



SAPIENZA
UNIVERSITÀ DI ROMA

Department of Chemistry and Technologies of Drugs

**PhD in MOLECULAR DESIGN AND CHARACTERIZATION FOR THE
PROMOTION OF HEALTH AND WELL-BEING: FROM DRUG TO FOOD**

XXXVII Cycle

High-Resolution NMR-Based Metabolomic Profiling in Plant-Based Foods and Cultural Heritage

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*A mia madre e mio padre,
due anime semplici dal cuore nobile.*

In the realm of chemistry, Nuclear Magnetic Resonance spectroscopy unlocks the hidden language of molecules, revealing the intricate structure and dynamics of matter that shape the world we live in.

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Thesis objectives

Nowadays, Nuclear Magnetic Resonance (NMR) spectroscopy has become one of the preferred instrumental methodologies in various scientific fields because it allows qualitative and quantitative chemical information to be obtained directly from measurements. NMR is capable to produce, with a single direct measurement, a very large amount of chemical information. However, this information is not always easy to interpret. In fact, the complexity of analyzing NMR spectral data is proportional to the number of compounds simultaneously present in the analyzed sample, as the resonances of different compounds overlap. One of the most extreme situations is the NMR spectra of samples from metabolomics studies, from which numerous compounds can be detected in a single measurement. In the study of chemical processes involving metabolites (metabolomics), the most commonly used NMR spectra are one-dimensional (1D ^1H) proton NMR spectra because they are relatively fast to acquire, and the proton sensitivity is the highest. Also ^1H - ^{13}C Heteronuclear Single Quantum Coherence (HSQC), ^1H - ^{13}C Heteronuclear Multiple Bond Correlation (HMBC), and ^1H - ^1H Total Correlation Spectroscopy (TOCSY), NMR spectra are frequently used in metabolomics for better structural characterization of detected metabolites. Additionally, in (NMR) metabolomics, data from multiple metabolites is simultaneously obtained for each sample. Interpreting this information from a biological standpoint can be challenging, not only due to the abundance of available data, as well as because the measured metabolite perturbations are directly influenced by various biological processes occurring at the same time within the studied organism.

Considering these situations, the two general purposes of this thesis are:

- Characterising the metabolomic profiles of different matrices in the food and cultural heritage fields;
- To investigate the metabolic processes involving the matrices studied under particular conditions such as environmental and biological conditions, or such as conservation, chemical safety and risk, and pedoclimatic effects.

These two general objectives can be broken down into specific objectives presented below:

- Design metabolomics experiments that provide relevant information on the metabolic status of the representative organisms analyzed;

- Develop different protocols for the preparation of specific samples;
- Define a data analysis workflow to study these NMR samples. This workflow will include alignment and normalization of NMR data, assignment and integration of resonances, Analysis of Variance (ANOVA) statistical analysis for significance, and analysis of the data set by chemometric methods, such as Principal Component Analysis (PCA);
- Establish a data analysis strategy to merge NMR spectroscopy data with those obtained from other analytical platforms with the aim of generating a more complete characterisation of metabolic perturbations in the organisms studied.

Thesis structure

This thesis is structured into General introduction, Part A and Part B, which will be briefly summarized below.

General introduction

This section is devoted to an introduction to *omics* science and specifically to the role of metabolomics, the methodologies and approaches involved in metabolomics analysis. The fundamentals of NMR spectroscopy are described in detail and specifically, with a focus on the phenomena underlying this methodology, and all the operations exploited to create different NMR pulse sequences. After a general description of the theoretical principles on which NMR spectroscopy is based, a brief explanation is given of the hardware and software that constitute the instrument used in NMR, so-called spectrometer, in order to get a more practical idea of all the steps that take place during an NMR experiment.

Finally, a concise brief overview is given of the main one-dimensional (1D) and two-dimensional (2D) NMR experiments that can be very useful for metabolomics studies

Part A

The common thread of this part is Foodomics, the concept of which is taken up for the description of metabolic approaches in food analysis (Chapter 1). First the general workflow to be followed for a foodomics matrix is specifically defined, then the various studies related to the samples analyzed are described.

In detail, Chapter 2 describes two case studies, that of medicinal plants and the second one concerning lemon balm from Corsican origin, *Melissa officinalis* subsp. *altissima*. In the use of officinal plants, NMR metabolomics has been recognised as a suitable approach to identify and quantify primary and secondary metabolites in plants, highlighting the effects of different altitudes and agronomic practices on the chemical profile. For *Melissa officinalis* subsp. *altissima*, in addition to the phytochemical profile by applying NMR, the volatile component was investigated by GC/MS and biological assays for neuroinflammation were performed.

Chapter 3, in the AgriTech background, is devoted to the characterization of matrices subject to high quality risk, such as apples (Golden Delicious) and tomatoes (Pomodoro Riccio). In the

apple case, bio-packaging effects during fruit storage were evaluated, whereas in the tomato “Pomodoro Riccio” metabolomic differences between two growing years were investigated. In Chapter 4, enzyme hydrolysates were obtained from artichoke and cauliflower waste. These were characterised and proposed for use as plant biostimulants.

Part B

In this section, in the context of cultural heritage, issues related to black lichens are described. In particular, this type of lichen is responsible for the biodeterioration of cultural heritage monuments, as in the case of the archaeological site on the Motya island (Sicily). In this instance, metabolomic analysis has made it possible to define the composition of these specific symbiotic organisms, and to quantitatively assess metabolites under the conditions when black lichens come in contact with water.

General introduction

Chapter 1

The metabolomics

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1.1 The role of metabolomics in *omics* science

Omics sciences are disciplines that focus primarily on the universal detection of genes (genomics), mRNAs (transcriptomics), proteins (proteomics) and metabolites (metabolomics) in a specific biological sample in an unbiased manner (Vailati-Riboni et al., 2017), **Figure 1.1**.

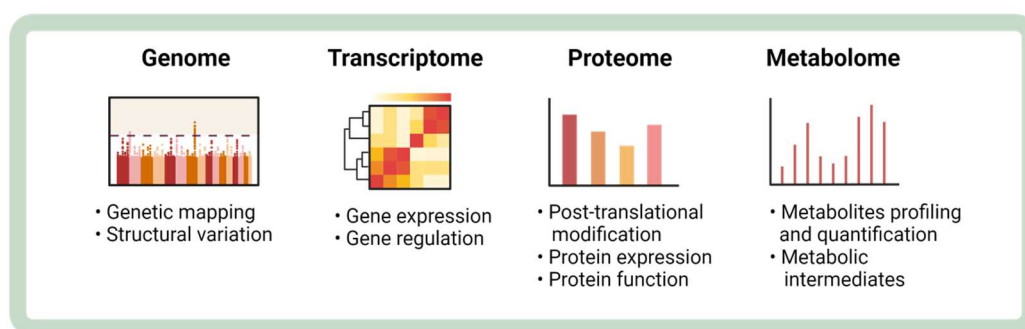


Figure 1.1 Illustration of *omics* technologies.

The key aspect of an *omics* approach is to allow a deeper understanding of a complex system when considered as a whole. Thus, it is useful for verifying and demonstrating the connections and interrelationships between the various components of a complex physiological state and for discovering missing pieces in our current knowledge.

Specifically, metabolomics involves a comprehensive analysis of small molecule metabolites (< 1 kDa) from a specific organism or biological sample and is based on a wealth of biochemical knowledge developed over many years (Klupczyńska et al., 2015).

The last of the so-called *omics* research fields focuses on the intermediates and products of metabolism, which include carbohydrates, fatty acids, amino acids, pigments, nucleotides, organic acids, sugars, antioxidants and many other classes of compounds. In analogy to the terms transcriptomics and proteomics, the complete set of metabolites synthesized by a biological system constitutes its metabolome. The metabolome is the set of metabolites present in a cell, tissue, organ or entire organism (Vignoli et al., 2019).

Metabolomics is a research approach that involves the study of a wide range of living organisms, from microbes to complex biological systems such as mammals. Although the number of metabolites in the human body is relatively small compared to genes, transcriptomes and proteins, there are numerous factors that influence metabolite levels in biological fluids and tissues. The metabolome is dynamic and capable of being influenced by

natural fluctuations and external influences, and metabolomics offers valuable information about the cell (Klupczyńska et al., 2015).

Metabolite levels are considered the ultimate response of an organism to various factors including environmental, and genetic, in gut microflora, and in enzyme activity changes (Vermeersch & Styczynski, 2013). Genomics, transcriptomics and proteomics do not fully explain the complex response of organisms to physiological and pathophysiological stimuli, whereas metabolomics is more closely related to phenotype. Indeed, the latter research may offer the opportunity to study the influence of genetic variation, disease, applied treatment or diet. Several metabolomics experiments aim to search for metabolites that discriminate samples from individuals with a disease from those from healthy subjects (Ambroselli et al., 2023).

In the field of metabolomics, researchers have the option of following three distinct approaches: fingerprinting, profiling, and targeted metabolomics.

Fingerprinting metabolomics involves rapidly assessing the reproducible fingerprint of a biological sample on a global scale (Ellis et al., 2007). This method favors capturing a broad spectrum of metabolites without the need for their specific identification. The goal is to encapsulate different classes of compounds of potential interest without prior knowledge of their identity. Used primarily for sample classification rather than quantitative analysis, metabolic fingerprinting seeks to distinguish between different biological states by identifying unique patterns that characterize a metabolomic state within a specific tissue or biological fluid. By simultaneously examining a wide range of metabolites, metabolic fingerprinting embodies a holistic "*omics*" approach to metabolomics.

1.2 Analytical methodologies used in metabolomics

The most common analytical platforms used in metabolomics are nuclear magnetic resonance (NMR) and mass spectrometry (MS) (Vailati-Riboni et al., 2017). Both spectroscopic methodologies allow for the analysis of numerous small molecules in a sample, enabling the identification and measurement of metabolites, even though each has strengths and limitations.

Advantages of NMR spectroscopy include minimal sample preparation and no need to separate analytes, non-destruction of analyzed samples, which can then be recovered and used

for further study. The nondestructive nature of NMR is particularly useful for the analysis of metabolites from intact cells or tissues (biopsy samples) (Klupczyńska et al., 2015).

Moreover, the NMR signal intensity is minimally influenced by the components of the sample matrix compared to MS, making NMR an efficient tool for analysing complex biological samples. However, the main disadvantage of NMR is its low sensitivity. The primary limitation of NMR is its low sensitivity. In metabolomic studies, mass spectrometry typically involves separating metabolites from analyzed biofluids. As a result, mass spectrometry is commonly used in conjunction with high-resolution techniques as liquid chromatography (LC), gas chromatography (GC), or capillary electrophoresis (CE).

Mass spectrometry requires only small sample amounts (μL) for analysis, despite being a destructive technique. In GC-MS, compounds are heated to the gas state, and nonvolatile substances require derivatization. Liquid chromatography-mass spectrometry (LC-MS) enables the analysis of a wide range of metabolites (Putri et al., 2013), and ultra-high performance liquid chromatography (UHPLC) has improved chromatography significantly. Capillary electrophoresis (CE) allows the analysis of polar, ionic molecules but has low repeatability (Li et al., 2012).

The biomarkers identified by NMR and MS are different, highlighting the complementary nature of these two analytical platforms. Therefore, a comprehensive characterization of an organism's metabolome can only be achieved through a combination of various analytical methodologies and data from multiple instruments (**Table 1.1**).

Table 1.1. Comparison of the main analytical techniques used for metabolomic profiling.

Property	Technique					
	NMR	HPLC-MS	UHPLC-MS	SFC-MS	CE-MS	GC-MS
Sensitivity	-	++	+++	+++	+	+++
Reproducibility	High		Lower than NMR			
Resolution	Low		Higher than NMR			
Quantification	Yes		Yes, internal standard needed			
Time for sample preparation	Low	Medium	Medium	Medium	Medium	Extensive
Sample volume for analysis	100-500 μL	2-10 μL	2-10 μL	2-10 μL	1-20 nL	1-2 μL

Range of metabolites	Polar and non-polar	Polar and non-polar	Polar	Volatile polar and non-polar
Metabolites identification	Easy	Difficult (databases need to be improved)		Easy

1.3 Metabolomic workflow

Proper sample collection, storage, and preparation are essential for the success of a metabolomics experiment (Klupczyńska et al., 2015). Inconsistencies in how samples are taken from patients, stored, or processed can lead to invalid data from the analysis. Sample preparation methods vary depending on the type of sample and the analytical platform used, and may involve processes such as extraction, derivatization, or blotting.

