ± 0.003	0.5 ± 0.01	0.6 ± 0.001
C17:0*	17.0	3.3	0.2 ± 0	0.2 ± 0.002	0.2 ± 0.004	0.2 ± 0	0.2 ± 0.003	0.2 ± 0.003	0.2 ± 0.01
C17:1*	17.3	2.9	0.1 ± 0.01	0.1 ± 0.002	0.1 ± 0.01	0.1 ± 0.003	0.1 ± 0.01	0.1 ± 0.004	0.2 ± 0.02
C18:0*	17.7	3.3	4.5 ± 0.07	4.5 ± 0.01	4.5 ± 0.01	4.3 ± 0.001	4.2 ± 0.05	4.1 ± 0.004	4.6 ± 0.04
C18:1n9c*	18.0	3.0	62.6 ± 0.3	62.0 ± 0.3	61.8 ± 0.3	61.9 ± 0.03	63.1 ± 0.1	63.0 ± 0.1	61.5 ± 0.2
C18:2n6t*	18.2	2.9	0.2 ± 0.04	0.1 ± 0.02	0.1 ± 0.1	0.1 ± 0.05	0.2 ± 0.1	0.1 ± 0.01	0.2 ± 0.1
c18:2n6c*	18.4	2.8	14.2 ± 0.2	14.6 ± 0.01	14.6 ± 0.01	14.8 ± 0.03	14.7 ± 0.2	15.1 ± 0.2	14.4 ± 0.1
C20:0*	18.9	3.7	0.3 ± 0.003	0.3 ± 0.01	0.3 ± 0.01	0.3 ± 0.001	0.3 ± 0.004	0.3 ± 0.001	0.3 ± 0.002
C18:3n3*	18.9	2.5	1.8 ± 0.2	1.8 ± 0.004	1.8 ± 0.01	1.8 ± 0.003	1.9 ± 0.2	1.9 ± 0.03	1.9 ± 0.2
C20:1n9*	19.1	3.3	0.5 ± 0.003	0.5 ± 0.01	0.3 ± 0.1	0.5 ± 0.001	0.5 ± 0.01	0.5 ± 0.003	0.5 ± 0.001
C22:0*	20.0	0.0	0.6 ± 0.004	0.6 ± 0.01	0.6 ± 0.01	0.5 ± 0.004	0.6 ± 0.01	0.5 ± 0.01	0.6 ± 0.01
C20:4*	20.0	2.6	0.5 ± 0.004	0.5 ± 0.01	0.5 ± 0.01	0.5 ± 0.01	0.5 ± 0.001	0.5 ± 0.02	0.5 ± 0.003
C20:5*	20.5	2.4	0.2 ± 0.001	0.2 ± 0	0.2 ± 0.004	0.2 ± 0.002	0.2 ± 0.01	0.2 ± 0.001	0.2 ± 0.01
C24:0*	21.1	0.4	0.2 ± 0.001	0.2 ± 0.004	0.2 ± 0.01	0.2 ± 0.001	0.2 ± 0.001	0.2 ± 0.002	0.2 ± 0.001

C22:6n3*	21.9	2.9	0.6	±	0.01	0.6	±	0.01	0.6	±	0.01	0.6	±	0.01	0.6	±	0.02	0.6	±	0.04	0.6	±	0.02
SFA			18.7	±	0.2	19.0	±	0.2	19.4	±	0.2	18.7	±	0.02	17.7	±	0.1	17.3	±	0.1	19.4	±	0.03
MUFA			63.9	±	0.2	63.4	±	0.2	63.0	±	0.1	63.3	±	0.03	64.4	±	0.05	64.3	±	0.1	62.9	±	0.1
PUFA			17.4	±	0.02	17.7	±	0.01	17.7	±	0.1	18.0	±	0.01	18.1	±	0.1	18.4	±	0.2	17.8	±	0.1

Table 11. FAME Percentage profile of oat obtained using AOCS Ce 2b-11, AOCS Ce 2c-11, MAEH-HCl/MeOH, MAEH-BF_3, MAEH-BF_3, MASE-HCl/MeOH, MASE-BF_3, MAED.

Group	**1tr**	**2tr**	**AOCS Ce 2c-11**			**AOCS Ce 2b-11**			**MAEH-HCl/MeOH**			**MAEH-BF3**			**MASE-HCl/MeOH**			**MASE-BF3**			**MAED**		
C14:0*	14.9	2.8	0.2	±	0.003	0.2	±	0.02	0.2	±	0.01	0.2	±	0.003	0.2	±	0.001	0.2	±	0.01	0.2	±	0.004
C16:0*	16.3	3.1	11.6	±	0.01	14.1	±	3.4	11.6	±	0.1	11.7	±	0.1	11.9	±	0.2	11.6	±	0.4	11.9	±	0.05
C16:1*	16.7	2.8	0.2	±	0.002	0.2	±	0.03	0.2	±	0.01	0.2	±	0.004	0.3	±	0.001	0.2	±	0.003	0.2	±	0.001
C18:0*	17.9	3.2	7.3	±	0.04	7.4	±	0.6	6.7	±	0.2	6.6	±	0.1	6.8	±	0.02	6.6	±	0.1	6.9	±	0.1
C18:1n9c*	18.1	3.0	62.1	±	0.1	65.5	±	5.3	61.9	±	0.5	61.7	±	0.2	61.5	±	0.001	61.7	±	0.01	61.4	±	0.05
C18:2n6c*	18.4	2.7	13.4	±	0.1	13.8	±	0.03	13.9	±	0.3	14.1	±	0.1	13.9	±	0.1	14.2	±	0.3	13.9	±	0.04
C20:0	18.9	3.6	0.5	±	0.01	0.5	±	0.05	0.5	±	0.02	0.5	±	0.01	0.5	±	0.01	0.5	±	0.02	0.5	±	0.004
C18:3n6*	18.7	2.6	0.1	±	0.02	0.1	±	0.02	0.2	±	0.01	0.2	±	0.01	0.2	±	0.001	0.1	±	0.1	0.1	±	0.001
C18:3n3*	18.9	2.5	3.6	±	0.1	4.0	±	0.3	3.9	±	0.1	3.9	±	0.02	3.8	±	0.02	4.0	±	0.04	3.8	±	0.05
C20:1n9*	19.1	3.3	0.8	±	0.02	0.7	±	0.07	0.7	±	0.002	0.7	±	0.01	0.7	±	0.02	0.7	±	0.04	0.7	±	0.001
C22:0*	20.0	4.0	0.2	±	0.01	0.2	±	0.02	0.2	±	0.01	0.2	±	0.01	0.2	±	0.003	0.2	±	0.01	0.2	±	0.001
C22:1	20.2	3.6	0.2	±	0.01	0.2	±	0.01	0.2	±	0.02	0.2	±	0.01	0.2	±	0.002	0.2	±	0.004	0.2	±	0.01
SFA			19.7	±	0.03	22.3	±	2.8	19.0	±	0.2	19.1	±	0.05	19.5	±	0.1	19.0	±	0.2	19.6	±	0.03
MUFA			63.3	±	0.1	66.7	±	3.8	63.0	±	0.3	62.8	±	0.1	62.6	±	0.01	62.8	±	0.02	62.5	±	0.02
PUFA			17.1	±	0.03	18.0	±	7.8	18.0	±	0.1	18.2	±	0.1	17.9	±	0.1	18.2	±	0.2	17.9	±	0.004

Subsequently, water is added to create a biphasic system, separating polar and nonpolar compounds into an upper and lower phase, respectively [8,75]. However, even if modifications of Folch and Bligh and Dyer methods are frequently used, they can involve several steps in the sample preparation and high consumption of solvents, especially at the routine level. The use of microwave energy for lipid extraction has been demonstrated to be faster with less solvent involved, giving equivalent results to Soxhlet [73], it has been widely used in sample preparation also elsewhere [73,76,77] and is used also in this work for total fat determination. Fat was determined in all food matrices using two different methods, MAEH and MASE described in 2.3.9 and 2.3.10, respectively. The first method utilized a combination of sulfuric acid and cyclohexane. This approach allows for a more thorough breakdown of complex food matrices, facilitating the extraction of fat by disrupting both protein and carbohydrate structures. Sulfuric acid acts as a strong acid hydrolyzing agent, while cyclohexane serves as the nonpolar solvent to dissolve the liberated fats. With MAEH what is called the "Total fat" is obtained. The second method is simple solvent extraction, so without the aid of an acid catalyst, the result is a measure of what is called "crude fat" or free fats, which is not bound to complex molecules such as proteins and carbohydrates. Table 12 reports the value obtained with the two different methods and the value reported on the product labels. The results of total fat obtained from MAEH closely matched the value reported on the nutritional labels, except for a little overestimation of the total fat in the infant formula and cheese, while the information about the total fat of BIPEA samples (i.e., pastry and cordon bleu) were not disclosed. The variance observed for infant formula and cheese (gouda) can be attributed to the different methods used for the determination of the total fat or to the fact that food products can vary between production batches; in fact, differences in raw materials or production processes can cause variations in fat content. Additionally, changes in fat content may occur also during product storage, affecting the results compared to the values on the label [77].

Table 12. Comparison of the total fat, the crude fat, and the label values (g/100 g).

	Cheese	Pastry	Cordon bleu	Oat	Spreadable cream	Infant formula
TOTAL FAT	36.7 ± 1.4	25.3 ± 0.9	18.8 ± 2.8	7.6 ± 0.9	38.8 ± 1.6	29.3 ± 0.6
CRUDE FAT	19.0 ± 5.4	19.6 ± 0.2	13.3 ± 0.4	4.6 ± 0.4	21.6 ± 0.7	10.1 ± 0.2
LABEL	28	-	-	7	32	24

-: no information available

2.3.3.4 FAME profile

The percentage profile of FAMEs of the two derivatizations on the two different extracts is compared in Figure 16 in terms of SFA, MUFA, and PUFA and in Figure 17 in terms of the percentage profile of each FAME detected in each sample.

Comparing the same extraction method (i.e., MAEH or MASE, represented by yellow and orange, respectively, on the top of the bars) with the two different derivatization procedures (different color in the bottom of the bars, blue for HCl/MeOH and green for BF_3), no difference is observed for any matrix and any FAME (Figure17). The results showed that the HCl/MeOH solution can efficiently convert FAs into their methyl esters derivatives, providing comparable results to the overrated BF_3 while being safer for the operator, more stable, and cost-effective. In fact, the HCl/MeOH solution is much less hazardous to handle, and use compared to BF_3, which is highly toxic and corrosive. Moreover, it is known that BF_3 can be unstable and difficult to store, whereas HCl/ MeOH solutions are generally more stable and easier to manage over time [78].

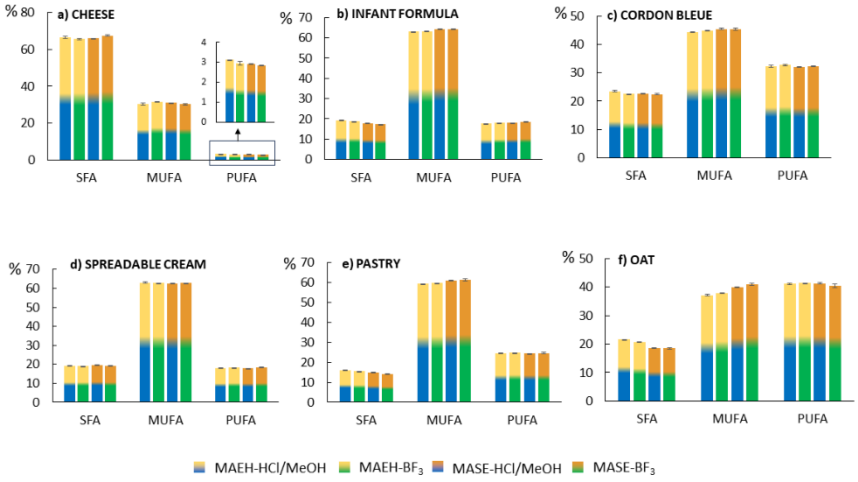


Figure 16. Percentage profile of Saturated (SFA), Monounsaturated (MUFA), and Polyunsaturated fatty acids (PUFA) in all matrices obtained with two different extractions (MAEH, yellowish top bar; MASE, orangish top bar) and derivatization (BF_3, greenish bottom bar; HCl/MeOH, bluish bottom bar) methods.

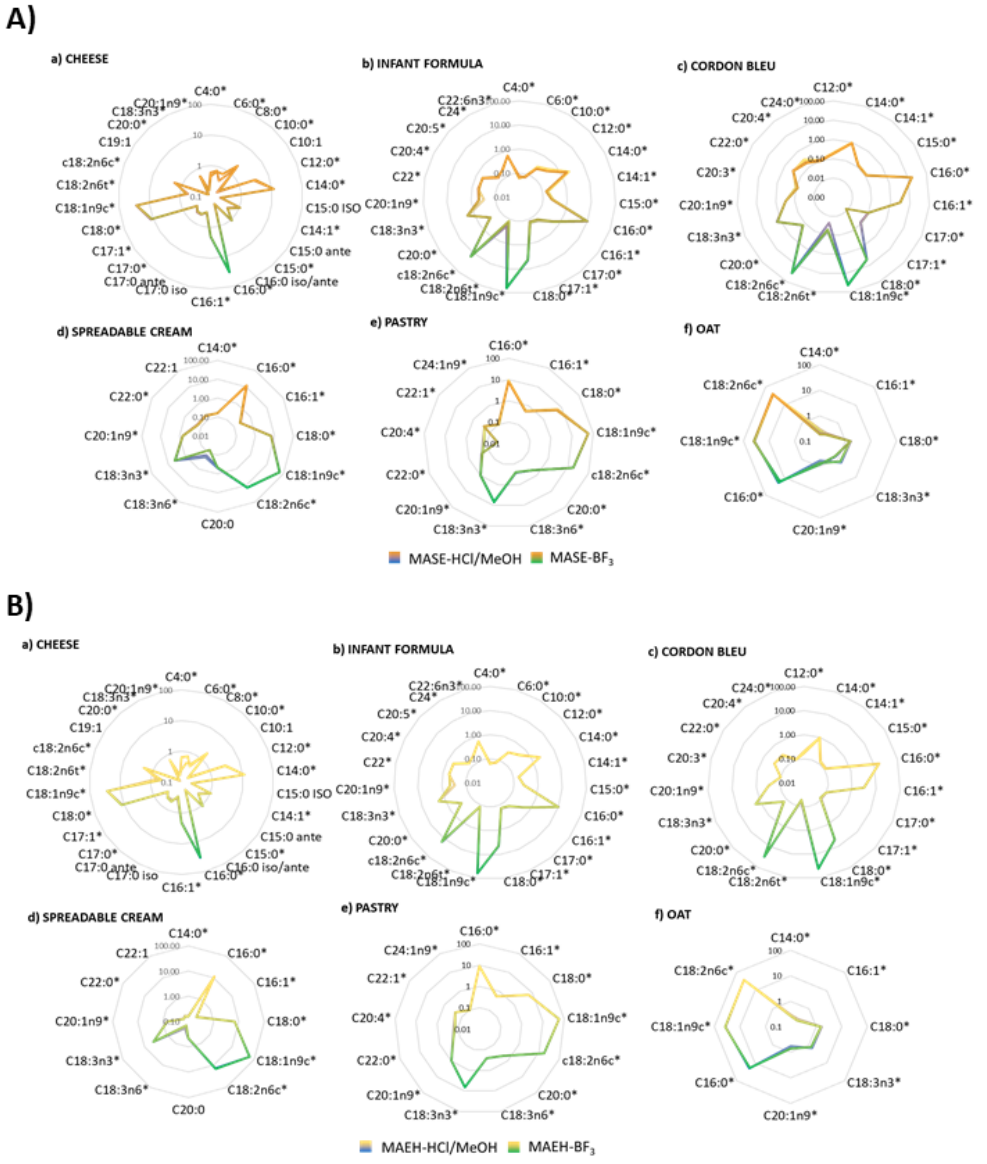


Figure 17. Percentage profile of FAMEs in all matrices obtained with A) the MASE extraction followed by the BF_3 or HCl/MeOH derivatization; B) MAEH extraction followed by the BF_3 or HCl/MeOH derivatization.