The purpose of data analysis is to identify important changes in metabolites or groups of metabolites. Prior to statistical analysis, the output data undergoes pre-processing including deconvolution, alignment, baseline correction and normalization in order to achieve more comparable characteristics. Given the large amount of data produced by metabolomics research, multivariate statistical analyses are frequently employed (Madsen et al., 2010). Common strategies for identifying significant metabolites include principal component analysis (PCA), hierarchical cluster analysis (HCA), partial least squares discriminant analysis (PLS-DA), and orthogonal projection to latent structure discriminant analysis (OPLS-DA) (Putri et al., 2013). After identifying metabolic biomarkers, additional experiments are needed to validate and test them. Differences in the concentration of distinguishing metabolites need to be confirmed using a new set of samples. Finally, internal cross-validation tests verify the prediction accuracy of the analysis and confirm the reliability of the identified biomarker.

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Chapter 2

NMR spectroscopy fundamentals

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2.1 Nuclear Magnetic Resonance

Nuclear Magnetic Resonance in the condensed phase was discovered in 1946 independently by two groups of scientists, Bloch, Hansen and Packard at the University of Stanford (California, USA) (Bloch et al., 1946), and Purcell, Torrey and Pound at the University of Cambridge (Massachusetts, USA) (Purcell et al., 1946).

Right from the beginning, NMR has been approached through two distinct perspectives. Bloch and coworkers envisioned NMR in classical terms, considering the orientation of microscopic magnetic moments with respect to an external magnetic field, whereas Purcell and coworkers pictured NMR in terms of quantum transitions.

The first conception affords one a rather simple and efficient way to describe the NMR phenomenon, providing insight regarding fundamental issues such as magnetization precession and nuclear relaxation. However, the classical description becomes rapidly limited and the second approach, the so-called spectroscopic description, relying mainly on quantum mechanics, becomes indispensable as soon as a deeper understanding of the interactions that rule nuclear spins is sought.

A detailed treatment of both theories would require too long space and would turn out to be out of the context of this work. For these reasons, an indicative description of classical NMR theory will be provided in this chapter. Anyway, in the spectroscopic description, NMR phenomenon is explained in terms of quantum mechanical frameworks such as the theory of stationary perturbations.

2.1.1 Nuclear magnetic moment

The NMR phenomenon is related to the existence of nuclear paramagnetism caused by the orientation of the individual nuclear magnetic moments $\vec{\mu}$ in the presence of an external static magnetic field $\vec{B}_0$. The magnetic moment of a nucleus $\vec{\mu}$ is proportional to the nuclear spin angular momentum $\vec{I}$ according to

$$\vec{\mu} = \gamma \hbar \vec{I} \quad (1)$$

where γ is the magnetogyric ratio of the nucleus and $\hbar$ is Planck's constant divided by 2π .

In contrast with the electron, whose spin number I always equals $\frac{1}{2}$, the nuclear spin number I of a nuclei ${}^A_Z X$ depends on the mass number A and the charge number Z such as:

- If A is odd, then I is an half integer (${}^1\text{H}$, ${}^{13}\text{C}$, ${}^{27}\text{Al}$, ${}^{23}\text{Na}$);
- If A is even and Z is odd, then I is an integer (${}^{14}\text{N}$, ${}^2\text{H}$);
- If both A and Z are even, then I is zero and the nucleus is not active in NMR (${}^{12}\text{C}$, ${}^{16}\text{O}$).

The application of an external magnetic field $\vec{B}_0$, which we suppose in this thesis to be oriented along the z axis only, leads to a motion of precession of all nuclear magnetic moments around the z axis at the Larmor frequency (**Figure 2.1**) given by

$$\omega_0 = \gamma B_0 \text{ (rad s}^{-1}\text{) or } \nu_0 = \frac{\gamma B_0}{2\pi} \text{ (Hz)} \quad (2)$$

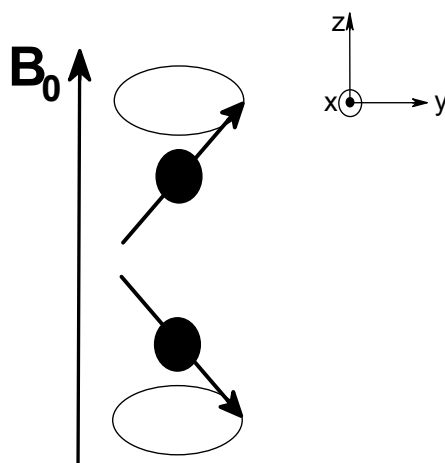


Figure 2.1. Motion of precession of the nuclear magnetic moments around the axis along which the external magnetic field is applied (by convention, the z axis).

In the classical description of NMR, the motion of precession does not affect the isotropic distribution of nuclear magnetic moments, and hence there is no net resulting magnetization. In contrast, the interaction of the nuclear magnetic moments with their environment (such as the thermal vibrations of the lattice) leads at equilibrium to an anisotropic distribution according to Boltzmann's law and hence gives a net longitudinal magnetization M_z (**Figure 2.2**).

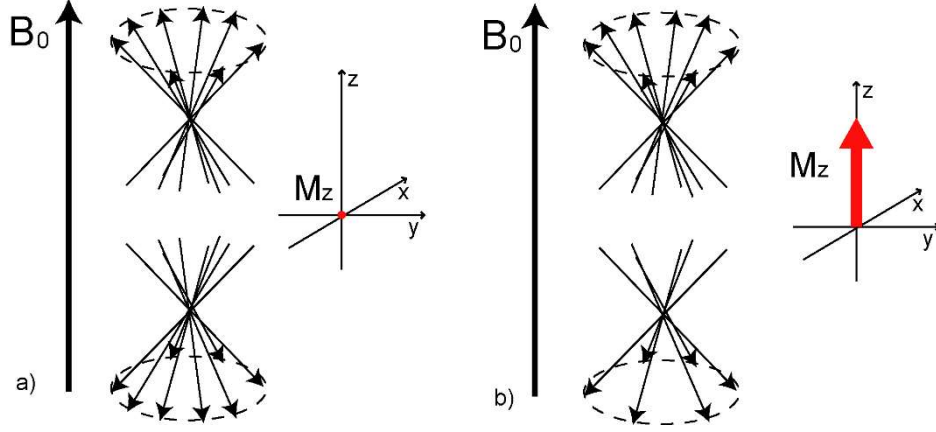


Figure 2.2. (a) Isotropic distribution of nuclear magnetic moments with no net resulting macroscopic magnetization; (b) Anisotropic distribution of nuclear magnetic moments with resulting net macroscopic magnetization, M_z , aligned the direction of the static magnetic field, $\vec{B}_0$.

The longitudinal magnetization appears progressively with time according to the following law

$$M_z = M_0 \left(1 - e^{-\frac{t}{T_1}} \right) \quad (3)$$

where T_1 denotes the spin-lattice relaxation time or the longitudinal relaxation time. Eq (3) implies that

$$\frac{\partial M_z}{\partial t} = -\frac{M_z - M_0}{T_1} \quad (4)$$

with M_0 the longitudinal magnetization at equilibrium at the temperature T given by Langevin's law

$$M_0 = N_0 \frac{\gamma^2 \hbar^2 B_0}{kT} \frac{I(I+1)}{3} = \chi_0 B_0 \quad (5)$$

where N_0 is the number of magnetic moments per unit volume, and $\chi_0 = \frac{M_0}{B_0}$ is the static magnetic susceptibility. At room temperature, the difference in population for the two orientations of the nuclear magnetic moments is very weak (e.g., it is of the order of 1 in 10^5 for ^1H spin in a magnetic field of 11.7 T) (Cavanagh et al., 2007).

2.1.2 Nuclear Magnetic Resonance phenomenon

The Nuclear Magnetic Resonance method consists in adding to the main static magnetic field $\vec{B}_0$, a secondary magnetic field $\vec{B}_1$, orthogonal to $\vec{B}_0$ and with $\|\vec{B}_1\| \ll \|\vec{B}_0\|$, which oscillates at the nuclear Larmor frequency. This second radio frequency field tips down the longitudinal magnetization to the xy plane, and hence generates a transverse magnetization M_{xy} . Then, the radiofrequency field is switched off and the magnetization progressively returns to its initial equilibrium position according to relaxation processes. The motion of precession of the magnetization while it returns to equilibrium induces a voltage in a coil placed perpendicularly to the main static field. This voltage constitutes the NMR signal and is called a Free Induction decay (FID): free of the influence of the radio frequency field, induced in the coil, and decaying back to equilibrium (**Figure 2.3**) (Sanders & Hunter, 1994).

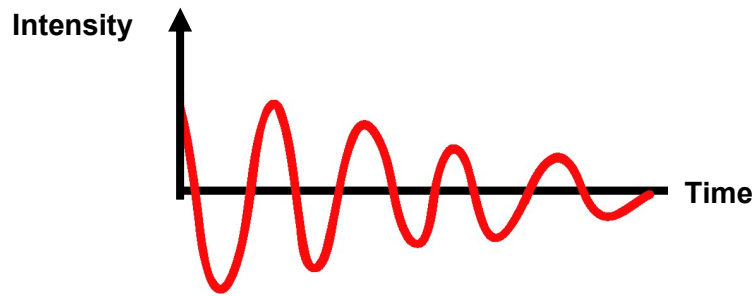


Figure 2.3. Schematic representation of a Free Induction Decay (off-resonance case).

Many relaxation mechanisms dictate the evolution of the transverse magnetization. To describe this evolution, a transverse relaxation time T_2 must be introduced. Transverse relaxation does not necessarily involve energy exchange but does however increase the entropy of the nuclear spin system. T_2 is defined by

$$\frac{\partial M_X}{\partial t} = -\frac{M_X}{T_2} \text{ and } \frac{\partial M_Y}{\partial t} = -\frac{M_Y}{T_2} \quad (6)$$

The system of nuclear magnetic moments can be associated with a macroscopic or bulk magnetization vector $\vec{M}$. In turn, the bulk magnetization vector $\vec{M}$ is related to a bulk angular momentum $\vec{J}$ through the nuclear magnetogyric ratio γ according to

$$\vec{M} = \gamma \cdot \vec{J} \quad (7)$$

Under certain conditions, the macroscopic behavior of the system of magnetic moments can be described by the laws of classical mechanics. The fundamental equation of motion is

$$\frac{\partial \vec{J}}{\partial t} = \vec{T} \quad (8)$$

where $\vec{T}$ is a torque. In the presence of a magnetic field $\vec{B}$, the torque exerted on the system of magnetic moments is given by

$$\vec{T} = \vec{M} \wedge \vec{B} \quad (9)$$

Thus, by combining eq (7), (8), and (9), we obtain

$$\frac{\partial \vec{M}}{\partial t} = \gamma \cdot \vec{M} \wedge \vec{B} \quad (10)$$

In this NMR experiment, the total magnetic field $\vec{B}$ is composed of two distinct components: an intense, static magnetic field oriented along the z axis, $\vec{B}_0$, and a weaker, oscillating field, $\vec{B}_1$.

Formally, the oscillating field $\vec{B}_1$ is linearly polarized along an axis that belongs to the xy plane. In the following, we assume this axis to be the x axis without loss of generality (Cavanagh et al., 2007). As described elsewhere (Sanders & Hunter, 1994), a linearly polarized magnetic field of amplitude $2B_1$ is equivalent to two counter rotating magnetic fields of amplitude B_1 rotating along the z axis. To 1st order, only one of these two counter rotating components can affect the bulk magnetization vector $\vec{M}$ (Cavanagh et al., 2007), and hence the other component will not be further considered here. In other words, the linearly polarized magnetic field $\vec{B}_1$ can be pictured as a magnetic field that rotates along the z axis with a frequency ω , an amplitude B_1 and an initial phase Φ . By definition, Φ is the angle formed between the x axis and the polarization axis of the $\vec{B}_1$ field, which, in our case, implies that Φ is zero.

In these conditions, if we suppose $\gamma > 0$, the total effective field acting on the bulk magnetization $\vec{M}$ is given in the laboratory frame $(\vec{e}_x, \vec{e}_y, \vec{e}_z)$ by

$$(B_1 \cos \omega \cdot t) \vec{e}_x + (-B_1 \sin \omega \cdot t) \vec{e}_y + B_0 \vec{e}_z \quad (11)$$

Thus, in the laboratory frame, the Bloch equations are given by

$$\begin{aligned} M'_X &= \gamma \cdot [M_Y B_0 + M_Z B_1 \sin(\omega t)] - \frac{M_X}{T_2} \\ M'_Y &= \gamma \cdot [-M_X B_0 + M_Z B_1 \cos(\omega t)] - \frac{M_Y}{T_2} \end{aligned} \quad (12)$$

$$M'_Z = \gamma \cdot [-M_Y B_1 \cos(\omega t) - M_X B_1 \sin(\omega t)] - \frac{M_Z - M_0}{T_1}$$

Additional simplification can be achieved by describing the NMR phenomenon in the so-called rotating frame, i.e. in an orthonormal frame that rotates along the z axis at the frequency of the radiofrequency field $\vec{B}_1$. In this case, if we further suppose the frequency of the radiofrequency field to equal the Larmor frequency of the nucleus (on-resonance case), both $\vec{M}$ and $\vec{B}_1$ appear fixed in the rotating frame. In other words, we have mathematically (and artificially) removed their time dependence.

We now focus on pulsed NMR and do not consider continuous wave NMR (see reference 4 for a more complete presentation). In pulsed NMR, an intense radiofrequency field $\vec{B}_1$ is applied for a very short period of time, typically a few μs . Because the relaxation times T_1 and T_2 are much larger than the pulse duration τ (note that, rigorously, T_1 and T_2 are not mere times but time constants), relaxation processes can be safely neglected during the pulse. Therefore, in the rotating frame, the effect of a radiofrequency pulse of duration τ and phase x (i.e. applied along the x axis) can be pictured as the rotation of the bulk magnetization vector $\vec{M}$ around the x axis through an angle θ of $\gamma B_1 \tau$ (**Figure 2.4**).

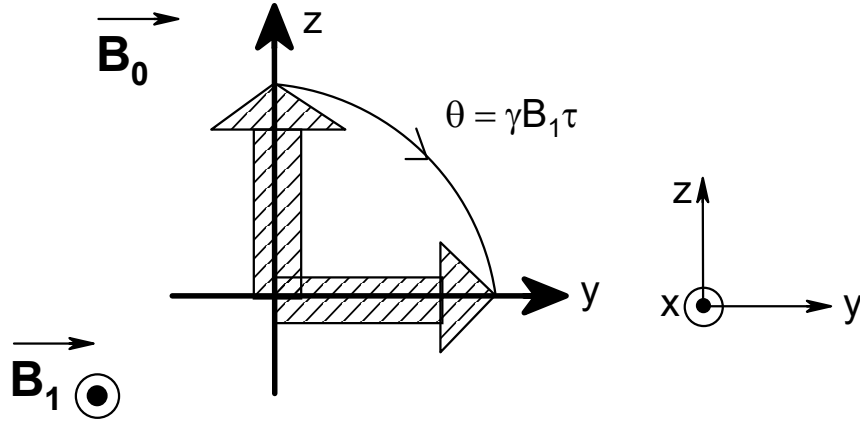


Figure 2.3. Schematic representation of the effect on the longitudinal magnetization vector $\vec{M}$ (represented by the large dashed arrow) of a radiofrequency pulse of duration τ applied with a x phase: $\vec{M}$ is rotated along the x axis of an angle θ that depends on the radiofrequency pulse amplitude B_1 and duration τ . Here γ is supposed to be positive.

One of the advantages of the pulsed method with respect to the continuous wave method, relies on the possibility to repeat each measurement in a faster way (typically every second).

It then becomes possible to coherently add the results of n measurements, hereby increasing the signal-to-noise ratio by a factor of $\sqrt{n}$ (Ernst et al., 1989).

2.2 The NMR spectrometer

After the general description of the theoretical principles on which NMR spectroscopy is based, in this section a brief explanation of the hardware and software that constitute the NMR spectrometer is provided, in order to have a more practical idea of all the steps that occur during an NMR experiment. The description is very generic and intuitive, since a detailed description of the hardware devices and the phenomena associated with their utilization would require a very longer space.

In Figure 2.5 a schematic representation of a single-channel NMR spectrometer, including a one pulsed field gradient channel, is presented:

- The magnet: NMR experiments require a homogeneous magnetic field that must be maintained over the entire volume of the sample, and also be extremely stable with respect to time. A perfectly uniform magnetic field avoids inhomogeneous broadening of the NMR signal and allows the resolution of small differences in the nuclear Larmor frequency, due to chemical shifts, spin-spin couplings, or other molecular interactions. All NMR spectrometers employ superconducting magnets, capable of supporting large electric currents without any external source of power. Once charged with current, a superconducting magnet runs almost indefinitely, providing an extremely stable magnetic field with no outside interference. Superconducting materials (Nb-Sn) must be cooled at the boiling point of liquid He (4.18 K) to support large magnetic fields without electrical resistance. The liquid He bath is itself insulated by a large reservoir of liquid N₂ (77 K). In the center of the cylindrical can, and through the center of the superconducting coil, there is a large hole called the bore. The sample is mounted in a cylindrical device called a probe, that is inserted into the bore to position the sample at the point of maximum field.
- The radio frequency (r.f.) synthesizer (1): this component of the spectrometer produces an oscillating electrical signal with a well-defined frequency. This electronic signal is usually subjected to several frequency conversion and

electronic filtering steps. The result is a r.f. wave that oscillates at the spectrometer reference frequency ω_{ref} . The phase of an oscillating wave indicates the position ($0, \pi/2, \pi, 3\pi/2$) of the oscillation at the time origin. In many NMR experiments, the r.f. phase is rapidly jumped between different values. In the NMR spectrometer, these phase jump events (2) are controlled by a timing device called pulse programmer (4).

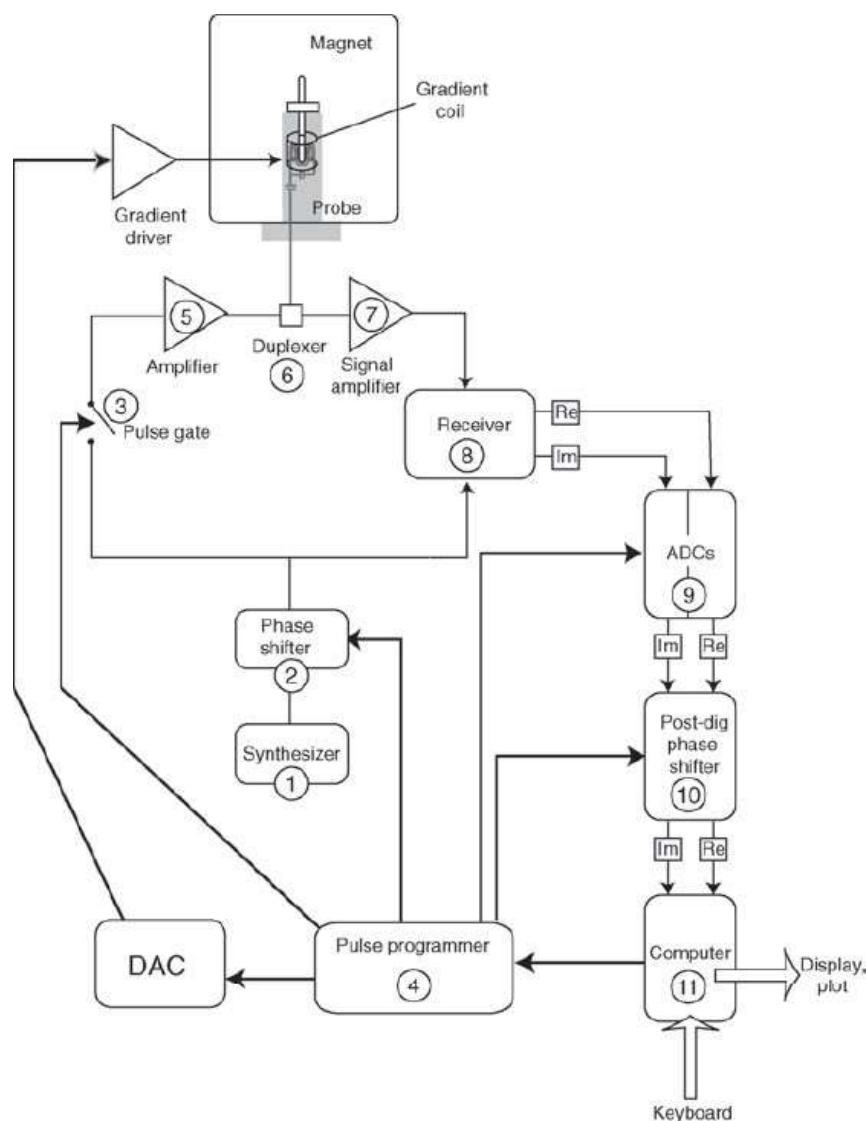


Figure 2.5. Schematic overview of a single-channel spectrometer, incorporating one pulsed field gradient channel.

- The pulse gate (3): this is a fast switch that is opened at defined moments to allow the r.f. reference wave to pass through. The effect is to chop a 'time-slice' out of the r.f. waveform. The duration of an r.f. pulse is sometimes referred to as the pulse width. These events are controlled by the pulse programmer.

- The amplifier (5): the function of the r.f. amplifier is to scale up the gated waveform so as to produce a large-amplitude r.f. pulse for transmission to the probe. Typical r.f. amplifiers for NMR applications have ratings between several watts and about 1 kW of peak output power.
- The duplexer (6): the amplified r.f. pulse travels down a cable into the duplexer. From the duplexer, two more cables emerge. One cable leads to the probe, which is mounted inside the magnet, and which contains the sample. The second cable leads to the receiver section, which is used to detect the weak r.f. signals generated by the nuclear spins. When a strong r.f. pulse arrives from the amplifier, the duplexer diverts it down the cable leading to the probe, not into the sensitive signal detection circuitry. On the other hand, the tiny NMR signal travels down the same cable from the probe to the duplexer and it is diverted into the signal detection path.
- The probe: the probe is the most specialized part of the NMR spectrometer and is often the only part that needs to be exchanged when switching between different NMR experiments. This piece of the apparatus has several functions such as locating the sample in the region of homogeneous magnetic field, includes r.f. electronic circuits for irradiating the sample with r.f. waves, rotating the sample. The probe electronic is formed by a r.f. circuit consisting of a coil and two capacitors. The function of the coil is twofold. First, during the r.f. pulse, electrical currents in the coil generate an oscillating magnetic field at the sample; second, when the r.f. pulse has terminated, the precessing nuclear magnetization generates electrical currents in the coil that give rise to a detectable signal travelling back down the cable again towards the duplexer. In a very general way, the two capacitors have the function of making stable the incoming and the outgoing electronic signals.
- Signal preamplifier (7): the NMR signal first arrives at the duplexer, that diverts it down the cable towards the signal preamplifier. This is a low-noise r.f. amplifier that scales up the tiny signal to a more convenient voltage level.
- The quadrature receiver (8): the NMR signal must be fed into the computer for interpretation and presentation. Therefore, it is necessary to convert the NMR signal (an oscillating electrical current) into digital form (a sequence of 'ones' and

'zeros'). The process of converting a continuous current or voltage into digital form is called analogue-to-digital conversion. This task is accomplished by specialized electronic circuits called analogue-to-digital converters (ADCs). Anyway, the 'raw' NMR signal oscillates at many hundreds of megahertz, that is too fast for current ADCs. Therefore, it is necessary to 'down-convert' the frequency of the NMR signals, so that they may be handled by the digital electronics at the next stage. The quadrature receiver accomplishes the signal frequency conversion by comparing it with a reference wave of frequency, as supplied by the r.f. synthesizer. The quadrature receiver combines the NMR signal, that oscillates at the Larmor frequency, with the reference signal, to generate a new signal that oscillates at the relative Larmor frequency, usually of the order of 1MHz or less. In order to distinguish between spins that rotate faster or slower respect to the Larmor frequency, the receiver supplies two output signals that are interpreted as the real and imaginary components of a single complex signal.