In the end, considering the throughput of an analytical laboratory, using an HCl/MeOH solution can be a more economical choice for routine use. Indeed, the HCl/MeOH derivatization can also be performed in the microwave, speeding up the

overall derivatization procedure. Considering that the derivatization procedure did not impact the results at all, to simplify the visualization the two extraction modes (i.e. MAEH and MASE) were compared (considering only one derivatization method, i.e., HCl/MeOH for consistency) with the one-step procedure (Figure 18). Similarly, for the one-step procedure the MAED is retained, considering that all the matrices gave perfectly comparable results (except for oat discussed later) with the two official AOCS methods. The results showed a very good comparability, except for oat, for which a more thoughtful discussion is presented in the next section. For all the other matrices, a slight difference can be observed for the percentage of C20:4 in pastry, where the MASE-HCl/MeOH method showed lower extraction. This is clearly the effect of the extraction method, harsher on one side, while only capable of extracting the crude fat (alias "free lipids") in the case of the MASE. Additionally, the C18:2n6t in the cordon bleu showed a discrepancy among the methods. A lower amount was found with the two-step method. Unfortunately, this FA is presented at a very low concentration, ~ 0.1 % and it is not reported in the BIPEA certificate. The value found using the MAED is comparable with both official AOCS methods, suggesting the reliability of the result compared to the two-step methods. It is not clear what could have caused this discrepancy, but the occurrence of this FA was also very low, impacting the reliability of the results. Generally, the methods resulted in equivalent results, pointing toward flexibility in choosing the method based on specific laboratory requirements and available resources.

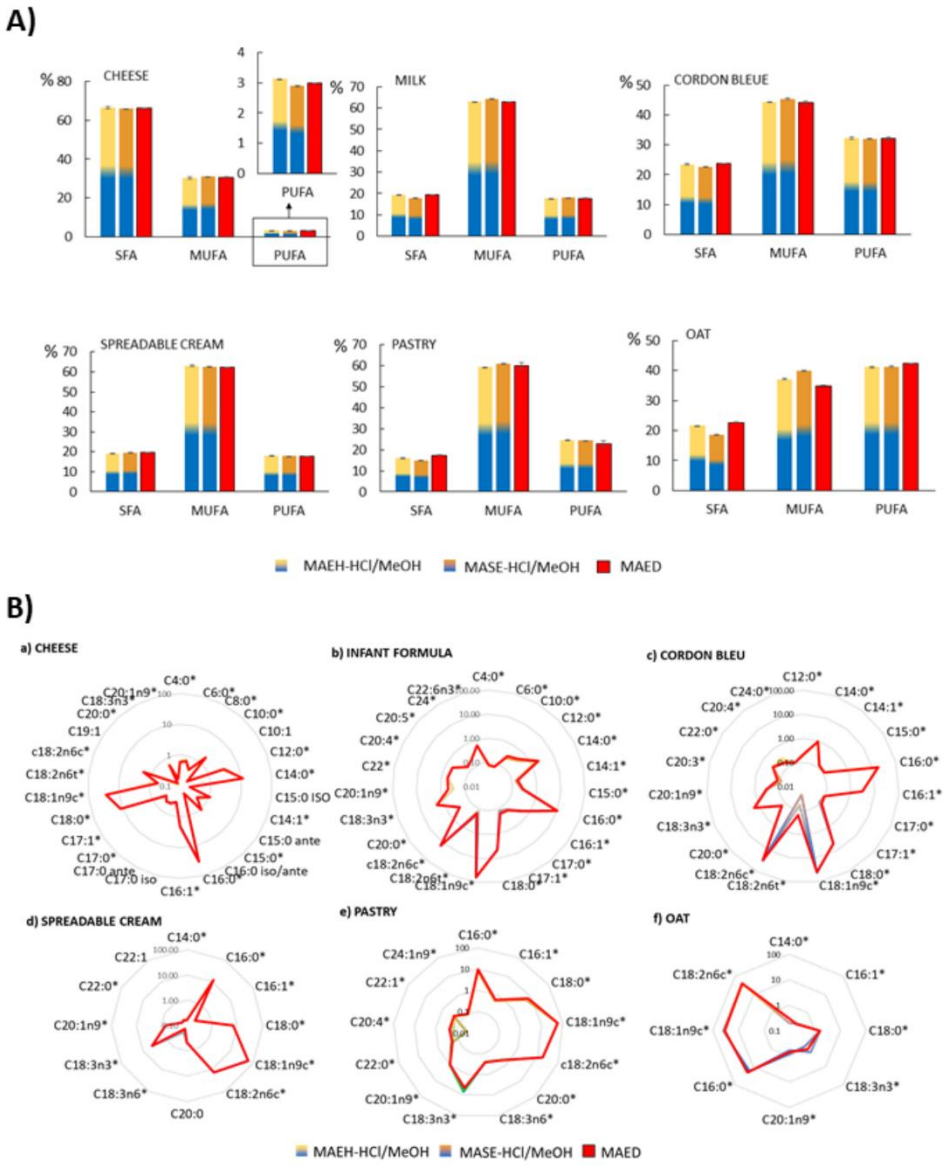


Figure 18. A) Percentage profile of Saturated (SFA), Monounsaturated (MUFA), and Polyunsaturated fatty acids (PUFA) in all matrices obtained with two different extractions and HCl/MeOH derivatization methods. B) percentage profile of FAMEs in all matrices obtained with two different extractions (MAEH, yellowish line; MASE, orangish line) and the same derivatization (HCl/MeOH, blue), and the MAED one-step procedure (red line)

2.3.3.5 The special case of oat

Interestingly, in the case of the oat-based food, the MAED method did not align with the two AOCS methods (Figure 15A). According to the AOCS protocols, the AOCS Ce 2c-11 should be used in this case. The PUFA obtained with MAED were similar to the results obtained using AOCS Ce 2b-11, but both were lower than those using the AOCS Ce 2c-11. For the MUFA, the MAED was very similar to AOCS Ce 2c-11, while AOCS Ce 2b- 11 was higher, while MAED gave higher amount of SFA compared to the other two reference methods, with the lowest value obtained using the AOCS Ce 2b-11. The same trend is observed in the three major FAMEs, namely C16:0, C18:1n9c, and C18:2n6c (Figure 19) and Table 11. A clear difference is also shown in the two-step methods, but this was expected as MASE is not efficient in breaking the complex interaction of the lipids with the matrix in this kind of sample. Indeed, in the case of oats, the simple solvent extraction is not sufficiently effective in breaking the complex structures of oat cells. In fact, in oats, lipids are bonded to other macromolecules, such as proteins and carbohydrates, thus requiring harsher conditions to be released [79]. The use of MAEH allowed breaking down these structures and releasing lipids for a more comprehensive analysis. Figure 19 compares the AOCS Ce 2c-11 Official method suggested for the oat, which implicates acidic pre-treatment, MAEH-HCl/MeOH, and MAED.

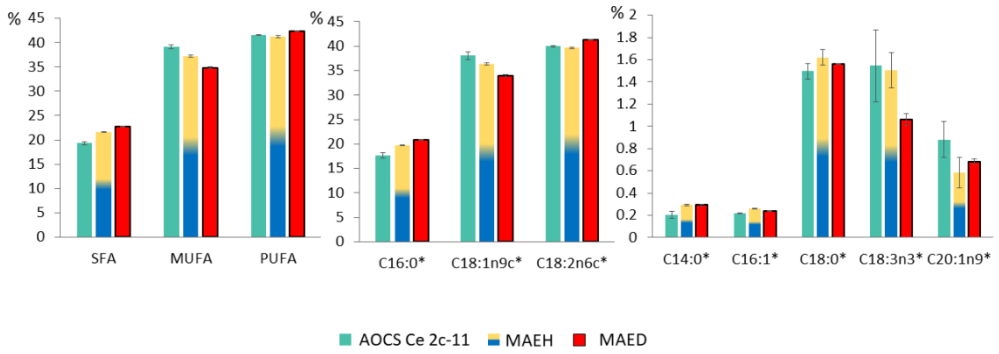


Figure 19. a) Comparison of Official Method Ce 2c-11, two- and one-step MAE Percentage profile of Saturated, Monounsaturated, and Polyunsaturated fatty acids in Oat; b) Comparison of Official Method Ce 2c-11, two- and one-step MAE Percentage profile of FAMEs.

As aforementioned, the MAED method was not capable of liberating the lipids comparably to acid hydrolysis with H_2SO_4. However, the MAEH-HCl/MeOH also showed some differences in the FAMEs profile. Differently than MAED, it gave

similar results than the AOCS Ce2c-11 method for C18:2n6 and C18:3n3, while it gave higher results for C16:0 and lower for C18:1n9. In Sahasrabudhe's work [80], the lipid class composition of oats is analyzed with particular attention to the various lipid classes present and their fatty acid distribution. According to this work, oats contain a significant amount of lipids compared to other cereals, generally ranging from 4 % to 9 % of the dry weight, with triglycerides representing the majority of the lipid content, followed by phospholipids and glycolipids while free FAs and sterols are present in smaller amounts. Referring to the fatty acids distribution, the work shows that C14:0 and C18:3 are more present in glycolipids, C16:0 is more present in phospholipids, C18:0 is equally distributed in glycolipids and phospholipids, and C18:2 in triglycerides, glycolipids and phospholipids. It appears evident that the different methods are not equivalent for the different classes of lipids, and this appears evident in samples where other classes than triglycerides do not represent only trace amounts. Nevertheless, it is difficult to draw a definitive conclusion on the efficiency of extraction of the different classes. A dedicated study should be performed considering the absolute quantity (and not the percentage profile) and also to perform the quantification of the different lipid classes as intact lipids and of the FAMEs after transesterification.

2.3.3.5 Green analytical evaluation

All the methods applied were evaluated for greenness and blueness using two specific tools (Figure 20). To assess the greenness of the sample preparation methods, the AGREEprep metric [81] was used. The software generates pictograms that display the score assigned to the method based on the 10 criteria of green sample preparation and taking into account the importance of criteria by assigning them specific weight.

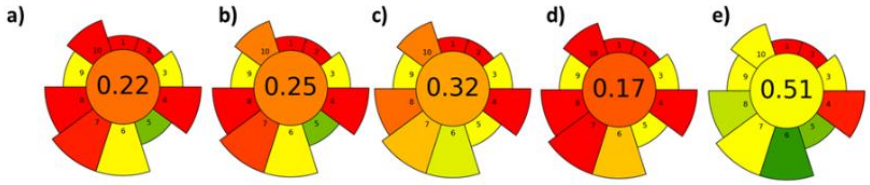


Figure 20. Comparison of the greenest of the methods: a) AOCS Ce 2c-11; b) AOCS Ce 2b-11; c) MAEH-HCl/MeOH and MAE-HCl/MeOH; d) MAEH-BF_3 and MAE-BF_3; e) MAED.

The default weight was kept except for criteria 2 (use of safer solvents and reagents), 6 (maximizing sample throughput), and 7 (promote automation and integrated steps) to align with the previous evaluation [2,5] in comparison with the AOCS Ce 2b-11. The MAED demonstrates excellent performance in maximizing sample throughput (Criteria 6). This criterion refers to the number of samples prepared in 1 h. MAED enables the preparation of 12 samples every 15 min, adding up to 48 per hour or even

more if the system is equipped with a rotor with 24 positions (96 per hour), compared to 8 per hour with the official methods. This efficiency is the result of the integration of multiple steps in MAED, making it as automated as possible, as required by Criteria 7. Additionally, MAED leads to a reduction in power consumption (Criteria 8). The final score obtained for all the methods is shown in Figure 20A. It is evident that the lower score is assigned to the methods that use BF_3 for the derivatization, and the higher is assigned to the MAED approach. The two-step methods had an intermediate value if the derivatization is the HCl/MeOH, or much lower, also of the official one if the BF_3 is used. HCl/MeOH derivatization can be preferred over BF_3 for the transesterification of lipids to form FAME and there are several reasons for this preference, including safety, ease of use, effectiveness, and compatibility with different matrices. Specifically, methanolic HCl is less hazardous and easier to handle compared to BF_3, which is corrosive and requires special handling precautions. Methanolic HCl provides an effective and complete transesterification reaction for converting fatty acids into FAME and works efficiently for most lipid matrices, whereas BF3 can lead to undesirable side reactions, such as the formation of artifacts in the presence of sensitive functional groups. Moreover, HCl/ MeOH is more stable and easier to store. Criteria 9 is based on the choice of the greenest possible postsample preparation configuration for the analysis. In general, for a greener analytical approach, GC-FID is preferably used on the GC–MS, however, in addition to the sustainability impact, one should also consider the method's performance. In this regard, the proposed method, which utilized GC×GC-FID, is capable of providing detailed information while remaining more sustainable than MS. Through the utilization of GC×GC, the interpretation capabilities are improved, and the structured chemical pattern obtained from the 2D plot enables detailed characterization of FAMEs profile based on specific positions in the chromatogram assuring a reliable identification without the need to use an MS. Moreover, a noteworthy sustainable aspect of the proposed analytical methods is the utilization of a flow modulator instead of a cryogenic one.

2.3.3.6 Blue analytical evaluation

On the other hand, we used the Blue applicability grade index (BAGI) [82] based on the blue principles of white analytical chemistry to evaluate the practicality of the methods. For a method to be considered practical, a final score above 60 is recommended. All the methods used achieved a score higher than 60 as shown in Figure 20B; however, the highest score was assigned to MAED, namely 75.0. The two crucial criteria that made the difference were criteria 5 and 6, namely the ability to process many samples within 1 h and the multi-step sample preparation merged into one single step. Regarding the two-step methods, they scored slightly over 60 (62.5) but less than the official methods (67.5). However, in addition to providing information on the FAMEs profile, the two-step methods also provide information on total fat; output that can be considered practical to many laboratories.

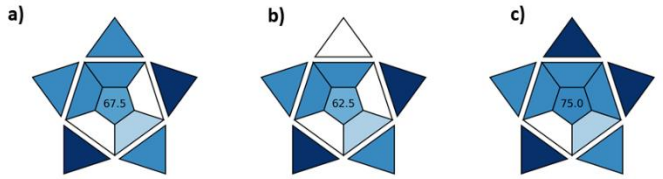


Figure 21. Comparison of the blueness of the methods: a) AOCS Ce 2c-11, AOCS Ce 2b-11; b) MAEH-HCl/MeOH, MAE-HCl/MeOH, MAEH-BF_3 and MAE-BF_3; c) MAED.

2.3.3.6 White analytical evaluation

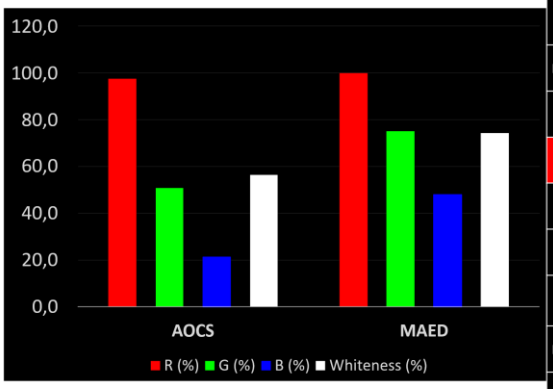
The AGREEPrep metric was employed to assess compliance with the principles of green sample preparation, while the Blue Applicability Grade Index (BAGI) was used to quantify practical efficiency in terms of throughput, automation, and operational simplicity. Although these evaluations provide valuable and complementary information, they address individual dimensions of method performance separately and do not fully capture the overall balance between analytical quality, environmental impact, and practicality.

For this reason, an additional assessment based on the concept of White Analytical Chemistry (WAC) was performed to integrate these aspects into a single framework. The WAC approach, grounded in the RGB model, simultaneously considers analytical performance (red), environmental sustainability (green), and practical efficiency (blue), expressing their combined contribution through the whiteness parameter. This integrated evaluation is particularly relevant in the context of FAMEs analysis, where methods must not only be greener and faster, but also analytically reliable and suitable for routine implementation.

As illustrated in Figure 22, the RGB-based evaluation highlights clear differences between the approaches investigated. While all methods exhibit strong red contributions, confirming their analytical suitability, the MAED workflow shows a markedly more balanced distribution of red, green, and blue components. This balance results in a substantially higher whiteness value compared with the AOCS reference method. In contrast, methods relying on more labor-intensive procedures or hazardous reagents achieve lower whiteness scores, reflecting an imbalance between analytical performance and sustainability or practicality.

Overall, the whiteness assessment complements the greenness and blueness evaluations discussed throughout this chapter by providing a unified measure of suitability method. Within this framework, the MAED approach emerges as the most balanced solution for FAMEs analysis, achieving a favorable compromise between analytical performance, environmental sustainability, and operational efficiency. This confirms that the proposed methodology is not only greener and more practical, but

also well aligned with the broader principles of sustainable and fit-for-purpose analytical chemistry.