- Analogue-digital converter (9): each of the two outputs of the quadrature receiver are connected to their own ADC. This is an electronic circuit that rapidly measures the voltage level of an input signal and presents the information as a digital signal, by repeating the measurement at a set of consecutive time points and storing the information in the computer as an ordered set of values. This is called digitization.
- Post-digitization phase shifter (10): this device which multiplies the signal by a complex phase factor before it is passed to the computer. This operation corrects the NMR signal phase variations that can occur during the experiment phases.
- Digital-to-analogue converter (DAC) and gradient driver: many NMR experiments require the application of pulsed static magnetic fields, as well as r.f. fields. In most spectrometers, each magnetic field gradient is controlled by a separate channel, that resembles the one used in the r.f. section. Each gradient channel consists of a digital-to-analogue converter (DAC) and a gradient driver. The DAC converts timed digital instructions from the pulse programmer into an analogue voltage level. It therefore performs the inverse function of the ADCs used in the receiver circuit. The gradient driver is a powerful amplifier that

converts the analogue input voltage into a strong, well-controlled current. This electric current is driven through the gradient coils, generating the desired magnetic field gradient pulse. These circuits allow each gradient pulse to be shaped accurately as a function of time.

2.3 NMR experiments

All the operations described in the previous paragraph are exploited to create different NMR pulse sequences, on the basis of which it is possible to generate different types of experiments and thus obtain different types of information about the analyzed sample (Jacobsen, 2007). In this section, a brief description of the main NMR mono (1D) and two-dimensional (2D) experiments that can be very useful for metabolomics studies is proposed:

- 1D ^1H : the principle of all mono-dimensional experiments is based on the theory and the practical description previously described. Basing on the selected nucleus, the proper r.f. pulse and field adjustments are carried out. ^1H NMR experiments are of sure the most used and the most indicative, since basing on the chemical shifts, signal shape, and J -coupling constants it is possible to obtain much information on the sample chemical features.
- 1D ^{13}C : together with ^1H , ^{13}C nucleus is very informative for structural determination. Anyway, since the isotopic abundance of this nucleus is very low and the coupling interactions with ^1H generate high-lined multiplets in the spectra, the NMR experiments for this nucleus are slightly sensitive and the signal to noise ratio is very low. For these reasons, ^{13}C experiments are carried out by applying particular pulses that enhance the signal intensity namely decoupling and NOE (Nuclear Overhauser Effect).
- 2D CORRELATION SPECTROSCOPY (COSY): this is the first and the simplest 2D experiment. It correlates one proton to another via a single J coupling that may be 2-bond (geminal), 3-bond (vicinal) or in rare cases 4-bond or 5-bond (long range).
- 2D TOTAL CORRELATION SPECTROSCOPY (TOCSY): Provides ^1H - ^1H spin-

spin coupling connectivity for all hydrogen atoms within a spin system that forms an unbroken chain of couplings, and not just to the protons connected by a single J coupling, thereby unravelling the number and nature of the molecular fragments.

- 2D HETERONUCLEAR SINGLE QUANTUM COHERENCE (HSQC): this experiment shows correlations between hydrogen atoms and the carbon atom to which they are attached.
- 2D HETERONUCLEAR MULTIPLE BOND CORRELATION (HMBC): provides correlations between hydrogen and a heteronucleus generally two or three bonds away.

2.3.1 NMR data processing

After finishing acquiring the NMR data, it is necessary to process the data into a spectrum and plot that spectrum on paper with a ppm axis, and possibly with integrals, peak lists, and other features. Each NMR instrument has its own software for data processing, and it can be daunting to try to learn all of the different commands and operations. The actual data processing task, however, is the same in all cases. Starting with a FID (raw time-domain data), the following operations are generally applied:

- With zero filling the size of the data set can be artificially increased by adding zeroes to the end of the list of FID data. This process of zero filling has no effect on the peak positions, intensities, or linewidths of the spectrum, but it does increase the digital resolution (fewer hertz per data point) in the spectrum.
- Multiply the FID by a multiplier (line-broadening parameter) or window function. This operation allows to increase the signal to noise ratio but on the other hand, broader signals are obtained. So, this operation has to be regulated basing on the quality of the obtained spectrum.
- Fourier transform converts the time domain data to a frequency domain spectrum. In fact, the raw NMR data obtained after an experiment is in the form of a series of intensity values collected as a function of time that die out gradually as the nucleus recover from the pulse and relax. In this form, the FID

cannot be interpreted because of the complexity of the FID. Fourier transform is a mathematical function that converts the time-domain data into a frequency-domain data, the NMR spectrum. The obtained spectrum allows to observe each type of detected frequency as a single signal well distinguished from the others thanks to the chemical shift phenomenon. Considering a mono-dimensional experiment, these signals.

- Phase adjusting of certain unavoidable instrumental errors involved in acquiring the FID.
- Find a reference standard peak and set its chemical shift to the reference value in parts per million. Generally, this operation is carried out by referring to the internal standard or residual solvent peak.
- Baseline adjustment. The “baseline” is the average of the noise part of your spectrum. Ideally, this would be a straight, horizontal line representing zero intensity. In the practice, this line not always reflect these characteristics so, basing on the used software, several correction operations can be applied to adjust this line.
- Integration of peaks area for quantitative measurements.

For further information about the theoretical and practical aspects of NMR, several texts have been developed during the time (Abragam & Hebel, 1961; Canet et al., 1991; Cavanagh et al., 2007; Ernst et al., 1989).

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PART A

Summary Part A

Nuclear Magnetic Resonance spectroscopy is a powerful methodology used to analyse food metabolites. It is helpful in determining the chemical composition, verifying food types and sources and detecting adulteration. Non-target NMR, which includes metabolic profiling and metabolic fingerprinting, enables a comprehensive analysis including specific and non-specific metabolites.

In particular, untargeted NMR-based metabolomics was exploited to acquire the complete metabolic profiles of burdock roots, lemon balm aerial parts and dandelion roots and aerial parts. This analytical method highlighted the impact of different altitudes and agronomic practices (spontaneous or organic cultivation) on the chemical profile. The analysis of plant samples revealed the presence and quantities of various compounds such as sugars, amino acids, organic acids, polyphenols, and fatty acids. Certain metabolites were found to be specific to particular tissues, for instance, arginine was exclusively found in the roots, while myo-inositol, galactose, glyceryldigalactose, pheophytin, and chlorophyll were identified in the aerial parts. Additionally, specific polyphenols, such as caftaric and chicoric acids, 3,5-di-caffeoylquinic acid, chlorogenic and rosmarinic acids were detected in dandelion, burdock, and lemon Balm, respectively. The quantities of metabolites varied significantly based on the plant's growth conditions, tissue type, and ecotype. Notably, all burdock ecotypes exhibited the highest amino acid content and the lowest organic acid content, while lemon balm displayed the opposite trend. Dandelion parts contained high levels of carbohydrates, with the exception of the aerial part, which exhibited the highest content of organic acids. These findings provided valuable insights into the chemistry of medicinal plants, thereby supporting further research in the nutraceutical and phytopharmaceutical fields.

A more in-depth study looked at wild lemon balm, named *Melissa officinalis* subsp. *altissima* (MOA), collected in Corsica. In this case, in addition to knowledge of the phyto-complex by NMR, studies were conducted on the obtained essential oil of the plant by applying GC-MS. Corsican lemon balm was found to be particularly rich in polyphenols (rosmarinic, chlorogenic, caffeic and caftaric acids), which express its antioxidant activity, and compounds such as Germacrene-D, with antibacterial and antifungal properties. Furthermore, the biological activity of plant extracts against neuroinflammation was evaluated. Specifically, hypothalamic Hypoe22 cells were exposed to MOA extracts under basal conditions and were tolerated, demonstrating good biocompatibility and efficacy in protecting cells from H₂O₂-induced cytotoxicity. The gene expression of inflammatory

and neurotrophic mediators such as TNF- α (Tumour necrosis factor), NOS-2 (Nitric oxide synthase-2) and BDNF (Brain-derived neurotrophic factor) in brain samples of C57/BL6 mice (prefrontal cortex and hypothalamus) treated with hydroalcoholic extracts of MOA was then assessed. In addition, the cortical tissues of the mice were exposed to β -amyloid to subsequently assess the expression of IL-6 (Interleukin-6) and ACHE (Acetylcholinesterase) genes during treatment with hydroalcoholic extracts of MOA. All extracts tested were effective in reversing the increased expression of TNF- α and NOS-2 and counteracting the down-regulation of the BDNF gene, suggesting potential protective effects on neuroinflammation. After β -amyloid stimulation, MOA extracts were efficient in inverting the increased expression of IL-6 and ACHE genes, showing potential protective effects on memory impairment and metabolic alterations in Alzheimer's disease.

Metabolomics plays a crucial role in monitoring food safety and quality by providing detailed information on the chemical composition of food products. By analysing metabolite profiles, harmful contaminants can be detected, spoilage markers can be identified, and the authenticity of food can be assessed. This approach allows early identification of potential food safety problems, such as pesticide residues or microbial contamination, thus ensuring consumer protection. Furthermore, metabolomics can help assess the nutritional quality of food, facilitating the development of healthier products. As part of Agritech consortium (National Research Centre for Agricultural Technologies), based on an extensive literature search on traditional and novel plant-based foods, fruit and vegetable matrices were listed and classified. Two products with high safety risks (pesticides, contaminants) were chosen as pilot matrices: tomatoes and apples.

Food packaging plays a critical function in the preservation of fresh food, protecting it from physical, chemical and biological contaminants. Although petroleum-based plastics such as polypropylene are commonly used due to their cost-effectiveness and effectiveness, their non-biodegradable nature poses risks to both humans and the environment. Alternatively, biodegradable materials made from biopolymers such as cellulose, chitosan, starch, polylactic acid (PLA), collagen and casein are being investigated for use in the food industry. These materials must fulfil specific criteria, such as non-toxicity and renewability, to be viable options for food packaging. In this specific case, non-target NMR analysis was used to obtain a complete metabolic profile of samples of Golden Delicious apples supplied by Consorzio Melinda S.c.a. (Cles (TN) - Val di Non - Italy).

The aim of the study was to evaluate the effect of two biodegradable packaging materials (ECO-PLA) on the quality and safety of fresh apples during cold storage (21 days, 5 °C) compared to packaging in conventional polymer reference bags (REF).

Metabolic profiling of the ^1H NMR spectra of the hydroalcoholic and organic extracts identified 23 compounds in the apple samples at 14- and 21-days cold storage in the three packages. The metabolic components identified included primary and secondary sugars, metabolites of amino acids and organic acids, fatty acids, sterols and other compounds. In principle, cold storage delayed ripening and senescence processes, in particular the reduction of catalytic activities, including enzymes involved in the respiration pathways, which influence primary and secondary metabolism.

This included the “Pomodoro Riccio” tomato, an old cultivar, which is particularly suited to growing on clay soils, with a low water demand. As water is a primary source of heavy metals and pesticide residues, this cultivar had the potential to improve tomato quality, which emerges as a priority issue for consumer safety. Samples of “Pomodoro Riccio” (whole tomato, tomato paste and tomato waste), collected in 2022 and 2023 years, were provided by the company ‘La Sbecciatrice’ (Caserta, Italy). Considering all the characteristics of the cultivar of interest, the aim of the study was to delineate the metabolic profile and possible identification of new and (re)emerging hazards of a particular tomato ecotype. Specifically, NMR analysis was applied to delineate the metabolic profiles of the same cultivar grown in two different years. Indeed, in addition to ripeness, which may be related to the time of harvest, factors influencing the nutritional composition of tomatoes may be variety, climate, location and agricultural practices. Higher concentrations of amino acids were found in tomatoes grown in 2022. In contrast, tomatoes from the second year of harvest had the highest sugar levels, the most abundant metabolite class measured in all samples. The organic acids total content was more or less similar between the samples according to the years. Considering that the content of sugars and organic acids are the main factors in the taste of tomato fruits, and that catabolites of organic acids are involved in the biosynthesis of amino acids, the “Pomodoro Riccio” harvested in the second year were sweeter than those grown in the first year. Furthermore, although in lower concentrations, the tomato waste had significant amounts of bioactive compounds, which could be used to improve sustainability, e.g. in fermentation processes.

In a different study, NMR metabolomic was applied for the analysis of protein hydrolysates of cauliflower and artichoke waste, which were exploited for the formulation of biostimulants. Specifically, hydrolysates (HY) were characterised chemically and biologically, identifying and

quantifying amino acids, organic acids, amines, polyols, mineral elements, phenols, tannins, flavonoids and sulphur compounds by NMR, inductively coupled plasma Mass Spectrometry and UV-visible analysis. The HY of cauliflower leaves and flowers showed the highest concentrations of free amino acids, while the stems were rich in calcium. In the case of artichoke, asparagine, glutamine and aspartic acid were found exclusively in the stems, while the leaves showed high levels of magnesium and manganese, as well as significant antioxidant activity. Diluted HYs were tested as biostimulants at five concentrations to evaluate their effects on seed germination and early growth of clover, alfalfa, durum wheat and maize. It was observed that the artichoke biostimulant positively influenced the germination speed of alfalfa, clover and durum wheat, whereas the cauliflower biostimulant significantly increased the germination speed of maize.

Chapter A1

Metabolomics in food science

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A1.1 NMR metabolomics in food science

A1.1.1 The Foodomics

Foodomics is a discipline that studies the worlds of food and nutrition through the application and integration of advanced omics technologies (Cifuentes 2009). Specifically, it is concerned with assessing the effect of different factors on the genome, transcriptome, proteome and metabolome of biological food sources, starting with the growth and development of the corresponding species, and then going on to define the evolution of food during digestion, absorption and interaction with humans, looking at nutrition and health from a food perspective (León, Cifuentes, and Valdés 2018). Many works demonstrated the possibilities of Foodomics to boost food science investigations, including those related to accelerating the resolution of food safety problems, improving food quality and traceability (Laghi, Picone, and Capozzi 2014).

A1.1.2 NMR approaches for food analysis

Characterization of food metabolites by NMR spectroscopy is a very powerful tool, as the application of this methodology can address several food-related issues, such as characterization of chemical composition, authentication (variety, geographic origin, year of harvest/production), and potential adulteration (Sobolev et al. 2017).

Following a targeted approach, NMR analysis is limited to the identification and quantification of specific compounds, chosen based on prior knowledge of the system under investigation. For this reason, sample preparation and extraction are carried out in order to concentrate targeted metabolites. This approach is mainly applied to identify the compounds responsible for certain peculiar characteristics of the food product analysed, thus characterizing its origin, variety or identifying adulterations (Monakhova et al. 2015).

Among untargeted NMR approaches, metabolic profiling, metabolic fingerprinting, and metabolomics has been developed as complex samples characterization approaches. Metabolic profiling consists in identification and quantification of a number of selected metabolites, belonging to various classes of compounds, in a given sample often without a separation procedure. Metabolic fingerprinting is used when classification of samples without recognition of individual specific metabolites is sufficient: in this case, the NMR spectrum can

be considered as a fingerprint of the foodstuff and all the NMR resonances are measured without any identification.

All these approaches require a specific and well-studied methodology that comprise not only the NMR experiment but also the numerous phases that occur before and after the NMR analysis (Sobolev et al. 2019). **Figure A1.1** schematically explains all the phases that constitute a typical NMR metabolomics workflow for the study of a complex matrix such as food.

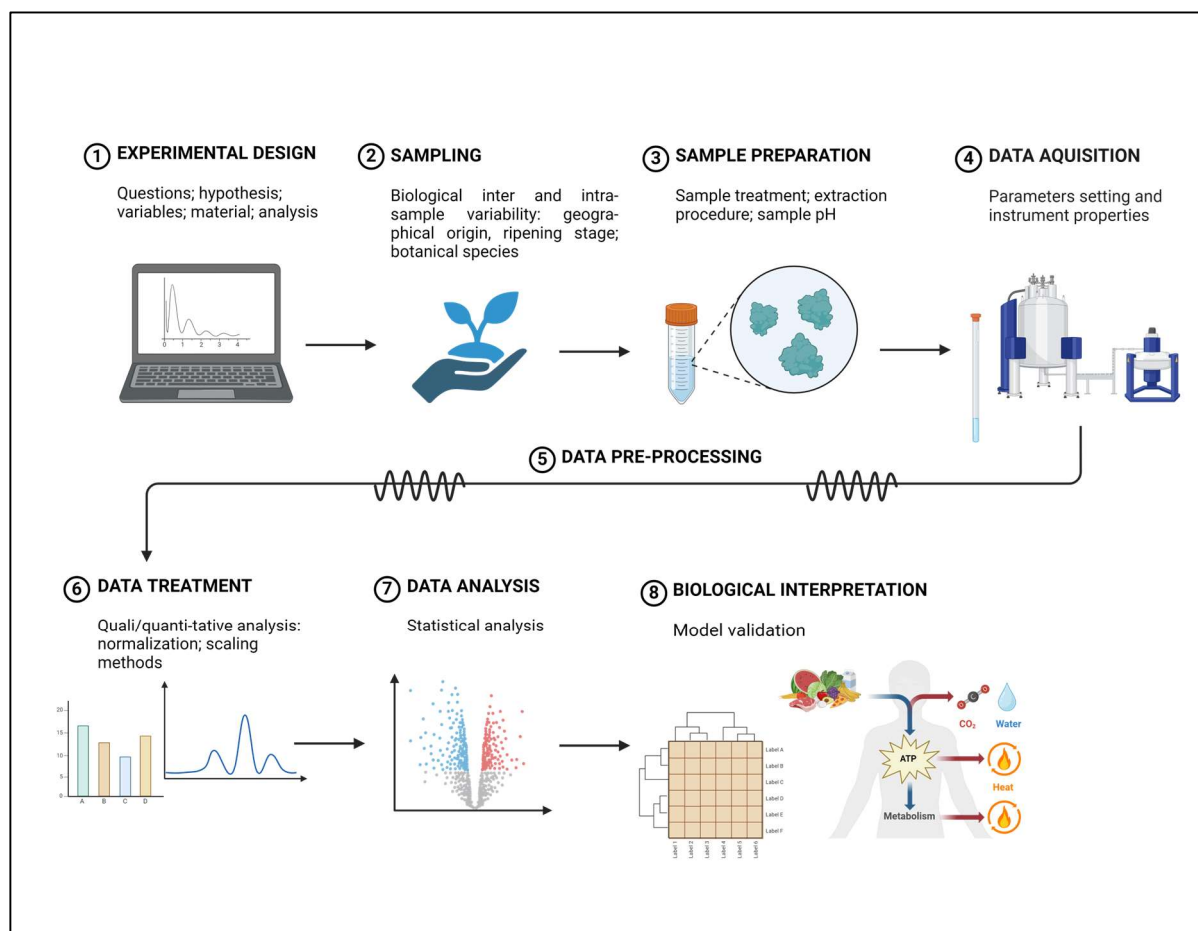


Figure A1.1. Representative NMR metabolomics workflow and sources of variability for the study of a complex matrix such as food.

A1.2 NMR metabolomics workflow

A1.2.1 Experimental design

The first step involves experimental design, which is very important because all experimental aspects, from sampling and collection size to data analysis, must be considered to maximize the chances of answering the research questions of interest as clearly and efficiently as

possible. Well-designed experiments, together with appropriate analytical data and statistical analysis, enable researchers to increase the robustness and validity of experimental results (Smolinska et al. 2012; Dunn and Ellis 2005).

A1.2.2 Sampling

Proper sampling is a crucial point of any analytical protocol. In the case of food products, from the choice of sample characteristics, in-depth knowledge of metadata, including growing conditions, production practices, botanical characteristics, storage, and so on, is important, as the final results will be closely related to the properties of the sample (Ben-Zvi, Bakker, and Makar 2015). Storage after sampling requires proper care to preserve food quality and composition. Indeed, some operations such as harvesting for vegetables and slaughtering for animals may be followed by enzyme attack and other degradation processes. Freezing in liquid nitrogen followed by dark storage at low temperature (-20° C or -80° C) are the most common procedures used to avoid chemical or enzymatic degradation. The second aspect of sampling to consider concerns sample processing within the laboratory, prior to extraction or direct analysis. Indeed, as pointed out earlier, food is a complex matrix in which the spatial distribution of metabolites may not be homogeneous.

If it is necessary to analyse just a part of sample, since it is impossible to put in the instrument the sample as a whole (this is the most common case), it is important to perform the sampling in a correct way. For instance, it can be chosen to take different parts of the foodstuff to have a “representative” sample to analyse, or the whole sample pool can be homogenised to uniformize the whole foodstuff. This concept has to be mainly applied for solid samples, since liquid ones are homogeneous matrices in each “point”. Liquid samples such as olive oils, vinegars, wines, fruits juices are generally analysed without any treatment, whereas dilution for a viscous liquid such as honey is recommended. Anyway, in some cases, liquid samples can be dried with nitrogen flux or lyophilization in order to eliminate the solvent presence (water or alcohol), whose NMR signals could cover the signals of other components (Zhu et al. 2020; Hu et al. 2020). In the case of solid foodstuffs, after sample homogenization and/or disruption, an extraction with a suitable solvent has to be performed.

A1.2.3 Sample extraction

The choice of the solvent is very important since it determines the metabolites that are extracted and thus analysed. If a targeted analysis has to be carried out, the proper solvents and/or pH values represent a crucial point. Acetic acid/H₂O or alcohol/H₂O solutions are generally used for extracting specific target compounds, such as polyphenols (Sridhar et al. 2021; McDougall, Martinussen, and Stewart 2008). Moreover, chloroform/ethyl acetate/2-butanol mixture is used to extract aromatic compounds (Sridhar et al. 2021). Solid phase extraction (SPE) also represents an important strategy to concentrate and isolate specific compounds, eliminating interfering molecules. For instance, in the case of a NMR study of blueberries, the SPE has allowed to reduce the sugar content and to provide NMR characterization of anthocyanins not easily identifiable in aqueous solution (Capitani et al. 2014). On the contrary, when an untargeted NMR analysis has to be carried out, the extraction of the whole metabolite pattern is recommended. In this case, a biphasic extraction system, generally formed by a methanol/H₂O/chloroform mixture (BLIGH and DYER 1959) in different proportions is often used to extract both hydrophobic and hydrophilic compounds. This extraction protocol has been largely applied for the metabolomic study of several foodstuffs such as vegetables and fruits (Sobolev et al. 2018; Capitani et al. 2014; Piccioni et al. 2009) and animal derived matrices (Jung et al. 2010; Mannina et al. 2008).

A1.2.4 Sample preparation for NMR analysis

Sample preparation for NMR analysis represents one of the steps that have to be normalized and clearly indicated. In the case of both liquid and solid samples, there are some important aspects that have to be considered to obtain reproducible ¹H spectra without undesirable chemical shift variations between samples. Among these aspects, variation in pH between aqueous samples can cause a significant difference in the chemical shifts of signals belonging to organic acids, amino acids, and other metabolites with acidic or basic functional groups (Fan 1996). Two different approaches to solve this problem can be applied: control of pH (buffered solution or pH adjustment) and specific data processing. Phosphate buffer is frequently used to stabilise pH in the range between 6 and 7, whereas oxalate buffer used for more acidic solutions (pH ~ 4). Both buffers do not give any additional proton signals in the

spectrum. The other approach to control the pH is represented by the addition of small volumes of hydrochloric acid or sodium hydroxide solutions basing on the starting pH value (Avenozza et al. 2006). The data processing approach to eliminate pH induced variations consists in using exponential multiplication of free-induction decays with high values (10–50 Hz) of line broadening factor (Duarte et al. 2004). Anyway, this last method causes a decrease in spectral resolution and only small chemical shifts differences can be levelled.

The liquid state NMR requires the presence of deuterated solvent in the sample to provide an independent from proton channel field-frequency lock signal and a signal on which resolution adjustment can be carried out. Liquid food samples can be submitted to NMR analysis upon the addition of a small fraction of deuterated solvent to provide the lock signal for NMR. When samples are viscous or dry, they have to be dissolved in deuterated solvents. The deuterated solvents commonly used in NMR food analysis are D₂O, methanol-d₄, DMSO-d₆, acetone-d₆, acetonitrile-d₃, and chloroform-d (Fulmer et al. 2010). The choice of solvent is determined by the nature of the sample, its preparation and solubility. For polar extracts usually D₂O, methanol-d₄ or a mixture of both of them are used. Deuterated chloroform is usually used for non-polar extracts. It is important to consider that, when a different solvent is used, metabolites chemical shifts variations occur. It is noteworthy that the presence of paramagnetic cations (Fe³⁺, Mn²⁺) in plant derived aqueous extracts and foodstuffs can affect the signals of several organic acids (citric, malic) capable of chelating metal, causing a strong broadening of signals. This source of broadening of NMR signals can be avoided by adding ethylene diamine tetra-acetic acid (EDTA) to the supernatant to capture the paramagnetic ions.