Method: AOCS					
R1: Scope of application	100,0	G1: Toxicity of reagents	50,0	B1: Cost-efficiency	70,0
R2: LOD and LOQ	90,0	G2: Amount of reagents and waste	40,0	B2: Time-efficiency	10,0
R3: Precision	100,0	G3: Energy and other media	40,0	B3: Requirements	5,0
R4: Accuracy	100,0	G4: Direct impacts	73,3	B4: Operational simplicity	0,0
97,5		50,8		21,3	

Method: MAED					
R1: Scope of application	100,0	G1: Toxicity of reagents	50,0	B1: Cost-efficiency	30
R2: LOD and LOQ	100	G2: Amount of reagents and waste	80	B2: Time-efficiency	85
R3: Precision	100,0	G3: Energy and other media	80	B3: Requirements	10
R4: Accuracy	100,0	G4: Direct impacts	90	B4: Operational simplicity	66,7
100		75		47,9	

Figure 22. Comparison of the whiteness of AOCS and MAED methods with relative scores.

2.3.4 Conclusion

The microwave-assisted processes proved to be suitable for FAs analysis in foods, and comparable to Official methods both when performing the one-step method (MAED) and the two-steps methods (MAEH and MASE). It is evident that whether the total fat data is needed, the MAEH procedure should be preferred over the one-step methods, but otherwise no major differences were noticed in the FAs profile of the samples considered in the present study (except for oat) for which acid hydrolysis is required. From the viewpoint of the derivatization step, it is worth stressing that the still overrated BF_3 derivatization procedure can be successfully replaced with the microwave-assisted HCl/MeOH derivatization, obtaining the same performance but improving the safety of the operator using less harmful reagents, being overall greener, and ensuring a higher throughput. To increase the greenness of the MAED method, in the future, more focus can be directed towards reducing solvent volumes, aiming to further enhance the method's environmental friendliness. Nevertheless, the representativeness of the sample must always kept in mind. Moreover, further studies are necessary to design an optimal MADE procedure alternative to the official method involving preliminary acid hydrolysis (AOCS Ce 2c-11).

3 Conclusions on the use of the MAED approach for fatty acid profiling

This chapter brings together two complementary studies that collectively address both the analytical performance and the practical applicability of microwave-assisted methodologies for fatty acid analysis in complex food and marine matrices. The first study demonstrated the robustness and suitability of a one-step microwave-assisted extraction and derivatization (MAED) approach coupled with comprehensive two-dimensional gas chromatography (GC×GC–FID) for the detailed characterization of fatty acid methyl esters (FAMEs) profiles in edible marine organisms. The method proved capable of handling lipid matrices rich in omega-3 polyunsaturated fatty acids, providing high chromatographic resolution, reliable identification, and accurate nutritional index calculation while significantly simplifying sample preparation. Building upon this foundation, the second study expanded the methodological scope by critically comparing one-step and two-step workflows across a broader range of food matrices with differing compositional and structural characteristics. By benchmarking microwave-assisted extraction and derivatization strategies against official AOCS reference methods (Ce 2b-11 and Ce 2c-11), this work demonstrated that microwave irradiation can be effectively integrated into both extraction-only and extraction–derivatization workflows without introducing significant bias in the resulting FAME profiles. Importantly, the results confirmed that the final fatty acid composition is largely independent of the specific sample preparation route employed, provided that the process is properly controlled, thereby reinforcing the analytical reliability of microwave-assisted approaches. From a broader perspective, the combined findings of these studies support the concept of decision-driven analytical workflows in lipid analysis. Rather than enforcing a rigid, one-size-fits-all protocol, the proposed strategies allow the analytical pathway to be tailored to the information required—total fat determination, detailed fatty acid profiling, or both—while relying on a single, coherent technological platform. This flexibility represents a significant advancement for routine laboratories, where regulatory, nutritional, or industrial needs often dictate different analytical depths for different samples. A great plus is represented also by the GC×GC, while it is often perceived as a technically demanding and resource-intensive technique, the results obtained clearly show that the analytical benefits offered by this approach largely outweigh the initial investment required for its implementation.

In particular, the superior separation power of GC×GC proved essential for resolving highly complex FAME mixtures, where conventional one-dimensional GC approaches are inherently limited by peak overlap and restricted peak capacity. The structured nature of GC×GC separations enabled a more confident identification of fatty acids, improved quantitative accuracy, and the reliable calculation of nutritionally relevant indices, even in matrices characterized by high lipid complexity and elevated omega-3 PUFA content. These advantages are not merely incremental but represent a qualitative improvement in the level of information that can be extracted from a single analytical run. Overall, this chapter demonstrates that microwave-assisted extraction and derivatization, when combined with comprehensive two-dimensional gas chromatography, constitute a mature and

versatile analytical framework for fatty acid analysis. The work not only validates MAED as a fast, green, and reliable alternative to conventional protocols but also positions microwave-assisted strategies as enabling tools for adaptive, decision-driven lipid analysis. These findings lay a solid methodological foundation for future developments aimed at further automation, broader matrix coverage, and integration into standardized analytical workflows.

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Chapter 4

Microwave-assisted methods for sterols characterization

1. Introduction

Sterol analysis relies on a sequence of well-established preparative steps, as already mentioned in Chapter 2. Saponification represents the first key step and is typically carried out under reflux using KOH/EtOH solution. This process hydrolyzes sterol esters and triglycerides, releasing free sterols and converting the bulk lipid fraction into water-soluble soaps. Following saponification, the unsaponifiable fraction is isolated by liquid–liquid extraction (LLE), commonly using non-polar solvents such as hexane or diethyl ether. This step often requires multiple extraction cycles, contributing to long processing times, high solvent consumption, and increased variability. Purification of the unsaponifiable matter is then required to remove co-extracted interferences prior to chromatographic analysis. Thin-layer chromatography (TLC) remains the reference approach in official methods, despite being labor-intensive and poorly suited to routine, high-throughput analysis. Official sterol determination methods, such as those established by the IOC [1], are well recognized for their reliability and regulatory acceptance; however, they are also characterized by lengthy and labor-intensive procedures and by the use of large volumes of organic solvents.

Over the years, a variety of analytical workflows have been proposed with the aim of simplifying the sterol preparation procedure while preserving the analytical performance and reliability of official reference methods, such as the IOC protocol.

In this context, Mascrez et al. [2] introduced microwave-assisted saponification (MAS) to significantly shorten reaction times, followed by conventional liquid–liquid extraction and purification of the unsaponifiable fraction by silica-based SPE. Although effective, the method retained multiple steps for the unsaponifable extraction. Moreover, Mascrez have optimized an SPE fractionation for the simultaneous determination of additional analytes (i.e., dialkyketones), neglecting the presence of interferences that hindered the accurate quantification of a few sterols. Gorassini et al. [3] proposed an alternative strategy based on reduced sample size and SPE-based extraction and isolation of the unsaponifiable fraction, partially replacing TLC; however, the workflow still relied on oven-assisted saponification, multiple conditioning and washing steps, and relatively high solvent consumption. Mathison et al. [4] further streamlined sample handling by employing silica SPE for purification, achieving good reproducibility and equivalence with the IOC method, albeit through a multi-step protocol involving long extraction, washing, and large volumes of organic solvents.

Although these approaches demonstrate that individual steps of the sterols preparation procedure can be optimized, none of the proposed workflows fully achieves the level of simplification, integration, and operational efficiency required for routine, high-throughput laboratories, while maintaining strict equivalence with official methods. Hyphenated LC-GC-FID methods have also been proposed over the year, and they are for sure the most efficient and robust method. Nevertheless, after the publication of the work of Mascrez et al. [2], we received a request from a boarder control laboratory. The person responsible for the lab highly appreciated the rapidity and

simplicity of the method, but highlighted the necessity of a complete purification from the coeluting interferences as they hindered the quantification of critical sterols for the authentication assessment of olive oil. They considered the use of an LC-GC-FID as an investment not affordable for them. Therefore, the objective of the present work was to develop a sterol preparation workflow specifically tailored to routine analytical laboratories with limited economical availability, capable of delivering IOC-equivalent results through a reduced number of steps, shorter processing times, and lower solvent consumption. To this end, microwave-assisted saponification was combined with an integrated unsaponifiable extraction and SPE-based purification strategy, with the goal of improving robustness, reproducibility, and greenness without compromising regulatory compliance.

A central element of the proposed method is the integration of saponification and extraction within a single sealed microwave vessel. This configuration minimizes solvent losses, reduces sample handling, and limits the risk of contamination or analyte loss. Accordingly, the extraction solvent and saponification mixture were added in the same vessel to enable simultaneous microwave-assisted saponification and extraction (MASE). In parallel, SPE was optimized to replace the TLC-based purification step employed in the IOC method. Compared with TLC, SPE offers markedly lower solvent consumption and easier implementation in routine laboratories, making it a more suitable purification strategy for high-throughput sterol analysis.

The IOC method was adopted as the reference procedure against which the proposed method was consistently evaluated. In parallel, the studies by Mascrez [2], Gorassini [3], and Mathison [4], discussed in detail below, provided both conceptual inspiration and a solid methodological foundation for the optimization strategy pursued in this work. In the following sections, these studies are outlined, and their respective strengths and limitations are critically examined, thereby establishing the context for the subsequent optimization steps that ultimately led to the development of the final methodology presented herein.

2.Materials and Methods

2.1 Official IOC Method for Sterol Determination

The reference method for our workflow was the method of the International Olive Council (IOC) [1], used to determine sterol composition and content. Two internal standards, α-cholestanol and betulin, were added to a flask at a final concentration of 50 mg/kg and evaporated under a gentle nitrogen stream.

Five grams of olive oil and 50 mL of 2 M KOH in EtOH/H_2O (80/20, v/v) were added to the flask. The mixture was boiled for 20 minutes. After cooling, the saponified mixture was transferred to a separatory funnel and diluted with 50 mL of ultrapure water.

The unsaponifiable fraction was extracted by liquid–liquid extraction (LLE) using three aliquots of Et_2O (80, 60, and 60 mL). The combined organic phases were washed repeatedly with 50 mL of water until neutrality was achieved, confirmed using phenolphthalein as a pH indicator (total wash volume: 200 mL). The ether fraction was filtered over anhydrous Na_2SO_4 and evaporated to dryness using a rotary evaporator.

The dried extract was dissolved in 1 mL of hexane; 300 µL were loaded on a TLC plate previously basified with 0.2 M KOH in EtOH/H_2O 98/2, v/v. Development was carried out using Hexane/Et_2O (65/35, v/v). The plate was sprayed with 2,7-dichlorofluorescein solution, and the sterol band was scraped, eluted with 10 mL of hot ethyl acetate, and filtered. The residue was washed three times with 10 mL of ethyl acetate.

The extract was derivatized with BSTFA and pyridine at room temperature for 15 minutes. After evaporation, the residue (the trimethylsilyl ethers) was dissolved in 1 mL of hexane and injected into a GC-FID system.

2.2 Mascrez's method

In the method optimized by Mascrez et al. [2], two internal standards (α-cholestanol and betulin) were added directly into microwave vessels at 50 mg/kg and evaporated under nitrogen.

Then, 2.5 g of sample and 25 mL of 2 M KOH in EtOH/H_2O 80/20 v/v were added. Saponification was performed in a microwave system at 90 °C for 10 minutes.

After cooling, the unsaponifiable fraction was extracted with LLE using Et_2O (40, 30, 30 mL) following the same washing procedure as in the IOC method. The hexane extract was filtered over Na_2SO_4, evaporated, and prepared for purification using a laboratory-packed SPE cartridge.

A 6 mL cartridge was packed with 1 g silica gel (60 Å, 70–230 mesh) and conditioned with 6 mL hexane. The extract dissolved in 1 mL hexane was loaded onto the cartridge.

Since this method also targeted dialkyl ketones (DAK), two washing and two elution steps were used:

- Wash 1: 5 mL Hexane/Et_2O (98/2, v/v)
- Elution 1 (DAK fraction): 5 mL Hexane/Et_2O (96/4, v/v)
- Wash 2: 25 mL Hexane/Et_2O (95/5, v/v)
- Elution 2 (sterol fraction): 7 mL Hexane /Et_2O (30/70, v/v)

The sterol eluate was evaporated, derivatized with BSTFA and pyridine at room temperature for 15 minutes, evaporated again, dissolved in 1 mL hexane, and analyzed by GC-FID.

2.3 Gorassini's method [3]

In the method developed by Gorassini et al. [3], α-cholestanol was added to a 20 mL vial (50 mg/kg) and evaporated under nitrogen. Then, 0.25 g of olive oil and 2.5 mL

of 2 M KOH in MeOH were added. Saponification was carried out in an oven at 70 °C for 60 minutes.

The saponified mixture was diluted with MeOH/H_2O (80/20, v/v) and loaded onto a StrataX SPE column previously conditioned with 5 mL MeOH and 5 mL MeOH/H_2O (80/20, v/v).

The column was washed with 10 mL of the same mixture and dried for 30 minutes under an air stream.

The unsaponifiable fraction was dissolved in 1 mL n-hexane and loaded onto a 3 mL cartridge packed with 0.5 g of silica scraped from a TLC plate. Conditioning was performed with:

- 2.5 mL hexane
- 0.25 mL 2,2-diethoxypropane
- 0.25 mL glacial acetic acid

followed by washing with 10 mL hexane.

After sample loading, the column was washed with 45 mL C6/Et_2O (95/5, v/v). Sterols were eluted with 5 mL C6/Et_2O (30/70, v/v), evaporated to dryness, derivatized with BSTFA and pyridine, and analyzed by GC-FID.

2.4 Mathison's method [4]

Sample preparation was performed by adding 40 µg of internal standard (IS) to each screw-capped test tube, followed by complete solvent evaporation under a gentle stream of nitrogen. An aliquot of approximately 0.200 g of oil (220 µL) was then introduced into the tube, and the exact mass recorded to the nearest 0.1 mg. Saponification was carried out by adding 2 mL of 2 N ethanolic KOH, capping the tubes, and heating them in an oven at 80 °C for 40 minutes. Upon completion of the reaction, 18 mL of water was added, and the tubes were gently inverted to ensure homogenization of the saponified mixture.

The unsaponifiable fraction was isolated by loading each saponified sample onto a diatomaceous earth cartridge (Chem Elut, 20 mL). After a 15-minute absorption period, the unsaponifiable matter was eluted with 60 mL of diethyl ether, and the eluate was passed through approximately 5 g of anhydrous sodium sulfate to remove residual moisture. The solvent was then evaporated to dryness under nitrogen, and the residue was reconstituted in 5 mL of hexane for immediate purification on silica cartridges.

Purification of 4-desmethylsterols and triterpenic diols was carried out using silica solid-phase extraction cartridges (Agilent Mega BE-1, 1 g, 6 mL), which were conditioned with 10 mL of hexane. Each cartridge was pre-treated by loading 1 mL of 0.2 N ethanolic KOH, immediately followed by a 5 mL hexane wash. The sample extract was then applied to the cartridge, which was washed with 100 mL of hexane/diethyl ether (98:2, v/v) to remove interfering components. The sterol and diol fraction was subsequently eluted with 10 mL of hexane/diethyl ether (60:40, v/v).

The collected eluate was evaporated to dryness under nitrogen, reconstituted in approximately 1 mL of acetone, evaporated again, and heated at 100 °C for 10 minutes to ensure complete removal of residual solvent. Derivatization was performed with 250 µL of pyridine/BSTFA (3:1, v/v), and the reaction mixture was heated at 80 °C for 15 minutes prior to GC–FID analysis. Chromatographic conditions and quantification procedures were identical to those employed for the IOC reference method.

2.5 GC-FID conditions

GC–FID analyses were performed using a Thermo Fisher Trace Ultra 1300 gas chromatograph equipped with an Optima-5-Accent capillary column (30 m × 0.25 mm i.d. × 0.25 µm film thickness). The GC operating conditions were as follows: injector temperature set to 300 °C, injection volume of 1.0 µL in split mode with a split ratio of 1:10, and helium used as carrier gas at a constant flow rate of 1.3 mL min^{-1}. The oven temperature program started at 80 °C, increased to 160 °C at 20 °C min^{-1}, then ramped to 340 °C at 5 °C min^{-1}, and held at 340 °C for 1 min.

The flame ionization detector (FID) was operated under the following conditions: data acquisition frequency of 50 Hz, detector temperature of 350 °C, air flow rate of 350 mL min^{-1}, hydrogen flow rate of 35 mL min^{-1}, and makeup gas flow rate of 30 mL min^{-1}. Quantification of sterols performed using the internal standard method, with α-cholestanol employed as the internal standard.

2.6 GC-MS conditions

For identification purposes, selected analyses were performed using a Shimadzu GCMS-TQ8050 NX system, consisting of a GC-2030 gas chromatograph equipped with an AOC-20i autosampler. Separations were carried out on an SLB-5MS capillary column (10 m × 0.10 mm i.d. × 0.10 µm film thickness). The GC–FID method was translated with EZGC methos translator of Restek and adapted to the GC–MS system in order to achieve chromatographic separation comparable to that obtained under GC–FID conditions.