Finally, the addition in the solvent a soluble internal standard, such as tetramethylsilane (TMS), trimethylsilylpropanoic acid sodium salt (TSP), and sodium trimethylsilylpropanesulfonate (DSS), can be useful to have chemical shift reference and/or to make a quantification analysis if the standard concentration is well known.

A1.2.5 Data acquisition, pre-processing and pre-treatment

In order to avoid chemical shift variations as much as possible during NMR experiments, it is important to make considerations about temperature control and instrumental drift. Within a set of samples, it is important to perform the NMR experiments using the same temperature,

with most of the ^1H NMR spectra of foodstuffs being acquired in the 25-27° C range of temperatures. When the temperature used in NMR experiment is not reported, the value of the results is drastically reduced. Instrumental drift (small gradual diminishing of the magnetic field strength of an NMR instrument), except in the most extreme circumstances, does not impact comparability of data. A modern instrument can expect to have a magnetic field drift of less than 1 Hz per hour (Evans et al. 2013).

Regarding the NMR experimental acquisition and process parameters that have to be properly adjusted before and after an NMR experiment, these aspects have been discussed previously. The identification of metabolites represents one of the most critical phases of the NMR metabolomics study. While the structural assignment of isolated organic compounds can usually be successfully performed by NMR, the NMR analysis of foodstuffs is challenging because these samples, as all complex biological matrices, include a myriad of compounds (saccharides, polysaccharides, lipids, peptides, amino acids, organic acids, etc.), that are present in a large range of concentrations. These very complex mixtures inevitably give rise to ^1H NMR spectra that are highly crowded due to the presence of numerous, often multiplet-resonances due to scalar couplings. In this context, the NMR analysis of complex mixtures represents an active field of research, not exclusively restricted to the study of foods samples. Various approaches for the structural assignment of compounds present in complex mixtures have been developed (McKenzie et al. 2011; Novoa-Carballal et al. 2011) and, regarding the NMR analysis and structural assignment of foodstuffs samples, two main approaches are generally applied: spectral comparison using databases of NMR spectra (database matching approach) or application of 1D and 2D/nD NMR experiments (NMR elucidation approach). In the database matching approach, similarly to what happen with other analytical methods, each compound in a mixture can be identified by its characteristic signals in the NMR spectrum after comparison with the NMR spectrum of the pure compound. One of the main limitations of this approach is that an exhaustive database should be available, otherwise it would require an enormous number of compounds to be synthesized or isolated from natural sources. In order to solve this limitation, in the last years, several publicly available databases of NMR metabolite standard measurements (HMDB, MMCD, $^1\text{H}(^{13}\text{C})$ -TOCCATA) have been developed (Tulpan et al. 2011). Anyway, further limitations are represented by the variation of NMR spectral parameters (chemical shifts, relaxation times, etc) that can occur when the compound is analysed in a mixture, as opposed to alone in solution, due to interactions with

other molecules or, more simply, due to changes in experimental conditions (ionic strength, pH, temperature, solvent, etc.). In this case, it is clear that some chemical shift values can be different from the values found in the library database, thereby precluding unambiguous identification.

In the NMR elucidation approach, together with 1D experiments, correlation 2D experiments between the proton signals of the same molecule (^1H - ^1H COSY, ^1H - ^1H TOCSY, ^1H - ^1H NOESY) or between ^1H and other nuclei, generally ^{13}C (^1H - ^{13}C HSQC and ^1H - ^{13}C HMBC), are carried out, in order to identify the chemical structure of the considered compound and so its identity. This type of approach is time consuming since these 2D experiments require more time to be acquired, as well as the compound structure elucidation. The correct identification of a compound can be very difficult, mainly when its presence in a given foodstuff has not been previously documented. For this reason, when possible, it is useful to confirm assignments by directly adding an appropriate amount of a pure chemical standard to the investigated sample, once its presence has been inferred or suspected. Moreover, it is recommended to compare the NMR assignment with data achieved by other techniques (mass spectrometry, chromatography, UV/Vis and IR spectroscopy) on the same analysed foodstuff, as only the combination of several analytical techniques can reliably unravel the complexity of foodstuffs (Bernal et al. 2011).

Apart from qualitative and structural information, robust quantitative data can be obtained from NMR spectra. The main advantage of NMR for quantification purposes is the possibility to quantify, with a good accuracy and reproducibility, a large number of metabolites in a given sample using a single experiment, without the necessity of creating calibration curves (Dais et al. 2007). Indeed, ^1H NMR is a quantitative technique owing to the proportionality of signal integral to the molar concentration of the corresponding metabolite. Generally, two quantification strategies can be applied: absolute quantification provides the amount of metabolites in a given sample, whereas relative quantification provides the percentage of a given metabolite in the mixture.

When an absolute metabolite quantification is required, the duration of recycle time and number of scans to reach a suitable signal to noise ratio have to be optimised in each experiment to obtain reliable and accurate results. NMR relaxation properties of metabolite nuclei are the key factors influencing the accuracy of metabolite quantification. Generally, a recycle time of at least five times the longitudinal relaxation time (T_1) of the slowest relaxing

nuclei is required to allow a complete relaxation of all nuclei in metabolites and internal standard molecules. When extremely accurate quantitative measurements are required, T1 measurements are usually performed on the sample of interest to set up the proper recycle time which essentially provides a complete nuclear relaxation. On the contrary, when a comparison between samples is needed without accurate quantifications, a recycle delay of 5-10 s is generally applied, in order to have a good signal to noise ratio. The traditional widely used quantification method is the integration of a specific signal area vs. the signal area of a reference compound, whose exact quantity has to be known. In aqueous solutions, trimethylsilylpropanoic acid sodium salt (TSP), and sodium trimethylsilyl propanesulfonate (DSS) have been mainly used, whereas hexamethyldisilane has been proposed for organic solutions, since tetramethylsilane (TMS), generally used as a chemical shift reference is not stable because of its high volatility. Anyway, other “new” internal standards have been proposed (Rundlöf et al. 2010). An alternative to the use of internal standard is represented by the Electronic REference To access In vivo Concentrations (ERETIC) method (Akoka, Barantin, and Trierweiler 1999) that provides a reference signal synthesised by an electronic device. A particular attention in the integration procedure has to be taken when the ^1H spectrum of a foodstuff sample is dominated by the water peak. In this case, it is difficult to perform a correct quantitative analysis of the signals close to the water signal tail. Moreover, water suppression procedures can also partially saturate the resonances near the water signal affecting their quantification. So, water suppression experimental parameters must be accurately calibrated. Other factors that can represent a source of error during integration are signals overlapping, solved with deconvolution or 2D J-resolved experiments, and chemical shifts variations. In this case, manual integration or bucketing approaches can be useful in solving these issues. Manual integration is a time-spending approach, mainly when many signals and many samples have to be considered.

Finally, another approach consisting in the measure of the signal height, reported as signal intensity, is used instead of the integral for quantitative measurements, since, as in the case of the integral, signal intensity is proportional to the compound concentration. In order to use this approach, the signal linewidths of each specific resonance have to be the same in all the compared spectra. The measurement of the intensity can be performed manually or in the case of many signals using an automatic peak peaking procedure.

A1.2.6 Data statistical analysis

The NMR metabolic analysis generates a large amount of data from which it is necessary to extract the desired information using chemometric methods that can identify possible patterns among samples. In fact, since the number of identified metabolites and the number of studied samples can be very consistent, the only possibility of generating some final consideration or hypothesis from the obtained data is represented by the application of several statistical approaches. The nature of quantitative data extracted from an NMR spectrum (all data points, buckets, integral or intensity of selected resonances) influences the choice of the statistical approach for their elaboration. To be properly applied, any statistical approach has to take into account several important aspects of data, such as number of variables, the data redundancy, and possible noise. When a quantitative analysis is performed using all NMR spectral data points, the statistical elaboration has to face the problems of high data redundancy and noise introduction. The role of any single metabolite in statistical models is not easily deducible, but the data analysis is quick, and experimental data is not excluded a priori, thus avoiding the risk of losing any pertinent information. Bucketing reduces the number of variables and hence data redundancy, but again the variables (buckets) are not necessarily associated with single metabolites thus hindering the interpretation of statistical results in terms of individual metabolic contributions. In many cases, when data points or bucketing approaches are used after detecting the signals or spectral regions responsible for the most significant differences, the identification of corresponding metabolites can be carried out.

Finally, the statistical analysis of integrals or intensities of selected resonances requires time consuming assignment and quantification procedures. The selection of signals, avoiding the data-redundancy problem, is an advantage with respect to other quantitative approaches, but pertinent information deriving from non-selected resonances could be lost. Additionally, the interpretation of the statistical results in terms of individual metabolic contributions is straightforward.

Two types of statistical approaches can be used for data analysis, namely univariate and multivariate (Andre and Soukoulis 2020). Among these, multivariate statistical analysis is generally applied for the processing of NMR data of complex matrices such as foodstuffs, by means of unsupervised and supervised approaches. Explorative unsupervised analysis is often the first step of statistical treatment. Structure of data, presence of outliers and natural grouping of samples can be revealed at this step and in many cases, the obtained results are sufficient to distinguish different groups of samples according to metabolic differences. However, in the case of classification and prediction problems, the application of supervised multivariate techniques is necessary.

When multiple spectra are collected on a set of samples, after the necessary processing actions the obtained data are normally arranged into a rectangular matrix X , having as many rows as the number of samples and as many columns as the variables that have been chosen as descriptors for the system. This matrix represents the basis for the multivariate data analysis.

Explorative unsupervised analysis

The exploratory data analysis consists in trying to summarize the main characteristics of the data in an easy-to-understand way, mainly through the use of graphical tools and without the formulation of any statistical model or a priori hypothesis (Biancolillo et al. 2020). The main aims of unsupervised data analysis are to maximize insight into a data set, to uncover its underlying structure (e.g., spontaneous clustering, trends in data time series), to identify relevant variables, and to detect outliers and anomalies.

- Principal Component Analysis (PCA): The variables extracted from NMR spectra define a multidimensional space where every spectrum and corresponding sample can be represented by a single point. Considering that the number of variables typically used in metabolomic studies is always higher than 3, the data space cannot be simply visualised, but, using the PCA it is possible to transform the multidimensional space of experimental variables into a space with reduced mutually orthogonal dimensions

that are a linear combination of variables, called principal components. PCA is the main chemometric tool for exploratory data analysis since it allows the compression of the relevant information of a data set into a reduced set of abstract variables (the principal components). PCA is commonly used as an explorative technique in multivariate analysis, having proven to be a powerful and widely applicable technique. However, PCA may not be suitable if the effect of interest is not the main source of data variability and is masked in PCA by other factors with higher variability.

- Tree clustering analysis (TCA), also called hierarchical clustering analysis (HCA): It is another unsupervised exploration method used to discover the natural grouping (clustering) of samples. Tree clustering methods join together objects (samples) into successively larger clusters, using amalgamation rules for clusters and some measurements of the distance between single objects. The statistical results are reported as a dendrogram. All possible classifications in a prescribed number of groups can be obtained by cutting the tree at a suitable level.

Supervised classification/regression methods

In some situations, the spectral profiles recorded by NMR constitute the basis to build predictive models, i.e., models that allow not only the description of the main features of the samples under investigation, but also predict one or more characteristics of new samples. In particular, depending on the nature of the properties to be predicted, two families of methods can be distinguished: calibration methods, used to predict quantitative properties, and classification methods, that provide qualitative answers. Since the final aim of these methods is to be able to accurately predict the desired properties on new (unknown) samples, it is mandatory that, at least, two requirements are met: a sufficient number of training samples, and a careful validation step, to test the reliability of the approach providing also a quantitative estimate of the error. Validation (Westad and Marini 2015) is indeed an unavoidable step for any predictive model, as it guarantees the applicability of the methods to any future sample: in a very general way, it consists in applying the model to a new set of samples, that are treated as unknown by the model itself but for which the “true” values of the responses to be predicted are available to the experimenter.

Indeed, the aim of classification methods is to define a mathematical criterion to assign an individual (a sample) to one group (category or class) based on the measured variables. The main applied supervised methods in foodstuffs NMR metabolomics are:

- Partial Least Square (PLS) regression: this approach can be chosen to create a prediction model, in which the features from PCA and multiple regression are generalised and combined. Unlike PCA, PLS studies the relationship between two data matrices, X and Y. Matrix X contains the data extracted from NMR spectra for each sample, whereas matrix Y contains extra information on samples obtained using reference methodologies. PLS transforms the multidimensional space of variables from matrix X into a new coordinate system with axes corresponding to the highest covariance between measured NMR data (matrix X) and the response variables (matrix Y). In this way, the relationship between the NMR spectra and other properties of food samples can be used to predict these properties directly based on NMR data.
- Linear Discriminant Analysis (LDA): this method is based on linear combinations of original variables, that maximises the difference between a priori defined classes. In LDA the hyperspace of the variables is divided in as many regions as the number of available categories or classes. LDA is rather sensitive to data redundancy and a reliable model can be built only using a limited number of original non-correlated variables and a sufficient number of samples for each category.
- PLS-DA: taking advantages of both statistical approaches PLS and LDA, and avoiding their weaknesses, PLS-DA has found many applications in the analysis of NMR metabolomic data. In PLS-DA, the information about the variables responsible for the classification of samples is obtained by examining VIP scores (Variable Importance in Projection) and the regression coefficients.
- Soft Independent Modelling of Class Analogies (SIMCA): it is a class-modelling technique, that acts by defining the category space of one class at a time and is more focused on the similarities among samples of the same class than on the differences among the classes as in the case of discriminant analysis. Consequently, each sample can be assigned to one or more classes or to none of them.

A1.3 Application

A1.3.1 Food

The workflows and approaches to NMR analysis of food described above have proven to be very effective in solving various food-related issues, such as chemical characterisation, geographical origin, pedoclimatic effects, authenticity, and fraud traceability.

Regarding the chemical characterization of foodstuffs, NMR spectroscopy has been very useful for this purpose identifying within the limits of detection the whole metabolic profile in the same experiment in an unbiased manner. On the contrary, in a targeted technique such as chromatography, the chemical analysis of a foodstuff is generally focused on specific chemical classes that are chosen a priori before the analysis, thus limiting the possibility of identifying for new or unexpected metabolites. Therefore, untargeted NMR approach can be very useful especially for the chemical characterization of new matrices with perspective uses as foodstuffs. This is the case with matrices such as apple, and tomato, whose untargeted NMR metabolomic characterisations have shown the presence of classes of compounds such as sugars, organic acids, amino acids, polyphenols, and other metabolites.

Furthermore, NMR metabolomics is a recognised approach suitable for the identification and quantification of primary and secondary plant metabolites, as well as for the study of metabolic processes of these (Ingallina et al. 2020; Spano et al. 2021). Indeed, untargeted NMR-based metabolomics was applied to obtain the complete metabolic profiles of officinal plants, such as burdock, lemon balm and dandelion (Ambroselli et al. 2024). This analytical method revealed the effects of different altitudes and agronomic practices (spontaneous or organic cultivation) on the chemical profile.

A1.3.2 Metabolic Syndrome

The metabolome also plays a key role in the early prediction and diagnosis of metabolic syndrome (MetS) (Ambroselli et al. 2023). Individuals with MetS may present with latent metabolic disorders that can potentially evolve into clinical conditions such as type 2 diabetes. Delays in treatment may influence patient adherence and early identification of associated health problems. Therefore, continuous monitoring is crucial for accurate diagnosis. The

metabolome, comprising a number of metabolites, varies in MetS due to genetic and acquired factors. Using metabolomics to analyse these small molecules may facilitate the identification of MetS-related health problems, offering a promising approach for tracking the metabolic status of patients with MetS (**Figure A1.2**).

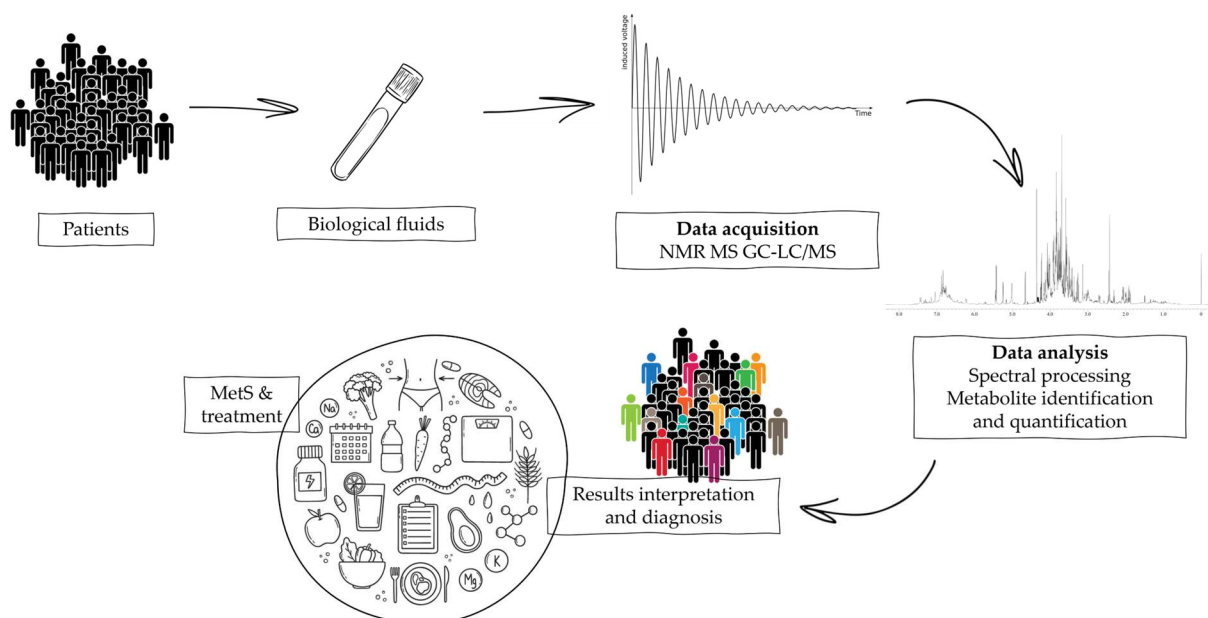


Figure A1.2. Experimental metabolomics work from biological samples of patients for the interpretation of results obtained by nuclear magnetic resonance (NMR) or mass spectroscopy (MS), coupled with targeted techniques such as gas chromatography and liquid chromatography (GC and LC).

In the diagnosis of MetS, the NMR approach should be a viable alternative for detecting an early stage of the condition compared to traditional methods; the latter are useful when the disease state is well defined. The biological fluids most commonly used in NMR metabolomic studies are plasma, serum, urine and faeces. For human studies, plasma and serum are preferred because they are easy to collect, and their metabolome reflects changes in metabolism at the individual level. Promising new biomarkers could be metabolites such as glucose, lactate, uric acid, citric acid, p -cresol sulphate, imidazole, histidine, branched-chain amino acids, aromatic amino acids, glutamate and glutamine, propionyl carnitine, lipids and many others. Glucose is a carbohydrate used as biomarker to diagnose a change in carbohydrate metabolism (glucose homeostasis) involved in insulin-resistance or type 2 diabetes, which are part of the multifactorial nature of MetS. In a recent study, NMR analysis of the urine profile revealed how patients affected by type 2 diabetes show metabolome variation in comparison with healthy subjects (Pasanta et al. 2019)

Thus, the metabolomics approach using NMR spectroscopy be applied to identify a number of metabolites that evolve with MetS or its individual components.

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Chapter A2

The role of officinal plants

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A2.1 Officinal plants

A2.1.1 Introduction

Officinal plants contain secondary metabolites and bioactive compounds such as polyphenols, flavonoids, carotenoids, and phytosterols, as well as vitamins and minerals, which have positive effects on human health and well-being (López-Rodríguez et al. 2022).

The return to natural products has led to the emergence of new problems and needs: first and foremost, the import of natural products/supplements from different countries around the world (Chaachouay and Zidane 2024). However, many of these medicinal remedies, mainly phytotherapeutic ones, have a largely unknown composition, which may represent a health risk factor for consumers (Davis and Choisy 2024). Furthermore, in the context of promoting and supporting sustainable agronomic practices for the protection and preservation of the territory, such as cultivation with organic methods, it is necessary to assess the actual difference in terms of metabolomic profile composition between ecotypes of the same wild species and those cultivated with organic methods, in order to have a greater knowledge and value of the product and to develop the agronomic technique (Noletto-Dias et al. 2023). In fact, the production of secondary metabolites in plant species is extremely variable, influenced not only by the species (genetic factor) but also by various environmental factors such as light, temperature, humidity, the nature of the soil and, finally, the method of cultivation (Cornara et al. 2023). In this context, it may be possible to support this field by carrying out phytochemical investigations aimed at characterising the composition of the new plant-based products that are becoming increasingly popular on the market.

Such investigations can also have a proactive aspect, helping to identify spontaneous and/or cultivated officinal plants and their preparations (hulled, peeled broken, pulverised or in the form of extracts) from our territory or imported from other countries. These may in fact be of interest to various sectors, including the food sector, for their greater richness in vitamins, mineral salts and antioxidants, the medicinal sector, for their greater beneficial effect or therapeutic value, and finally the agronomic sector, for their phytoiatric/bioherbicidal effect for use in organic farming. In the latter sphere, the growing interest in new strategies with a low environmental impact in the defence of agricultural crops opens up new and promising prospects for molecules of plant origin. Extracts from plant matrices have a very complex chemical composition, a characteristic that does not favour the emergence of resistance in

pests. However, they are active ingredients that are extracted from plants and then formulated into commercial products. The chemical characterisation of some plant extracts of particular interest could also lead to patenting some of these mixtures, with possible positive implications from an economic point of view. In Italy, moreover, there is a very strong tradition on the use of officinal plants and this use is guaranteed thanks to a directive 2004/24/EC ("EUR-Lex Document 32004L0024", 2004), on the protection of the traditional use of plants and medicinal products of plant origin, whereby their efficacy is evaluated and considered plausible on the basis of long-term use and experience. Even today, it is mainly rural populations that have maintained practices related to the use of wild plants, and this occurs most frequently in the more inland regions and territories of our country, still playing a marginal role compared to commercial products.

A further important issue concerning the chemical aspect of officinal plants is undoubtedly the post-harvest treatment (Kader and Rolle 2004). There are different extraction methods, not always sustainable, each of which has a different composition in primary and secondary metabolites. It is therefore of paramount importance to develop and standardise sustainable extraction procedures characterised by an interesting phytochemical composition.

Finally, processing is another step in the officinal plant supply chain that needs further investigation and support from scientific research. The extract obtained from officinal plants must be appropriately formulated according to its intended use (foodstuff, medical device, cosmetic, phytoiatric) in order to guarantee the stability of the phyto-complex and the control of its properties. In the field of cosmetics and cosmeceuticals, for example, there is great scope for innovation. Unlike the food sector, where there is a very precise regulation, to date there are no European reference standards or criteria to define a natural or organic cosmetic (i.e. containing raw materials derived from organic agricultural processes). Therefore, natural or organic cosmetics and cosmeceuticals, or in any case those based on components of plant origin, today represent an excellent opportunity for innovation and reconversion of cosmetic formulation as well as a form of incentive for environmental sustainability and a form of valorisation of the national and non-national agricultural heritage.

In the field of agriculture, the search for alternatives to pesticides with high efficacy, low cost and low environmental impact represents a real challenge for modern agriculture in an eco-sustainable key. Proposed alternatives include physical methods such as solarisation, bio-fumigation, biological soil disinfestation and the application of biocontrol agents, or the use of

natural products. In Italy, a number of products derived from the metabolites of officinal plants that can be used as plant protection products in organic farming have already been registered and are included by the European Community among the objectives for sustainable environmental and economic development. Therefore, the preparation of increasingly 'green' formulations would be a response to these needs and would provide new development opportunities both in the production field and as an incentive to search for 'new' functional ingredients derived from traditional plants.