The GC operating parameters were as follows: injector temperature set at 300 °C, injection volume of 1.0 µL in split mode with a split ratio of 1:35, and helium used as carrier gas at a constant flow rate of 0.61 mL min^{-1}. The oven temperature program started at 80 °C, increased to 160 °C at 20 °C min^{-1}, then ramped to 340 °C at 5 °C min^{-1} and held at 340 °C for 1 min.

Ionization was performed by electron impact (EI). The ion source temperature was set at 200 °C, and the interface temperature was maintained at 280 °C. The detector voltage was set according to the tuning file. Data were acquired in full scan mode over an m/z range of 50–700, with an acquisition frequency of 10 Hz.

3.Experimental Design for Microwave-Assisted Saponification and Extraction Optimization

To optimize the MASE, a central composite design (CCD) with two variables (k = 2) and $\alpha = 1/\sqrt{k}$ was applied. The variables were time (explored in a range of 10-30 minutes) and temperature (explored in a range of 60–140 °C) of simultaneous saponification and extraction.

A total of nine experimental points (Figure 1) were tested:

- 1 center point (performed in quadruplicate),
- 4 factorial points (single replicates),
- 4 axial points (duplicate).

Experiments were randomized to minimize uncontrolled variability. The mass of the residue (mg) obtained after saponification, extraction, and washing was used as the response variable in the response surface methodology.

Final conditions were selected to achieve a residue comparable to those obtained with the official IOC method.

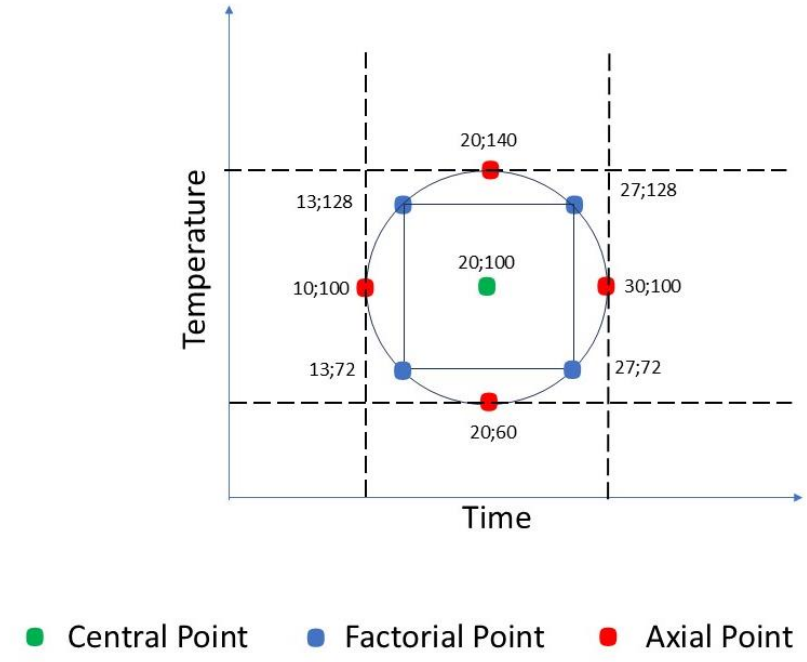


Figure 1. Central composite design (CCD) points.

4.Results and discussion

4.1 Optimization strategy

The development of the proposed workflow followed a two-step approach aimed at simplifying the sterol preparation procedure while maintaining equivalence with the IOC reference method.

The first objective was to reduce saponification time and integrate the liquid–liquid extraction step by exploiting microwave irradiation in a closed-vessel system and enabling simultaneous saponification and extraction.

The second objective was to replace the TLC-based purification step with a SPE protocol, selected for its lower solvent consumption, and improved suitability for routine, high-throughput laboratory analysis.

The IOC method [1] was adopted as the reference procedure for sterol determination, serving as the regulatory benchmark against which all workflow modifications were evaluated. The method developed by Mascrez et al. [2] was selected as the starting point for optimizing both the saponification and purification steps, as it streamlines several critical phases of the IOC procedure and offers a faster and more reproducible alternative. The workflows proposed by Gorassini [3] and Mathison [4] were used as sources of inspiration for further improving the purification stage, providing valuable insights into solvent reduction, procedural simplification, and enhanced standardization of SPE-based clean-up strategies.

4.2 Optimization of MASE

Under alkaline conditions, triglycerides (which represent ~98% of the total sample) are hydrolyzed, forming hydrophylic prod, whilst free and esterified sterols are transferred into the unsaponifiable non-polar fraction, which can be efficiently extracted in an organic solvent. Exploiting the closed-vessel configuration of the microwave system allows the saponification reaction and the partitioning of sterols into the organic phase to occur simultaneously, without solvent losses or safety concerns associated with volatile solvents. As a preliminary step, the composition of the saponification medium KOH was intentionally modified from the EtOH/H_2O (80:20, v/v) mixture employed in IOC method to an EtOH/H_2O (50:50, v/v) system. This modification was motivated by two complementary considerations. First, it allowed harmonization of the saponification procedure with one used for the analysis of mineral oil saturated hydrocarbons (MOSH) and mineral oil aromatic hydrocarbons (MOAH) performed in the lab, enabling the use of a unified sample preparation protocol in routine laboratory practice. Secondly, using a higher water content is inherently advantageous in terms of safety, cost, and sustainability, reducing ethanol consumption while maintaining effective alkaline conditions.

However, changing the ratio of EtOH/H_2O in the saponification medium and adding hexane in the same vessel significantly influenced the reaction. This change, therefore, necessitated a re-optimization of the saponification and extraction time and

temperature, providing the rationale for the following systematic optimization strategy. Application of Response Surface Methodology (RSM) enabled modelling of the relationship between residue and the independent variables (time and temperature), incorporating linear, quadratic, and interaction terms (Figure 2A). The high R-squared value (0.9215) and the non-significant lack of fit ($p < 0.001$) indicate that the model adequately captures the variability of the data. As illustrated in the Pareto chart (Figure 2B), temperature—both in its linear (B) and quadratic (BB) components—has a significant impact on the residue, while time exerts a comparatively minor effect within the explored range. The interaction term (AB) was not significant, indicating that the effect of temperature on residue is independent of time.

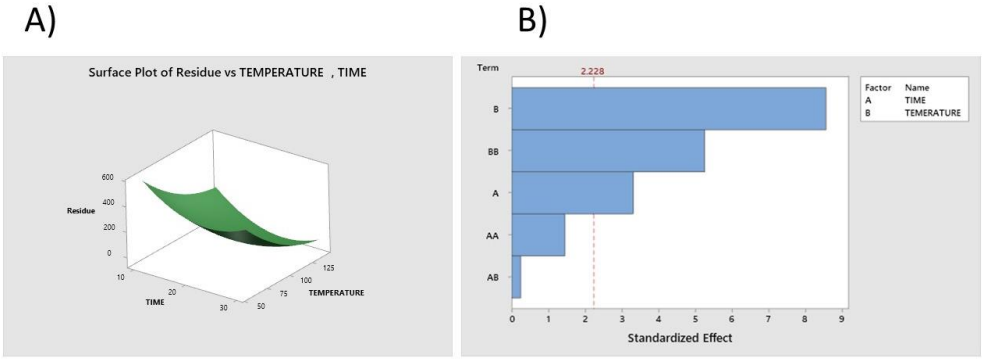


Figure 2. Response Surface Methodology (RSM) results: (A) response surface plot; (B) Pareto chart illustrating the relative influence of the investigated factors.

The model identified approximately 120 °C as the temperature associated with the lowest residue, with no further improvements observed at higher temperatures. The optimal condition was therefore selected as the one producing a residue closest to that obtained with the reference method, namely 120 °C for 20 minutes. Olive oil samples were processed in triplicate under these conditions, yielding an average residue of 13±2 mg, before undergoing the purification step. To isolate the effect of modifying only the saponification and extraction phase, the subsequent purification step was performed according to the official method, employing TLC as discussed before. The results obtained by altering the saponification step solely were quantitatively compared with the results obtained with the IOC method (Figure 3), and the quantification of all sterols was found to be comparable, as confirmed by a *t*-test for each sterol ($p > 0.05$).

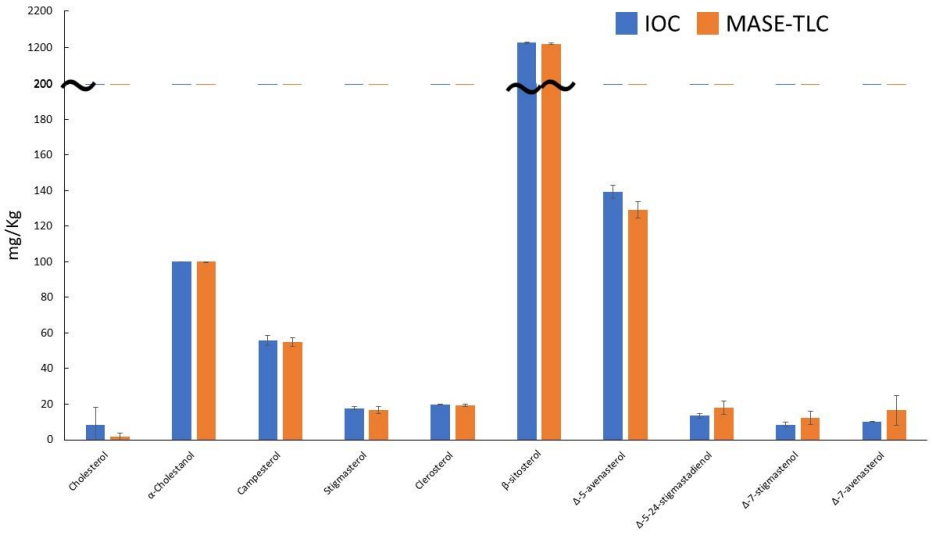


Figure 3. Comparison of IOC saponification and extraction method (blue), and MASE (orange).

4.3 Optimization of SPE procedure

Once the MASE step was optimized, the next step was to optimize the purification step. The optimization of the SPE procedure is addressed in the following section, with specific emphasis on selecting the most suitable stationary phase and optimizing solvent composition and volume. As a starting point, previously established methodologies developed by Mascrez et al.[2], by Gorassini et al. [3], and Mathison [4], as described in the Materials and Methods section, were adopted as reference or inspiration protocols.

Gorassini et al. [3] method involves the use of a 3 mL cartridge packed with silica scraped from a TLC plate. However, several limitations were encountered when applying this procedure. In particular, difficulties packing the scraped silica led to inconsistent elution behavior and required the application of negative pressure to facilitate solvent flow. Despite this, the overall purification step required approximately two hours to be completed. These drawbacks led to poor repeatability in the results. The lack of repeatability was mainly attributed to the challenges in achieving uniform packing. Further optimization of the purification step using this type of silica was deemed impractical.

In contrast, the Mascrez's method [2], which employs a commercially available silica gel (60 Å, 70–230 mesh, 63–200 μm) packed into a 6 mL cartridge, did not present any significant handling or operational issues. Consequently, this stationary phase was

selected as the basis for subsequent method optimization, with the focus placed on refining the elution conditions.

The SPE elution protocol used in Mascrez et al [2], included the elution of a dialkyl keton (DAK) fraction before the sterol one, which is not of interest in this work. Moreover, it failed to remove two 4,4′-dimethylsterols and one 4-monomethyl sterol, specifically cycloartenol, 24-methylenecycloartenol, and citrostadienol. Their presence (Figure 4) results in partial overlap with sterol and triterpene dialcohol signals, thereby interfering with the accurate quantification of several sterol components. In particular, cycloartenol is coeluting with the sterol Δ-7- stigmasterol and citrostadienol is coeluting with the triterpene dialchol erytrodiol. This observation highlighted the need to further refine the purification strategy. To rationally guide the optimization process, structural differences between sterols and interferences were taken into account. In contrast to sterols, cycloartenol, 24-methylenecycloartenol, and citrostadienol possess additional methyl substituents at the C4 position, resulting in a lower overall molecular polarity. These differences were exploited to modulate the polarity and strength of the washing solvent, with the aim of selectively eluting interfering compounds while retaining sterols on the stationary phase.

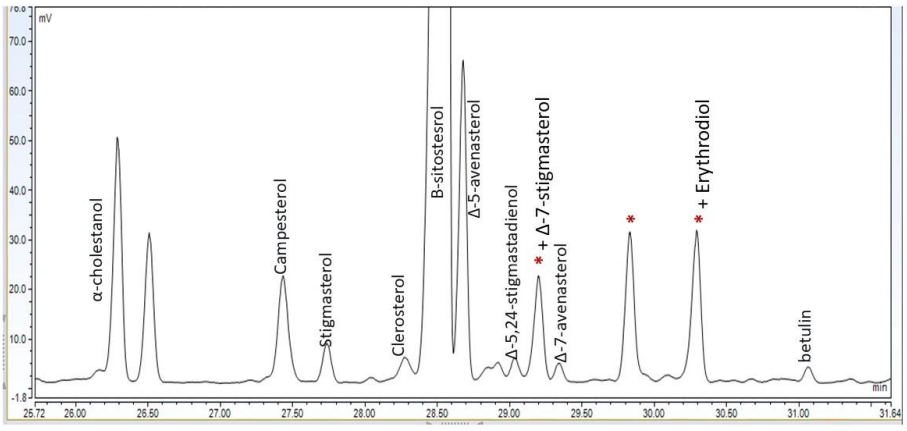


Figure 4. Presence of Interferences in the sterol fraction obtained with Mascrez's SPE method.

4.4 Study of the elution of sterols and interferences.

Starting from the Mascrez's method [2], the SPE procedure was optimized by eliminating the elution step dedicated to the DAK fraction and increasing the greenness of the method. To achieve this modification without compromising separation efficiency and to ensure effective removal of undesired compounds during

the washing step, the composition of the solvent mixture was adjusted. Specifically, n-hexane was systematically replaced with cyclohexane, a solvent with a more favorable environmental profile and higher elution strength. This substitution was expected to enhance washing efficiency, potentially allowing the removal of undesired compounds without the need for additional elution steps. Different cyclohexane/diethyl ether mixtures (Cyclo/Et_2O 75/5, 85/15, 90/10, 95/5 v/v) were therefore investigated for the washing step, while sterol elution was consistently performed using a CyHex/Et_2O (30/70, v/v) mixture. In all experiments, collected fractions were subdivided in smaller sections (es 20 ml in 4 fraction of 5 mL) and analyzed by GC–FID in order to monitor the elution behavior of sterols and interferences. Progressive modulation of the washing solvent polarity demonstrated that less polar mixtures improved selectivity, as evidenced by increasingly differentiated elution profiles (Figure 5). The mixture 75/25 Cyclo/Et_2O v/v is too strong and elutes too fast sterols together with interferences. Decreasing the polarity is possible to see a minimal improvement of the separation between sterols and interferences, to arrive at the condition 95/5 Cyclo/Et_2O v/v were they are almost completely separated. However not enough to consider the Cyclo/Et_2O 95/5 v/v mixture the optimal one.

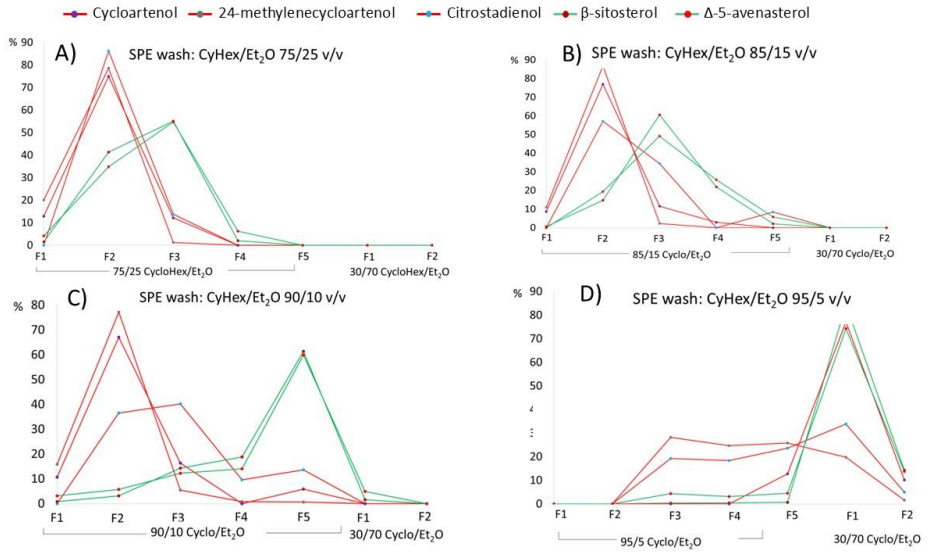


Figure 5. Elution of sterols and interferences using different washing mixture. A) CycloHex/Et_2O 75/25 v/v; B) CycloHex/Et_2O 85/15 v/v; C) CycloHex/Et_2O 90/10 v/v; D) CycloHex/Et_2O 95/5 v/v.

4.5 Basification of silica

The subsequent optimization step was inspired by the approach proposed by Mathison, which involves basifying the silica prior to the SPE procedure. Under these conditions, sterols are efficiently separated from interfering compounds, owing to modified surface interactions between the analytes and the stationary phase. While this strategy proved effective in improving selectivity, it presents a major drawback in terms of analytical sustainability. In particular, the method requires approximately 100 mL of n-hexane/Et_2O for the washing step and significantly longer elution times, which directly conflicts with the objectives of developing a rapid method with reduced solvent consumption. Consequently, silica basification was retained as a strategy to improve selectivity toward sterols, while efforts were directed at substantially reducing solvent consumption. To achieve this, the optimization focused on increasing the elution strength of the washing solvent, with the aim of maintaining effective removal of interfering compounds while minimizing the required solvent volumes. In line with this approach, n-hexane was replaced with cyclohexane, whose higher elution strength was expected to allow more efficient washing and elution using reduced solvent volumes.

The optimization was guided by a quantitative evaluation of elution strength. The elution power of the reference washing solvent (100 mL of hexane/diethyl ether, 98/2, v/v) was estimated and used as a benchmark to calculate an equivalent washing condition based on cyclohexane, exploiting its higher elution strength compared with n-hexane. On this basis, it was determined that 40 mL of a cyclohexane/Et_2O (98/2, v/v) mixture provides an elution strength comparable to that of the reference hexane-based system.

Accordingly, the SPE procedure was performed using basified silica, with a washing step consisting of 40 mL of cyclohexane/ Et_2O (98/2, v/v), followed by sterol elution with 7 mL of cyclohexane/ Et_2O (30/70, v/v). Under these conditions, an effective and well-defined separation between interfering compounds, sterols, and triterpenic diols was achieved, demonstrating that the combination of silica basification and solvent-strength optimization allows a substantial reduction in solvent consumption (Figure 6).

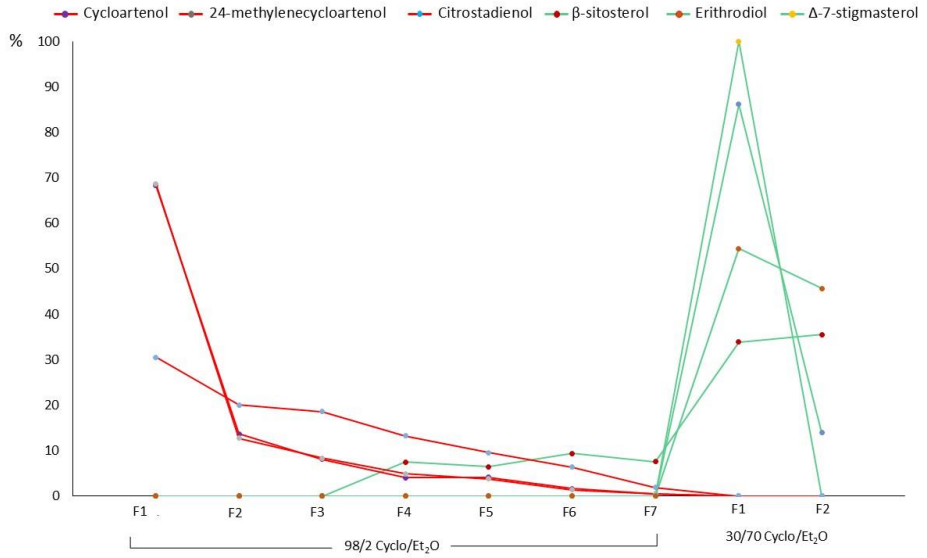


Figure 6. Elution of sterols, triterpene dialchols, and interferences with SPE with basification of silica. Washing with 40 ml of Cylohex/Et_2O 98/2 v/v and elution with 7 ml of Cylohex/Et_2O 30/70 v/v.

Although the optimized SPE protocol based on basified silica and reduced solvent volumes demonstrated a clear and effective separation between interfering compounds, sterols, and triterpenic dialchols, the quantitative results indicate that full analytical equivalence with the reference method was not achieved for all individual sterols under the investigated conditions. As shown in Figure 7, deviations in the quantified amounts were observed for specific compounds, suggesting that recovery and/or elution efficiency may still be influenced by the simplified washing and elution scheme.

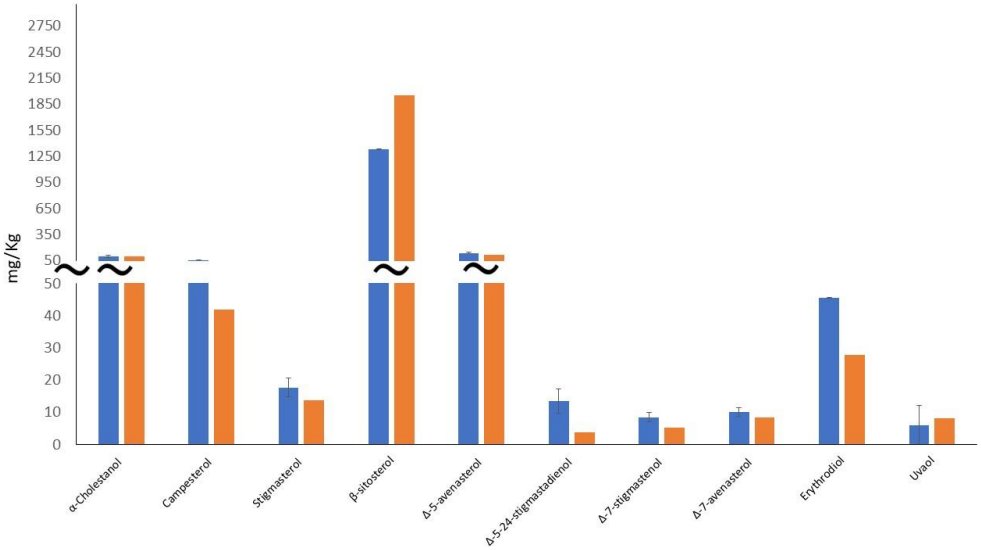


Figure 7. Comparison of IOC method (blue) and the proposed MASE-SPE method (orange).

Further experiments aimed at fine-tuning solvent composition, volumes, and fractionation would represent a logical continuation of this work and are expected to further improve quantitative agreement with the reference method. However, the data presented here provide a robust foundation for method implementation and support the conclusion of this chapter.

4.6 Description of the final proposed method

α-Cholestanol and betulin were added to the microwave vessel at a final concentration of 50 mg/kg and evaporated under nitrogen.
One gram of olive oil, 10 mL of 2 M KOH in EtOH/H_2O (50/50, v/v) and 10 mL of n-hexane were added.
Saponification and extraction were performed simultaneously using a Synthwave microwave reactor (Milestone S.r.l., Italy), equipped with a 5-position rack. Temperature was ramped to 120 °C within 2 minutes and held for 20 minutes under continuous stirring.
A nitrogen pressure of 35 bar was applied to seal the vessels and maintain an inert atmosphere.
After cooling, the hexane phase was recovered and washed until neutrality (phenolphthalein indicator). The organic phase was dried over anhydrous Na_2SO_4, filtered, evaporated to dryness, and reconstituted in 1 mL n-hexane for SPE purification.
A 6 mL cartridge was dry-packed with 1 g silica gel using CyHex as packing solvent. Conditioning was performed with 6 mL CyHex. The silica was basified with 1mL of

0.2 M KOH/EtOH.
The extract in 1 mL CyHex was loaded, washed with 40 mL of CyHex/Et_2O 98/2 v/v and sterols were eluted with CyHex/Et_2O 30/70, v/v.
The eluate was evaporated, derivatized with 150 µL BSTFA and 150 µL pyridine (15 min, room temperature), evaporated again, dissolved in CyHex, and analyzed by GC-FID.

4.7 Evaluation of the greenness and whiteness of the method

The greenness of the analytical workflows was evaluated using the AGREEprep metric [5], which provides an assessment of sample preparation procedures based on principles of green analytical chemistry. The evaluation was performed separately for the saponification/extraction step, the purification step, and the complete method, allowing a comparative analysis of the IOC reference procedure, selected literature approaches, and the proposed method.
As shown in Figure 9, the IOC method [1] exhibits consistently low AGREEprep scores, mainly due to the use of conventional reflux saponification, extensive manual handling, and large volumes of organic solvents. These characteristics led to high energy consumption, low throughput, and increased safety concerns related to prolonged heating in open systems. In contrast, the introduction of microwave-assisted saponification leads to a clear improvement in the greenness profile. Both the Mascrez's protocol [2] and the proposed MASE approach benefit from reduced reaction times, improved energy efficiency, and the use of closed vessels, which minimize solvent losses and operational risks. Notably, the proposed method achieves the highest score in the saponification and extraction stage, reflecting the further integration of extraction into the microwave-assisted process. A similar trend is observed for the purification step. The IOC method, based on TLC, is penalized by high solvent consumption and labor-intensive handling. SPE-based purification strategies consistently show higher AGREEprep scores, confirming the advantage of replacing TLC with cartridge-based approaches. Among these, the proposed method achieves the highest score, owing to the combined reduction in solvent volumes and simplification of the purification scheme, while maintaining effective separation performance. When considering the complete workflows, the proposed method shows a substantially improved greenness profile compared with the IOC reference method. In particular, the most significant color improvements are observed for Criterion 5 (sample size economy) and Criterion 6 (sample throughput). Criterion 5 reflects the reduction of sample mass from 5 g in the official IOC method to 1 g in the proposed workflow, contributing to lower material consumption. However, Criterion 6 represents the most impactful improvement from an operational perspective and is of paramount importance for routine analytical laboratories. In the case of the IOC method, the overall duration and complexity of the procedure make it difficult to complete a full analysis within a single working day, effectively resulting in a throughput of less than 0.5 samples per hour. This limitation severely constrains

laboratory productivity, particularly in contexts where multiple samples must be processed daily for quality control, regulatory compliance, or authenticity assessment. In contrast, the proposed MASE–SPE workflow dramatically increases sample throughput by integrating and accelerating the most time-consuming steps of the procedure. Microwave-assisted saponification and extraction significantly shorten reaction times, while SPE purification replaces labor-intensive TLC and minimizes manual handling. As a result, approximately 12 samples can be processed per hour under routine conditions. This improvement is not merely incremental, but transformative, enabling same-day analysis of large sample batches and rapid turnaround of results. Within this framework, Criterion 6 clearly highlights one of the main strengths of the proposed method, demonstrating its strong suitability for routine

Although the overall greenness scores taken as absolute value may suggest that the method does not excel in terms of greenness, it is essential to emphasize several key aspects that must be taken into account for an informed interpretation of these results. The metric does not adequately differentiate between substantially different absolute values, as exemplified by criterion 4 (waste), which assigns similar scores to methods generating vastly different solvent volumes. In fact, the IOC method generates 1400 mL of waste, while the proposed method generates only 120 mL of waste (11.7-folds less). With the proposed method, the waste is reduced of around 90% and still gets the same AGREEprep score. The same holds for other criteria, like criterion 2 (Hazardous materials), where we have 1200 mL for IOC versus 65 mL of the proposed. Moreover, sterol analysis inherently requires derivatization and the use of specific organic solvents to ensure analytical performance, making the complete elimination of certain penalizing factors unrealistic. Consequently, the absolute AGREEprep scores should not be interpreted in absolute but rather used for comparative purposes in a relative way.

Moreover, at first glance, it may appear counterintuitive that the AGREEprep score of the complete proposed method is lower than that obtained for the MASE step alone. This outcome reflects the cumulative contribution of all sample preparation steps. As shown in Figure 8, the microwave-assisted saponification and extraction (MASE) step achieves a relatively high score due to reduced reaction time, lower energy consumption, and improved throughput. In contrast, the subsequent SPE purification step, although significantly greener than TLC, still involve solvent use and additional handling steps, resulting in a comparatively lower individual score. For this reason, when the complete workflow is evaluated, the overall AGREEprep score represents an integration of the scores of the individual steps, and so the inclusion of the SPE purification step reasonably lowers the final score compared with MASE alone.

Overall, the AGREEprep assessment confirms that the proposed workflow represents a significant improvement in terms of environmental sustainability, efficiency, and suitability for routine application compared with the IOC reference method. While intrinsic constraints in sterol analysis limit the achievable greenness, the combined use of microwave-assisted saponification and SPE purification clearly advances the method toward a more sustainable and high-throughput analytical solution.

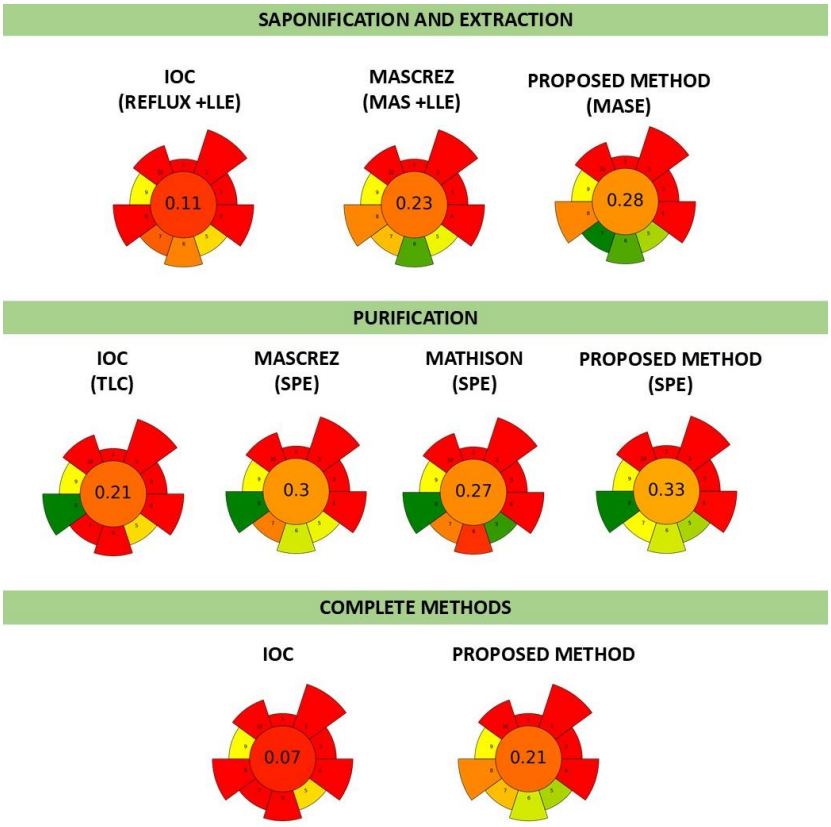
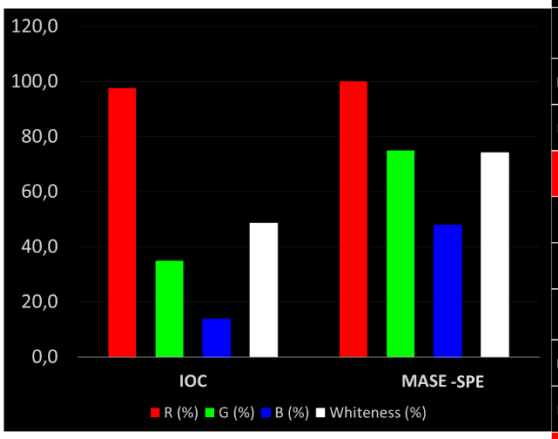


Figure 8. AGREEprep pictogram for saponification/extraction step, purification step and entire methods.

The IOC method and the proposed one were further evaluated in accordance with the principles of White Analytical Chemistry (WAC) [6]. As shown in Figure 9, both the IOC reference method and the proposed MASE-based workflow exhibit high red scores with a minimal difference, indicating that analytical requirements for sterol determination are fulfilled in both cases. This confirms that the proposed method does not compromise analytical reliability despite the substantial simplification of the workflow. However, pronounced differences are observed in the green and blue components. The proposed method shows a markedly higher green contribution, in agreement with the AGREEprep evaluation, reflecting the significant reduction in solvent consumption, waste generation, and energy demand achieved through microwave-assisted saponification and SPE purification. In parallel, the blue component is substantially enhanced, highlighting improvements in throughput, automation, and overall operational efficiency, all of which are essential in routine laboratories that require rapid turnaround times.



Method: IOC					
R1: Scope of application	100,0	G1: Toxicity of reagents	50,0	B1: Cost-efficiency	40
R2: LOD and LOQ	90,0	G2: Amount of reagents and waste	10,0	B2: Time-efficiency	10,0
R3: Precision	100,0	G3: Energy and other media	10,0	B3: Requirements	5
R4: Accuracy	100,0	G4: Direct impacts	70	B4: Operational simplicity	0,0
97,5		35		13,8	

Method: MASE-SPE					
R1: Scope of application	100,0	G1: Toxicity of reagents	50,0	B1: Cost-efficiency	30
R2: LOD and LOQ	100	G2: Amount of reagents and waste	80	B2: Time-efficiency	85
R3: Precision	100,0	G3: Energy and other media	80	B3: Requirements	10
R4: Accuracy	100,0	G4: Direct impacts	90	B4: Operational simplicity	66,7
100		75		47,9	

Figure 9. Comparison of whiteness between IOC and MASE-SPE proposed method with relative scores.

As a result of the more balanced contribution of the three dimensions, the overall whiteness index of the proposed method is significantly higher than that of the IOC reference procedure. In contrast, the IOC method, although analytically robust, is penalized by low green and blue contributions, resulting in a lower whiteness value. This integrated evaluation demonstrates that the proposed workflow achieves a more harmonized balance between analytical performance, sustainability, and practicality, in line with the principles of WAC. Overall, the whiteness assessment complements the greenness evaluation by providing a comprehensive perspective on method suitability. Within this framework, the proposed MASE–SPE workflow emerges as a more balanced and fit-for-purpose solution for sterol analysis, particularly in routine and high-throughput analytical contexts, where environmental considerations must be addressed alongside performance and efficiency requirements.

5.Conclusions

In this chapter, an optimized analytical workflow for sterol determination was developed to improve efficiency, robustness, and sustainability while maintaining quantitative performance compatible with routine and regulatory requirements. The work focused on the critical steps of saponification, extraction, and purification, which account for most of the time consumption, solvent use, and variability in conventional sterol analysis. Microwave-assisted saponification and extraction (MASE) was successfully implemented, significantly reducing reaction time and energy demand, thereby the overall cost, compared with the IOC reference method.

The modification of the saponification medium to an EtOH/H_2O (50:50, v/v) system, motivated by the need to harmonize sterol analysis with MOSH/MOAH workflows and to improve sustainability, required a dedicated re-optimization of reaction conditions. The systematic optimization demonstrated that efficient saponification and simultaneous extraction could be achieved under microwave irradiation, enabling a robust and integrated sample preparation step. In parallel, the traditional TLC-based purification was replaced by a SPE protocol specifically optimized for sterol analysis. The use of SPE, combined with solvent-strength modulation and silica basification, allowed effective separation of sterols from interfering compounds while substantially reducing solvent consumption. Although the method needs still some minor refining and full validation, the method still provides a reliable sterol profile suitable for routine analysis. The greenness evaluation of the developed workflow confirmed a marked improvement over the IOC reference method. In particular, solvent consumption was reduced by more than 90%, throughput was increased by an order of magnitude, and operator safety was improved through the use of closed microwave vessels and simplified handling. Beyond greenness, the application of the White Analytical Chemistry framework further demonstrated that the proposed method achieves a more balanced compromise among analytical performance, environmental sustainability, and practical efficiency than the official procedure. The higher whiteness score reflects not only improved sustainability but also enhanced suitability for high-throughput routine laboratories, without compromising analytical reliability. Overall, the results presented in this chapter demonstrate that the combination of microwave-assisted saponification and SPE purification constitutes a viable, high-throughput, and more sustainable alternative to conventional sterol analysis. The developed workflow provides a solid foundation for further refinement and validation and is well suited for implementation in routine analytical laboratories requiring rapid and reliable sterol determination.

6.References

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Chapter 5

Prototype of a supercritical CO_2 MW extractor

1. Introduction to Supercritical CO_2 extraction

In recent years, increasing environmental awareness and stricter regulations on the use of hazardous solvents have driven the development of sustainable and eco-friendly extraction technologies. Among these, Supercritical Fluid Extraction (SFE) has been revitalized as a promising green alternative to conventional solvent-based extraction methods, owing to its high efficiency, selectivity, and reduced environmental impact.

Supercritical carbon dioxide (SC-CO_2) is the most widely employed solvent in SFE processes due to its highly advantageous physicochemical properties. CO_2 is non-toxic, non-flammable, non- corrosive, and easy to handle, allowing supercritical operation at relatively low pressures and near-ambient temperatures. These operation conditions make SC-CO_2 particularly suitable for the extraction of thermolabile and oxidation-sensitive compounds, minimizing degradation and preserving product quality.

From a solvent performance perspective, SC-CO2 exhibits a strong ability to solubilize lipophilic substances, while its tunable density enables selective extraction through controlled adjustment of pressure and temperature. An additional advantage lies in the ease of solvent removal: after depressurization, CO_2 returns to the gaseous state, leaving solvent-free extracts without the need for further purification steps. From an environmental and regulatory standpoint, supercritical CO_2 is recognized as a safe and sustainable solvent and is classified as "Generally Recognized as Safe" (GRAS). Consequently, SFE using SC-CO_2 has gained significant interest in food, pharmaceutical, nutraceutical, and cosmetic applications. Overall, the combination of process efficiency, product quality, and environmental compatibility positions supercritical CO_2 extraction as a key technology for developing sustainable extraction processes [1].

2. Supercritical conditions

When a substance is subjected to temperatures and pressures exceeding its critical temperature (Tc) and critical pressure (Pc), it exists as a single-phase, non-condensable fluid. In this supercritical region (Figure 1) the fluid exhibits physicochemical characteristics that are intermediate between those of gases and liquids, including relatively high density, enhanced diffusivity, and low viscosity and surface tension. Specifically, a supercritical fluid density approaches that of a liquid, while its viscosity and diffusivity are comparable to those of a gas. As a result, supercritical fluid can function as an effective liquid-like solvent while benefiting from enhanced mass transfer kinetics. Sometimes the polarity of the supercritical fluid can be enhanced by adding a miscible polar co-solvent, such as ethanol, commonly referred to as a modifier.

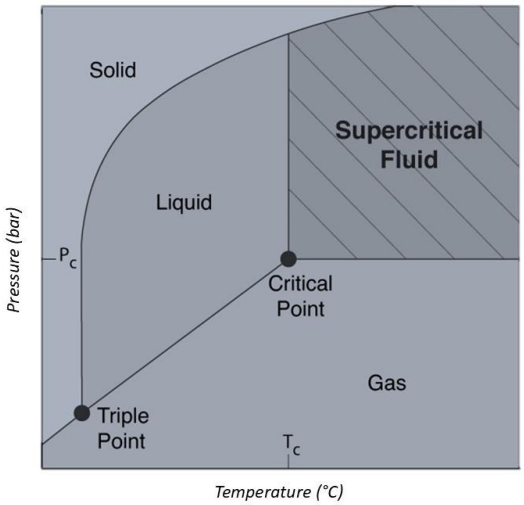

Figure 1. Phase diagram showing the solid, liquid, and gaseous regions, as well as the critical point and the supercritical fluid region.

In certain cases, effects comparable to those observed under supercritical conditions can be achieved at temperatures close to the critical point, where the substance exists in the liquid phase at pressures exceeding the critical pressure ($P > Pc$) and temperatures below the critical temperature ($T < Tc$), a regime commonly referred to as the subcritical state. Similar to conventional extraction methods, the efficiency of supercritical fluid extraction of target compounds is governed by multiple operational parameters, including the pretreatment of the raw material, particle size, extraction temperature and pressure, extraction time, solvent flow rate, and solvent-to-matrix ratio. These parameters collectively influence the extraction performance, which is typically evaluated in terms of extraction yield and recovery of the target compounds. Extraction yield is defined as the total amount of extract obtained per unit mass of initial feed material, whereas recovery represents the proportion of the target compound originally present in the feed that is successfully transferred into the extract [2].

3. Advantages of using CO_2 as supercritical fluid

As already mentioned, CO_2 is the most widely used extraction solvent due to its non-toxic, non-flammable, and non-corrosive nature, ease of handling, and ability to operate under mild pressure and temperature conditions. In addition, it is inexpensive, readily available at high purity, and preserves the bioactive compounds and their functional properties. Carbon dioxide reaches the supercritical state above its critical temperature (31.1 °C) and critical pressure (73.8 bar), conditions that are

readily achievable and particularly attractive for the extraction of thermolabile compounds.

A thorough understanding of solubility phenomena in supercritical CO_2 is essential for the rational design and optimization of extraction processes in food and natural product processing. The solubility of target compounds in SC-CO_2 is strongly influenced by operating temperature and pressure, as these variables directly affect the density and, consequently, the solvent strength of the supercritical fluid. Increasing pressure generally enhances CO_2 density toward liquid-like values, thereby promoting solute–solvent interactions and significantly improving solubility. Conversely, temperature exerts a dual effect: while higher temperatures reduce CO_2 density, it simultaneously increases the vapor pressure of the solute. Therefore, the overall solubility response is determined by the relative magnitude of these opposing effects. Near the critical region and at relatively low pressures, the density decrease dominates, leading to reduced solubility with increasing temperature. At higher pressures, however, the vapor pressure effect becomes predominant, resulting in an increase in solubility as temperature rises. This interplay gives rise to the characteristic crossover behavior observed in solubility isotherms of supercritical systems (the example of water is reported in Figure 2). In addition to process conditions, intrinsic solute properties such as molecular weight, polarity, and vapor pressure play a crucial role in determining solubility in SC-CO_2. Solute–solvent and solute–solute interactions, including hydrogen bonding, further influence extraction performance [2].

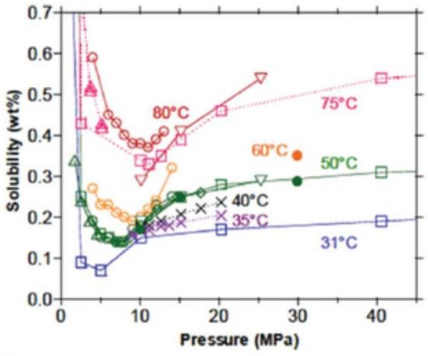

Figure 2. Solubility of water in supercritical CO_2 [3]

4. Apparatus

The components required for performing SFE in laboratory scale consist of a CO_2 (or a source of an alternative extraction fluid), a pump or a gas compressor, an oven or a temperature-controlled heating system, an extraction cell, a pressure-regulating outlet restrictor, and a collection unit, such as an extract accumulator (Figure 3).

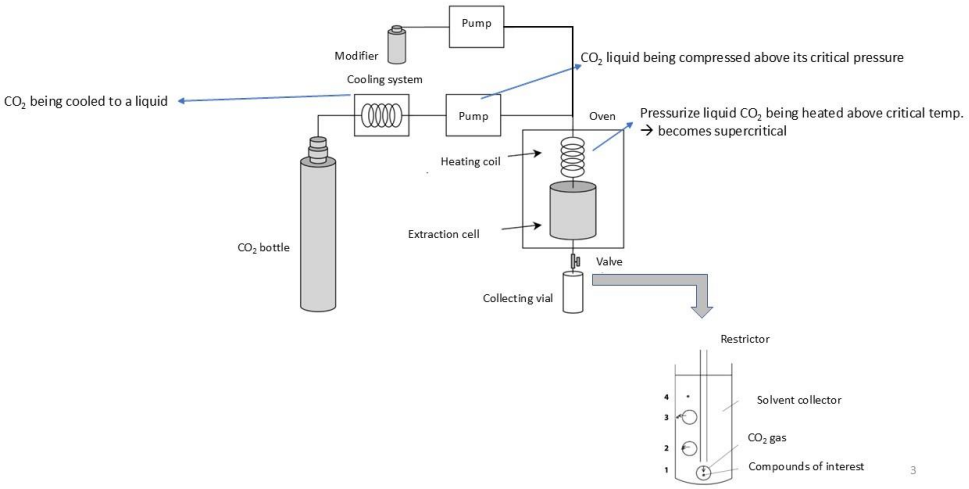

Figure 3. General representation of a supercritical fluid extractor [4]

The SFE system is equipped with a set of control valves designed to regulate the flow of the supercritical fluid throughout the process. An inlet valve controls the delivery of the pressurized fluid into a temperature-controlled extraction cell, while an outlet valve directs the stream toward a flow restrictor, where controlled depressurization takes place before the fluid is transferred to an appropriate collection device. During operation, the raw material is loaded into an extraction vessel fitted with precise temperature and pressure control systems to maintain the desired extraction conditions. The sample, adjusted to the required particle size, is typically packed into a sample basket equipped with filters at both ends, which retain the solid matrix while allowing supercritical CO_2 to pass freely through the extraction vessel. After the extraction vessel is properly sealed, the apparatus is pressurized with CO_2 at cylinder pressure while keeping the depressurization valve closed, in order to verify integrity of the system and the absence of leaks. Accurate temperature monitoring is achieved by positioning the thermocouple directly inside the extraction vessel, as external sensors are unable to detect the temperature rise associated with CO_2 pressurization.

Only when the desired temperature and pressure conditions within the extractor are reached and stabilized, the outlet valve is opened to initiate the flow of SC-CO_2 through the extraction cell.

The SF transports the solubilized target compounds to the collecting vial, where the extract is collected via a discharge port located at the bottom of the extraction cell. Upon depressurization, CO_2 reverts to the gas phase and leaves the extract behind for collection (a little volume of solvent can be used to facilitate this transfer). To prevent valve blockage caused by cooling during pressure release, the depressurization valve is typically heated, mitigating freezing effects associated with the Joule-Thomson phenomenon [5].

Generally, the gas CO_2 can be measured with the means of a flow meter, to understand the volume of CO_2 that has been used. And in some cases, especially in industrial scale apparatus, the possibility to recollect the exhausted CO_2 in a tank and recycle/sent again it to the extraction vessel is exploited. In lab scale apparatus, the CO_2 is instead vented and released to the environment.

5. When Microwaves Meet Supercritical CO_2: A Synergistic Extraction Concept

The development of the microwave-assisted supercritical CO_2 extraction (MA-SFE) prototype was driven by the vision of exploiting the intrinsic complementarity of two advanced technologies whose combined potential remains largely underexplored. Microwave irradiation and supercritical fluid extraction address different, yet synergistic, limitations of conventional extraction approaches, and their integration offers a unique opportunity to redefine extraction efficiency.
Microwave heating enables rapid and selective energy transfer directly within the extraction matrix, leading to enhanced internal heating, cell disruption (Figure 4), and accelerated release of target compounds.

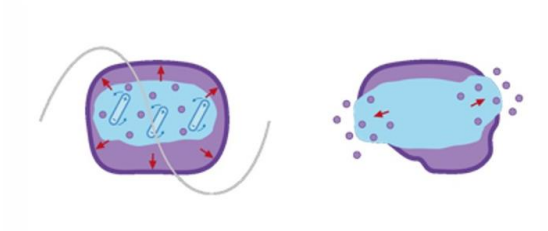

Figure 4. Extraction process promoted my MW irradiation

Unlike conventional heating, which relies on thermal gradients and surface-to-core heat transfer, microwaves promote uniform energy distribution and localized heating at the molecular level. This mechanism is particularly advantageous for solid matrices, where mass transfer limitations often dominate extraction kinetics. Supercritical CO_2 provides a highly tunable extraction medium, combining gas-like diffusivity with liquid-like solvent power. Its solvating capacity can be precisely modulated through pressure and temperature, enabling selective extraction under mild conditions while preserving thermolabile compounds. Moreover, the use of CO_2 eliminates solvent residues and aligns with the growing demand for cleaner and more sustainable extraction technologies. The rationale for combining these two approaches lies in the creation of an extraction environment in which microwave-induced internal heating enhances matrix permeability, while the supercritical fluid rapidly solvates and transports the released compounds. This synergistic interaction is expected to overcome diffusion and solubility limitations that constrain conventional extraction processes, potentially enabling shorter extraction times, improved yields, and reduced energy consumption.

Based on these considerations, a laboratory-scale prototype was designed to explore the feasibility of microwave-assisted supercritical CO_2 extraction. The aim in this case is not to achieve full process optimization, but rather to demonstrate the conceptual validity of the approach and to provide a foundation for future technological development.

6. Prototype's design and test

In the present section, a laboratory-scale prototype integrating MW heating with SC-CO_2 extraction was designed, assembled and evaluated. The system is based on the SynthWAVE, a last-generation MW reactor patented by Milestone s.r.l (Bergamo, Italy) and was specifically adapted to operate under supercritical CO_2 conditions.

6.1 SynthWAVE characteristics

The system is capable of performing single or multiple reactions at temperatures of up to 300 °C and pressures reaching 199 bar. Small-scale synthetic protocols can be readily translated to this platform, enabling straightforward scalability and methodological transfer. The reactor design enables batch and parallel reactions under controlled conditions, significantly enhancing experimental flexibility. The application of this technology results in substantially reduced reaction times, often decreasing processing durations from hours to minutes. Moreover, improved reaction selectivity and higher product yields are typically observed, accompanied by reduced by-product formation and sample contamination.

The synthWAVE system (Milestone Srl) is a high-performance microwave reactor specifically designed for the safe, reliable, and reproducible scale-up of microwave-assisted chemical processes. The instrument is capable of operating under extreme conditions, allowing reactions to be conducted at temperatures up to 300 °C and pressures up to 199 bar. These operating limits enable the use of low-boiling solvents, reactive gases, and pressurized media, extending the applicability of microwave-assisted synthesis beyond conventional boundaries. The reactor architecture allows experimental protocols developed at a small scale to be directly transferred to larger batch or parallel configurations without significant modification, facilitating process optimization and scalability. The system configuration is illustrated in Figure 5.

The synthWAVE is based on a Single Reaction Chamber (SRC) concept, in which a PTFE-lined stainless-steel vessel simultaneously functions as both the reaction chamber and the microwave cavity. This configuration ensures homogeneous microwave distribution and rapid, uniform heating of the reaction mixture, while integrated stirring systems promote efficient mass and heat transfer. Prior to irradiation, the chamber is pre-pressurized with an inert or reactive gas, a feature that prevents solvent boiling and allows reactions to be conducted well above the normal boiling point of the reaction medium.

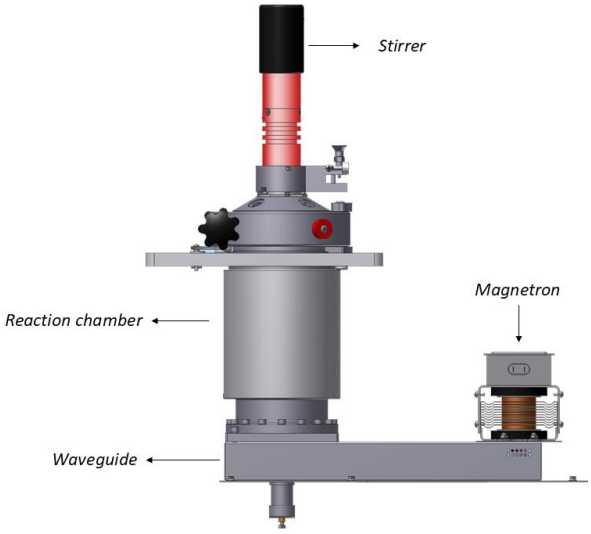

Figure 5. System configuration of synthWAVE system

The system supports both single-vessel and multi-vial operation, enabling parallel experimentation under identical temperature and pressure conditions. Compared to conventional thermal methods, microwave-assisted processing in the synthWAVE significantly reduces reaction times, often from hours to minutes, while improving reaction selectivity and product yields. Additionally, the controlled and efficient energy input minimizes by-product formation, sample contamination, and contributes to improved atom economy and sustainability of the process.

6.2 Modifications to the existing system

The synthWAVE, in its original configuration, is not fully compatible with operation under supercritical CO_2 conditions, for this reason, specific modifications were introduced to adapt the system . Prior to any modification, the pressure integrity of the system was verified, confirming adequate sealing performance under static conditions. The modifications primarily consisted of replacing the O-rings present in the system. In the synthWAVE, O-rings play a crucial role in ensuring pressure sealing of the closed vessels, allowing safe operation under high-temperature and high-pressure microwave-assisted conditions. However, the standard Viton O-rings are not specifically designed for prolonged exposure to pressurized CO_2 and low-temperature effects associated with depressurization; selected sealing components in direct contact with the reaction environment were replaced with silicone-based O-rings to ensure chemical compatibility and operational safety. Experimental validation tests demonstrated stable reactor performance under combined CO_2/N_2 atmospheres, both in the absence and presence of microwave heating. Controlled pressure release, particularly when performed through the automatic pressure valve at defined depressurization rates, prevented freezing phenomena associated with the Joule–

Thomson effect. Test inspections confirmed the mechanical integrity of the reactor components and sealing elements, indicating that the SynthWave platform could safely operate under CO_2-containing pressurized conditions following appropriate material adaptations. These results establish the technical feasibility of employing the SynthWAVE system as a prototype platform for exploratory studies involving pressurized CO_2 and microwave-assisted processes.

6.3 Prototype assembly

Initial modifications enabled the system to operate safely with CO_2 under high-pressure conditions; however, a limitation emerged due to the maximum delivery pressure of the CO_2 cylinder, which was insufficient to reach the critical pressure of pure CO_2 (73.8 bar). The possibility of supplementing the missing pressure with the regularly used nitrogen bottle was ruled out based on phase-equilibrium considerations of CO_2–N_2 mixtures. As reported by Blum et al. [6], binary mixtures of CO_2 and N_2 exhibit critical points that are strongly dependent on composition (Figure 6).

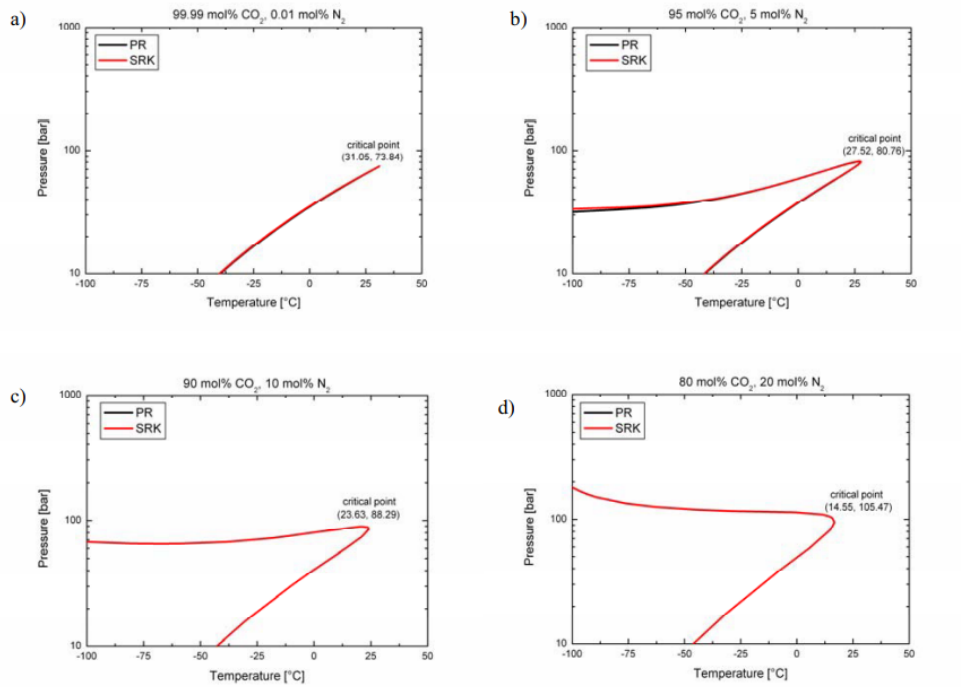

Figure 6. Phase diagrams for different mixtures of CO_2 an N_2.a.) 99.99 mol% CO_2, 0.01 mol% N_2; b) 95 mol% CO_2, 5 mol% N_2; c.) 90 mol% CO_2, 10 mol% N_2; d.) 80 mol% CO_2, 20 mol% N_2. [6]

In particular, for a mixture containing approximately 80% CO_2 and 20% N_2, the critical point shifts to significantly higher pressures (≈104 bar) and lower temperatures (≈15 °C), with further increases in N_2 content leading to an even greater displacement of the critical pressure. Consequently, although the total system pressure exceeded the critical pressure of pure CO_2, the gas mixture did not reach supercritical conditions, explaining the absence of extraction phenomena. To achieve true supercritical operation, it was therefore necessary to pressurize the reactor exclusively with CO_2. A high-pressure gas compressor recovered from decommissioned equipment was integrated into the system, allowing direct compression of CO_2 to pressures exceeding 50 bar. Although this solution enabled achieving pressures close to the critical point, the loading rate proved too slow for practical operation. To improve pressurization efficiency, a second CO_2 cylinder was subsequently connected to the synthWAVE's rear inlet valve, enabling faster reactor loading, while the compressor was used to fine-tune the pressure to 73.8 bar. This combined approach ultimately allowed reproducible access to 73.8 bar. Following extensive testing and optimization, stable supercritical CO_2 conditions within the microwave reactor were successfully achieved using pure CO_2.

A further challenge inherent to the prototype configuration was the extraction collection system. Unlike conventional supercritical fluid extraction systems, which operate under semi continuous-flow conditions, the microwave-assisted system developed in this work functions in a static mode. As a result, it was not possible to use conventional solvent flow to transport and collect the extract. To address this limitation, a plunger tube was introduced inside the reaction vessel to facilitate the removal of extracted compounds during depressurization. Upon controlled pressure release through the synthWAVE head valve, the expanding CO_2 effectively stripped the solubilized extract from the matrix and transported it toward the recovery zone, enabling extract collection despite the batch nature of the system. The configuration of the prototype is illustrated in Figure 7.

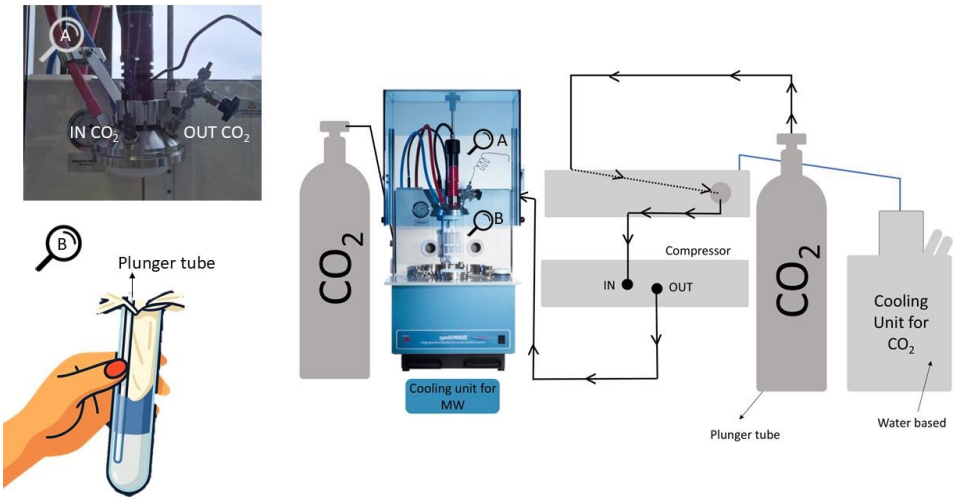

Figure 7. Units of the SC-CO_2-MW prototype. A) close-up of the connection at the head of the MW unit for the IN and OUT of CO_2, B) close-up of the vessel and plunger tube connected at the out valve.

Once the feasibility of operating under CO_2 supercritical conditions was established, the next objective was to evaluate whether the system could be operated at different temperatures. For this purpose, a series of heating tests were carried out to investigate the response of the system to microwave-assisted temperature ramps. After the system was loaded with CO_2, microwave heating was then applied with target temperatures set at 30, 40, 50, and 60 °C. The system accurately followed the programmed temperature profile up to 50 °C, demonstrating effective microwave heating and stable pressure behavior under near-supercritical conditions (Figure 8). However, when approaching 60 °C, the experiment had to be interrupted due to a rapid increase in pressure, which brought the system close to the instrument safety cut-off limit. This issue can be readily addressed during the system engineering phase through the implementation of appropriate pressure-control strategies and safety mechanisms. By the way, it is actually not expected to operate at higher than 50 °C as the properties of the SF are not suitable any longer for extraction.

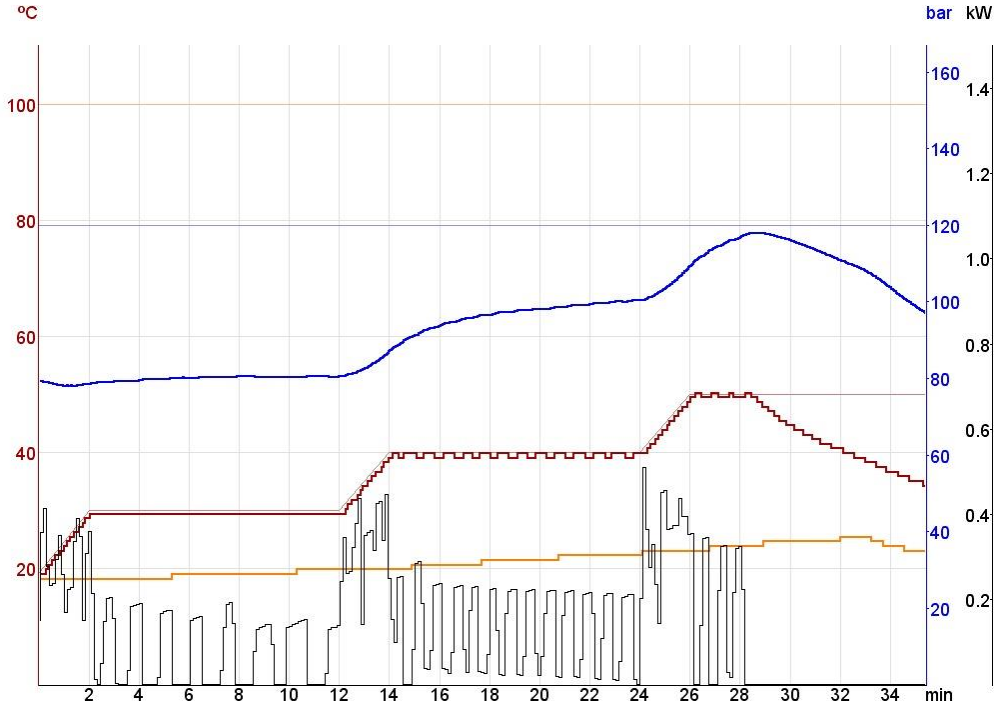

Figure 8. Measured temperature profile during microwave-assisted heating of the extraction cell pressurized with CO_2, demonstrating accurate control of the temperature ramp up to 50 °C. Temperature (red), Pressure (blue), MW power (black), safety temperature (orange)).

Once the ability of the system to operate under controlled temperature and pressure conditions was verified, a preliminary extraction test was performed to assess whether the prototype could effectively extract compounds from a real matrix. Peanuts were selected as a model sample due to their high lipid content, with the specific aim of extracting the fat fraction. This experiment was designed to verify the extraction capability of the microwave-assisted pressurized CO_2 system under the tested conditions.

Despite the constraints, measurable amounts of extract were successfully recovered. Under microwave-assisted CO_2 conditions, 23 mg of extract were obtained from 500 mg of sample, whereas a simple solid-liquid extraction with hexane yielded 49 mg from the same sample mass. This is clearly not an exhaustive extraction of the total fat from peanuts, but was used to evaluate whether, in the non-optimized conditions tested, the CO_2 extraction could at least simulate an easy hexane extraction. Although the extraction yield achieved with the prototype was lower than that obtained with an organic solvent, these results demonstrate the feasibility of the proposed approach and confirm that the system with pressurized CO_2 can perform the extraction similarly to simple hexane.

As the collection system of the prototype is far to be optimal, a second test was performed adding 1 mL of hexane in the bottom of the extraction vial (not in contact with the sample) to solubilize the extracted fat and allow easier removal of the liquid phase from the plunger tube during depressurization, with the ultimate purpose of trying to reduce losses on the pathway from the vessel to the collection vial. However, no significant difference in extraction yield was observed with or without the addition of hexane.

The observed limitations are mainly related to the prototype configuration and operating mode and highlight the need for further system optimization to enable operation under fully controlled supercritical conditions.

From an engineering perspective, further development of the system would require a comprehensive redesign to enable efficient extract recovery. A dedicated collection module would be essential to minimize the intervention of an operator as much as possible during the recovery of the extract. In addition, future work should consider the implementation of improved pressure and flow control to allow operation not only under supercritical conditions but also in subcritical regions, which may offer additional flexibility in terms of selectivity and operating stability. Overall, continued engineering development and systematic exploration of operating conditions are required to fully exploit the potential of the microwave-assisted CO_2 extraction concept.

7. Conclusions and future perspective

In this chapter, a novel microwave-assisted supercritical fluid extraction concept was explored through the design and construction of a laboratory-scale prototype. The primary objective was to evaluate the feasibility of combining microwave irradiation with supercritical fluid extraction as an innovative approach to enhance extraction efficiency from complex matrices. Using peanuts as a model system, the prototype successfully demonstrated the ability to extract target compounds, thereby confirming the technical viability of the proposed concept.

Beyond this initial demonstration, further optimization and systematic investigation were not pursued within the scope of this work. The prototype, while functional, was not sufficiently engineered to operate under fully controlled and optimized supercritical conditions, limiting the possibility of comprehensive performance evaluation, scale-up studies, or detailed comparison with established SFE systems. As a result, the experimental work was intentionally limited to demonstrating the approach's feasibility rather than achieving full method development or validation (which would not be feasible under the present conditions).

Nevertheless, the results obtained represent a significant starting point, demonstrating that the integration of microwave heating with supercritical fluid extraction is technically feasible and holds potential to improve mass transfer and extraction kinetics.

Future developments should focus on redesigning the system to allow easy loading of the CO_2 at the desired pressure, precise control of pressure, temperature, and what's more, design a proper recollection tool to isolate the extract.

Within this context, the present work establishes a solid proof of concept and provides a foundation for further research and technological development.

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Chapter 6

Conclusions and future perspectives

This doctoral research demonstrates that microwave-assisted sample preparation represents a versatile and highly effective analytical strategy for addressing modern challenges in food quality and safety analysis. By systematically investigating microwave-based approaches across different analytical targets and matrices, this thesis provides both methodological advancements and conceptual evidence supporting the integration of microwave technology within the frameworks of Green Analytical Chemistry (GAC) and White Analytical Chemistry (WAC).

Throughout the experimental work, microwave-assisted techniques significantly improved analytical workflows by reducing extraction times and solvent consumption while maintaining performance in terms of extraction efficiency, reproducibility, and robustness. These advantages were consistently observed across a range of applications, including fatty acid methyl ester (FAME) determination, sterol analysis, and additional emerging applications. In the field of lipid analysis, the methodologies developed here showed a high degree of robustness and adaptability. In particular, MAED approaches for FAME determination were successfully applied to complex, heterogeneous, and chemically diverse food matrices, confirming their suitability for real-world samples characterized by variable composition and structural complexity. The results obtained were fully comparable to those of conventional reference methods, both in terms of qualitative fatty acid profiles and quantitative accuracy, thereby confirming the analytical equivalence of the proposed strategies.
The application of these approaches to microalgal matrices—an area still relatively unexplored—further highlights their relevance within emerging analytical contexts. Despite the limited literature available, the results obtained for microalgae were in excellent agreement with established methods, demonstrating both reliability and adaptability. Similarly, the analysis of mussel samples confirmed the robustness of the methodology while enabling the effective determination of nutritional quality indices derived from fatty acid composition, emphasizing its applicability to nutritionally relevant studies.

Overall, these findings support the use of microwave-assisted sample preparation as a reliable and broadly applicable approach for FAME analysis. The methods also exhibited satisfactory repeatability, with consistent performance across different matrices and experimental conditions, reinforcing their suitability for routine, high-throughput analytical laboratories.

For both FAMEs and sterol applications, a key advancement lies in the integration of traditionally labor-intensive and time-consuming steps into simplified and streamlined workflows. In particular, extraction/derivatization and extraction/saponification steps, typically performed separately, were successfully merged into single microwave-assisted processes. This consolidation led to a substantial simplification of the analytical procedure, reducing preparation time and

manual handling while improving operational efficiency and throughput. Importantly, these gains were achieved without compromising analytical reliability, as the results remained fully comparable to those obtained using established reference methods in terms of accuracy, precision, and reproducibility. This integration of critical steps represents a major strength of the proposed approaches, demonstrating that faster and simpler methods can be developed without sacrificing analytical robustness.

A further important outcome of this work is the demonstration of the practical value of these methodologies in routine analytical laboratories, where rapid turnaround, high sample throughput, and operational robustness are essential. Food control, quality assurance, and regulatory laboratories are increasingly required to deliver reliable results within short timeframes and under high sample loads. In this context, the protocols developed in this thesis offer a clear advantage, enabling a significant acceleration of sample preparation while preserving data quality. The reduction of preparation time from hours to minutes, combined with the possibility of parallel processing in closed-vessel systems, makes these approaches particularly well suited for routine workflows requiring fast and reproducible responses.

At the same time, simplified procedures and reduced reliance on hazardous solvents contribute to improved laboratory safety and facilitate implementation, lowering the barrier to adoption in non-specialized environments. Within the framework of WAC, these characteristics strongly support the “blue” dimension of sustainability, ensuring a balance between analytical performance, environmental responsibility, and practical feasibility.

The exploratory integration of microwave irradiation with supercritical CO_2 extraction further highlights the forward-looking and transformative potential of this technology. Although presented as proof of concept, this approach conveys a broader perspective: microwave technology is not confined to a specific application but represents a flexible and enabling platform capable of adapting to evolving analytical needs. Its compatibility with intensified, solvent-reduced, and highly selective extraction strategies reflects a natural alignment with the future of analytical chemistry, where sustainability, efficiency, and innovation must coexist. More broadly, this work suggests that microwave-assisted approaches are likely to find their place across diverse laboratory environments, regardless of the specific analytical objective pursued. Whether applied to routine analysis, advanced method development, or technological integration, their adaptability positions them as a powerful driver for future innovation.

Looking ahead, the evolution of analytical chemistry will increasingly rely on the integration of advanced sample preparation strategies with high-resolution and multidimensional analytical platforms. In this context, the coupling of microwave-assisted workflows with techniques such as comprehensive two-dimensional gas chromatography (GC×GC) represents a particularly promising direction. The enhanced separation power and sensitivity of GC×GC systems, combined with rapid and efficient sample preparation, could enable a more detailed characterization of complex food matrices, including minor components and trace-level contaminants that remain challenging to resolve with conventional approaches.

More broadly, sample preparation is expected to evolve towards fully integrated, automated, and miniaturized systems, in which multiple steps are combined into seamless workflows with minimal human intervention. Microwave-assisted techniques are particularly well suited to this transition, as their ability to accelerate reactions, reduce solvent consumption, and operate in closed systems aligns naturally with the development of high-throughput, automated analytical platforms. Their integration with online or at-line analytical techniques, as well as with emerging technologies such as flow-based systems and digitally controlled laboratories, could further enhance efficiency and reproducibility.

In parallel, the growing emphasis on sustainability will continue to drive the development of environmentally responsible analytical strategies. Microwave-assisted methodologies, already aligned with the principles of Green and White Analytical Chemistry, provide a strong foundation for further progress in this direction. Their compatibility with alternative solvents, reduced energy requirements, and potential for process intensification make them particularly suitable for next-generation analytical methods, where sustainability is a fundamental requirement.

Ultimately, the future of analytical science will depend on the ability to integrate complementary technologies into coherent and efficient systems. Within this evolving landscape, microwave-assisted sample preparation emerges as a flexible and enabling platform, capable of bridging current methodologies with future analytical needs. Its adaptability, efficiency, and compatibility with advanced instrumentation suggest that it will continue to expand its role, contributing to the development of faster, greener, and more informative analytical workflows.

Chapter 7

List of publications and scientific production

1.Publications

D.Ferrara, M. Beccaria, C.E Cordero, G.Purcaro. Microwave-assisted extraction in closed vessel coupled to chromatographic techniques in food analysis. Journal of Separation Science. https://doi.org/10.1002/jssc.202300390

Chapter "Microwave-assisted extraction" for the Comprehensive Sampling and Sample Preparation, 2e . Authors Ferrara D, Purcaro G.

D.Ferrara, M.Cescon, G. Giacoppo, V. Costa, G. Purcaro, N. D.Spadafora, C. Cordero, L. Pasti, A. Cavazzini, M. Beccaria. Composition and nutritional values of fatty acids in marine organisms by one-step microwave-assisted extraction/derivatization and comprehensive two-dimensional gas chromatography - flame ionization detector.Journal of Chromatography B,2024, https://doi.org/10.1016/j.jchromb.2024.124074.

D. Ferrara, M.Beccaria, C. E. Cordero, G. Purcaro. Comprehensive comparison of fatty acid methyl ester profile in different food matrices using microwave-assisted extraction and derivatization methods and comprehensive two-dimensional gas chromatography coupled with flame ionization detection, Advances in Sample Preparation, https://doi.org/10.1016/j.sampre.2024.100124.

J. Carpentier, C. Martin, H. Luttenschlager, N. Deville, D. Ferrara, G.Purcaro, C. Blecker, F.Francis Rudy, C. Megido. Common soluble carbohydrates affect the growth, survival, and fatty acid profile of black soldier fly larvae Hermetia illucens (Stratiomyidae). Sci Rep 14, 28157 (2024). https://doi.org/10.1038/s41598-024-75730-5

G. Cafeo, D. Ferrara, A. Schincaglia, M. Beccaria, M. Russo, L. Mondello, P. Dugo, G.Purcaro. Microwave-assisted extraction of oxygen heterocyclic compounds from Citrus-scented creams using a deep eutectic solvent and their determination by HPLC-PDA. Journal of Chromatography A. https://doi.org/10.1016/j.chroma.2025.466085.

A. Gorska, D.Ferrara, P.Albendea, C.E.Cordero, G.Purcaro. Update on the microwave-assisted saponification conditions for mineral oil hydrocarbons determination in fats and oils. *Analyst*, 2025,150, 5190-5200. DOI: 10.1039/d5an00701a

Bellinghieri C, Giacoppo G, Schincaglia A, Ferrara D, Purcaro G, Cavazzini A, Pasti L, Stevanin C, Chenet T, Marchetti N, Maietti A, Franchina FA, Beccaria M. Applicability of MTBE-based lipid extraction methods assisted by microwave in food

analysis. Statistical comparison and greenness evaluation with Soxhlet and Matyash. Talanta. 2026 Mar 1;299:129124. doi: 10.1016/j.talanta.2025.129124.

2.Scientific production

2.1Oral presenations

D.Ferrara, A.Fina, C.Cordero, G.Purcaro. A new microwave-assisted extraction technology for the profiling of fatty acids in food matrices. (25th International Symposium on Advances in Extraction Technologies (ExTech 2023)). 18-21 July 2023, Tenerife, Spain.

D.Ferrara, C.Cordero, G.Purcaro. Microwave-assisted processes for Fatty Acids Methyl Esters analysis in food matrix: A comparative study. (AOCS annual meeting & expo 2024). 28 April - 01 May. Montreal, Canada

D.Ferrara, M. Beccaria , C.Cordero, G.Purcaro. Advantages of the use of Microwave-assisted technology for lipid analysis.(3rd European Sample Preparation Conference and 2nd Green and Sustainable Analytical Chemistry Conference). 15-18 September 2024. Chania,Creete

D.Ferrara, M. Beccaria , C.Cordero, G. Purcaro. Microwave-assisted methods for greener and more practical (blueness) fatty acid profiling in food. (11th International Symposium on RECENT ADVANCES IN FOOD ANALYSIS(RAFA)). 5-8 November 2024. Prague, Czechia.

D.Ferrara, G.Giacoppo, A. Cavazzini, L.Pasti, M.Beccaria,C. Cordero; G.Purcaro. One-step-microwave-assisted extraction and derivatization followed by comprehensive two-dimensional chromatography coupled with flame ionization detector to analyze fatty acid methyl esters (FAMEs) in complex food matrices. (16th multidimentional chromatography workshop).1 to 5 february 2025. Liege,Belgium

P.Albendea, D.Ferrara, G.Purcaro. A miniaturized microwave-assisted saponification and extraction method for MOH analysis.(3rd Advance in Separation Sciences workshop). 12 to 13 of June 2025. Gembloux, Belgium

D.Ferrara, N.Ruin, M.Beccaria, C.Cordero, G.Purcaro. Microwave and SPE: a recipe for faster and greener lipid analysis. (Euroanalysis 2025). 31 august - 4 september. Barcelona, Spain.

J.Carpentier, Z. Jullien,H.Luttenschlager, D.Ferrara,N Deville, A.Anglicus, F.Francis, R. Caparros Megido. Addressing variability in Hermetia illucens

bioassays: have all the key species-specific parameters been identified? (BSF CON 2025), Cambridge, United Kingdom

D.Ferrara, N.Ruin, C.Cordero, M.Beccaria, G.Purcaro. Enhanced Extraction through Microwave-Assisted Technologies: Exploring New Analytical Frontiers. (27th International Symposium on Advances in Extraction Technologies, ExTech 2025). 07/09/2025 - 11/09/2025Mulheim an der Ruhr, Germany

2.2Posters

D.Ferrara,G.Giacoppo, M.Beccaria, G.Purcaro. Comparison of different extraction methods for the determination of fatty acid methyl esters in mussles samples. (2nd Advance in Separation Science). 28 june to 29 june 2023. Gembloux,Belgium.

G.Bauwens, D.Ferrara, G.Purcaro. Improvement of the saponification process for mineral oil analysis using microwave assisted saponification/extraction. (25th International Symposium on Advances in Extraction Technologies (ExTech 2023)). 18-21 July 2023.Tenerife, Spain.

D.Ferrara, M.Cescon, G.Giacoppo, V.Costa, G.Purcaro, D.N. Spadafora, C.Cordero, L.Pasti, A.Cavazzini, M.Beccaria. One-step microwave-assisted extraction and derivatization for the evaluation of fatty acid methyl esters composition in marine organisms and their nutritional value. (AOCS annual meeting & expo 2024). 28 April - 01 May. Montreal, Canada.

D.Ferrara, M.Beccaria, G.Purcaro. Comparative evaluation of Microwave-assisted processes for fatty acid methyl esters analysis in food matrices. (18th International Symposium on hyphenated techniquest in chromatography and separation technology(HTC-18). 28-30 May 2024. Leuven, Belgium.

S.Mascrez, D.Ferrara, G.Purcaro. Vacuum-assisted and multi-cumulative trapping coupled with GC×GC for highly informative virgin olive oil volatile profiling. AOCS annual meeting & expo 2024. 28 April - 01 May. Montreal, Canada.

P.Albendea, D.Ferrara, G.Purcaro. Comparison of microwave-assisted saponification and extraction methods for MOH determination in meat. (3rd European Sample Preparation Conference (EuSP2024) and the 2nd Green and Sustainable Analytical Chemistry Conference (GSAC2024)). 15-18 September 2024. Chania, Greece.

D.Ferrara, N.Ruin, M.Beccaria, C.Cordero, G.Purcaro. Microwave-assisted extraction and saponification to increase the throughput in food quality analysis. (11th

International Symposium on RECENT ADVANCES IN FOOD ANALYSIS (RAFA)). 5-8 November 2024. Prague, Czechia

D.Ferrara, N.Ruin, M.Beccaria, G.Purcaro. Sterols analyis in olive oil by microwave-assisted saponification and extraction followed by flow modulation comprehensive two dimentional chromatography. (16th multidimentional chromatography workshop). 1-5 February 2025. Liege, Belgium

C.Bellinghieri, G.Giacoppo, D.Ferrara,G.Purcaro, A.Cavazzini, L.Pasti, F.Franchina, M.Beccaria. Lipid extraction assisted by microwave using methyl tert-butyl ether as based solvent for extraction and fatty acids characterization of pistachio oil by mono- and two- dimensional gas chromatography. 12/06/2025 - 13/06/2025. 3rd Advances in Separation Science Workshop. Gembloux, Belgium

D.Ferrara, N.Ruin, M.Beccaria, C.Cordero, G.Purcaro. Fast and green sterols analysis by microwave-assisted saponification and extraction followed by solid phase extraction.12/06/2025 - 13/06/2025. 3rd Advances in Separation Science Workshop. Gembloux, Belgium

A.Gorska, D.Ferrara, P.Albendea, G.Purcaro. Optimized saponification for MOSH and MOAH analysis in food matrices: addressing internal standard variability. (Euroanalysis 2025). 31 august - 4 september. Barcelone,Spain.

C.Bellinghieri, G.Giacoppo, D.Ferrara, A.Schincaglia, L.Pasti, A.Cavazzini, F.Franchina, G.Purcaro, M. Beccaria. Applicability of MTBE lipid extraction assisted by microwave in pistachio oil and fatty acids characterization by mono- and two-dimensional gas chromatography. (27th International Symposium on Advances in Extraction Technologies, ExTech 2025). 07/09/2025 - 11/09/2025Mulheim an der Ruhr, Germany

J.Carpentier, Z. Jullien,H.Luttenschlager,D.Ferrara,N Deville, A.Anglicus, F.Francis, R. Caparros Megido. Addressing variability in Hermetia illucens bioassays: have all the key species-specific parameters been identified?. BSF CON 2025. 08-09-2025. Cambridge, United Kingdom.

D.Ferrara, N.Ruin, M.Beccaria, C.Cordero, G.Purcaro. Analytical Method for the Analysis of the Sterol Fraction in Olive Oil to Detect Food Fraud. (27th International Symposium on Advances in Extraction Technologies, ExTech 2025). 07/09/2025 - 11/09/2025Mulheim an der Ruhr, Germany