A2.2 Case study 1

In the world of natural health, the careful selection and cultivation of medicinal plants is essential to obtain high-quality extracts that retain their beneficial properties. Three of these plants - burdock (*Arctium lappa*), dandelion (*Taraxacum officinale*) and lemon balm (*Melissa officinalis*) - are cultivated using innovative agronomic practices that enhance their efficacy, ensuring the best possible product for consumers. These plants are grown at different altitudes by a local company, played a significant role in their growth and chemical composition. Using advanced agricultural techniques, the company optimises the health and potency of the plants by taking advantage of the microclimates offered at the different altitudes. This practice not only promotes sustainable agriculture, but also contributes to the unique qualities of the plants, making them ideal for the production of high-quality herbal extracts. Once harvested, the plants are supplied to a specialised company that produces hydro-alcoholic extracts. These extracts are carefully processed to preserve the plant's active ingredients, which are highly valued in natural wellness products. The collaboration between the two companies ensures that only the best raw materials are used, maximising the therapeutic benefits of the extracts. Each of the plants has a long history of use in traditional medicine. Burdock is known for its detoxifying properties, particularly for supporting liver health and purifying the bloodstream. Dandelion, on the other hand, is revered for its diuretic and digestive benefits, often used to promote liver function and reduce inflammation. Finally, lemon balm is prized for its calming and anxiolytic effects, often used to promote relaxation and support the nervous system. By carefully selecting these plants, cultivating them at various altitudes and ensuring sustainable farming practices, the two companies work together to provide high-quality natural extracts that meet the growing demand for plant-based wellness remedies.

For all these reasons, a metabolomic NMR approach was applied to burdock, dandelion and lemon balm, on the different ecotypes (LSE, Land Spontaneous Ecotype, 150 m - MSE, Mountain Spontaneous Ecotype, 800 m - OE, Organic Ecotype, 150 m). In particular, several classes of secondary metabolites were identified by NMR analysis of hydroalcoholic and lipophilic extracts by Bligh-Dyer (**Figure A2.1**).

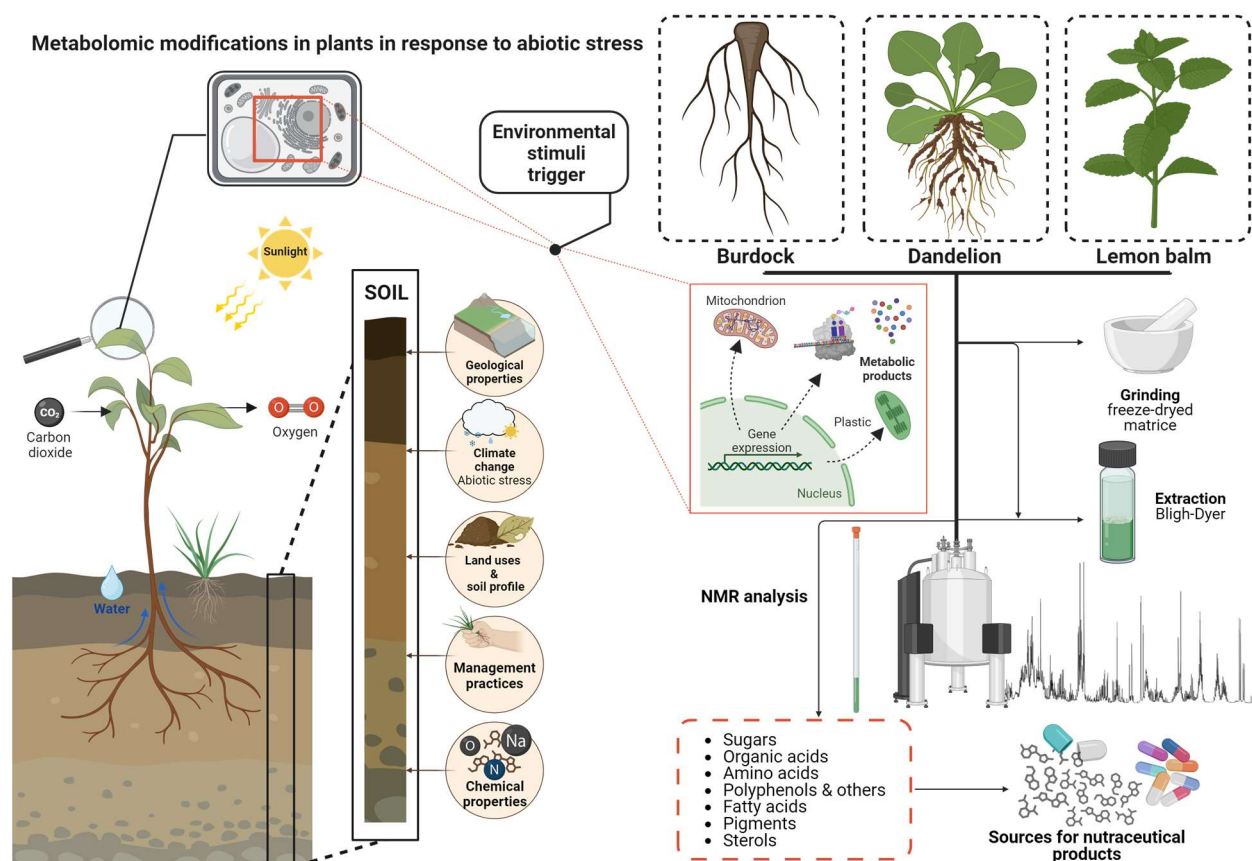


Figure A2.1. Graphical representation of the study on burdock, dandelion and lemon balm (Ambroselli et al. 2024).

The analysed matrices were examined on two distinct levels, each of which contributed to a deeper understanding of plant characteristics. The first level of investigation concerned agronomic practices and the altitude at which each plant grew. This aspect was particularly important because it influenced the chemical and physiological composition of the plants, leading to variations in their bioactive compounds and general properties. The second level, on the other hand, focused on the comparison between the different anatomical parts of plants,

with particular attention to the differences between the aerial parts (such as leaves and stems) and the roots. Each plant part contained a different metabolic and chemical profile, and the comparison between these parts was essential for understanding how resources are distributed and how plants adapt to various environments and growing conditions. **Figure A2.2** provides a visual representation of this diversity by comparing the profiles of Bligh-Dyer hydroalcoholic extractions. The comparison of the profiles of the analysed samples helped to illustrate the heterogeneity of the samples, providing important indications for future studies and the optimisation of agricultural practices based on the specific characteristics of the plants and their growth environment.

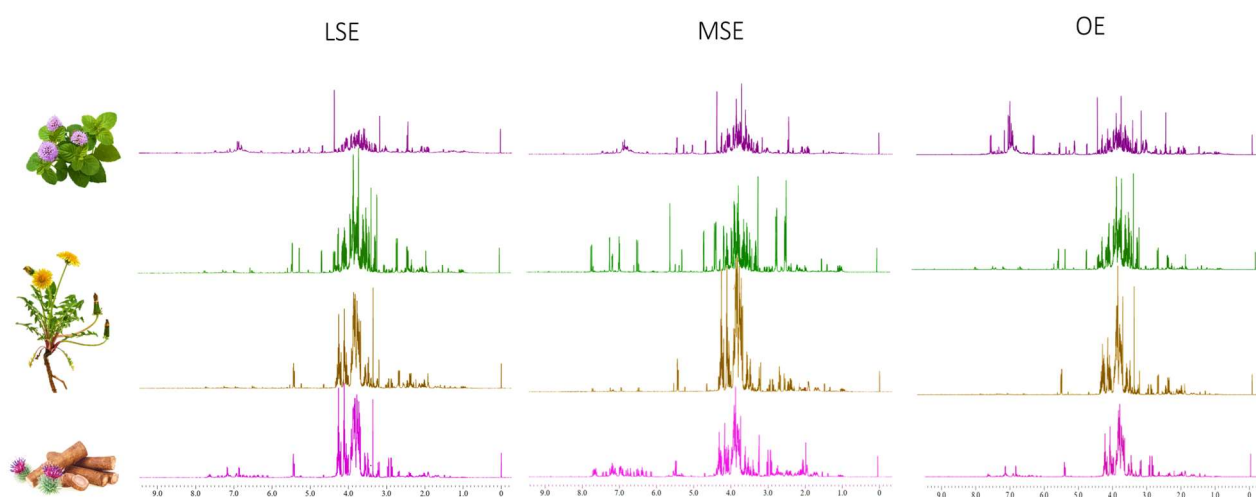


Figure A2.2. Overlapping proton spectra of hydroalcoholic extracts (^1H , H_2O suppression, ppm scale), starting from above, of lemon balm aerial part, dandelion aerial part, dandelion root part, and burdock root part, according to ecotypes (LSE, MSE, and OE).

Article

NMR Metabolomics of *Arctium lappa* L., *Taraxacum officinale* and *Melissa officinalis*: A Comparison of Spontaneous and Organic Ecotypes

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Abstract: Officinal plants are a source of metabolites whose chemical composition depends on pedo-climatic conditions. In this study, the NMR-based approach was applied to investigate the impacts of different altitudes and agronomical practices (Land, Mountain Spontaneous, and Organically Grown Ecotypes, namely LSE, MSE, and OE, respectively) on the metabolite profiles of Burdock root, Dandelion root and aerial part, and Lemon balm aerial part. Sugars, amino acids, organic acids, polyphenols, fatty acids, and other metabolites were identified and quantified in all samples. Some metabolites turned out to be tissue-specific markers. Arginine was found in roots, whereas myo-inositol, galactose, glyceroyldigalactose moiety, pheophytin, and chlorophyll were identified in aerial parts. Caftaric and chicoric acids, 3,5 di-caffeoylquinic acid, and chlorogenic and rosmarinic acids were detected in Dandelion, Burdock and Lemon balm, respectively. The metabolite amount changed significantly according to crop, tissue type, and ecotype. All ecotypes of Burdock had the highest contents of amino acids and the lowest contents of organic acids, whereas an opposite trend was observed in Lemon balm. Dandelion parts contained high levels of carbohydrates, except for the MSE aerial part, which showed the highest content of organic acids. The results provided insights into the chemistry of officinal plants, thus supporting nutraceutical–phytopharmaceutical research.

Keywords: officinal plants; pedoclimatic conditions; NMR spectroscopy; metabolomics



Citation: Ambroselli, D.; Masciulli, F.; Romano, E.; Guerrini, R.; Ingallina, C.; Spano, M.; Mannina, L. NMR Metabolomics of *Arctium lappa* L., *Taraxacum officinale* and *Melissa officinalis*: A Comparison of Spontaneous and Organic Ecotypes. *Foods* **2024**, *13*, 1642. <https://doi.org/10.3390/foods13111642>

Academic Editors: Runqiang Yang, Yongqi Yin and Liping Guo

Received: 29 April 2024

Revised: 18 May 2024

Accepted: 21 May 2024

Published: 24 May 2024



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1. Introduction

Officinal plants have been used as a nutrient in traditional cuisines and ethnomedicine throughout history, providing a rich source of nutrients, unique flavours, and cultural significance. Moreover, officinal plants represent a relevant source of bioactive compounds [1] with direct or indirect therapeutic effects [2,3], which are present in specific parts (roots, leaves, and flowers) and also throughout the plant, albeit in different concentrations.

Among several officinal plants, Burdock (*Arctium lappa* L.), Dandelion (*Taraxacum officinale*), and Lemon balm (*Melissa officinalis*) have been used for their edible and medicinal properties [4–9].

Burdock, scientific name *Arctium lappa* L., is a biennial herbaceous plant belonging to the Compositae (or Asteraceae) family. The Burdock root is part of the eating habits of Asian populations, who consume fresh roots in tea, salads, and soups after domestic pre-processing [7]. Burdock root contains a large amount of minerals, flavonoids, proteins, phenolic compounds, and polysaccharides, and it is commonly consumed as a vegetable

in East Asian cuisine such as stir-fries, soups, and pickles [10–13]. Due to its nutritional and nutraceutical properties [14], Burdock root is used to produce food supplements such as infusions, extracts, tinctures, and decoctions targeting digestive health, detoxification, and immune support [7,15]. Despite its culinary and medicinal benefits, Burdock may pose risks for individuals allergic to plants in the Asteraceae family. Moreover, its root contains bitter compounds called polyacetylenes, which can be toxic in large amounts [16]. Proper processing and preparation are necessary to mitigate these risks.

Dandelion, scientific name *Taraxacum officinale*, is a leafy perennial herb characterised by a rosette of markedly toothed leaves. Fresh leaves and flowers are used fresh in cuisines as food by being fried or boiled, as well as being consumed fresh in salads, while roots are generally used after being dried as additives in the preparation of jams, teas, and coffees [4,17–19]. In particular, it is mainly appreciated as a diuretic and bitter tonic to treat the stomach, liver, and gall bladder. Dandelion hydroalcoholic extracts are also used for their antioxidant and anti-inflammatory effects on cardiovascular risks [20,21], rheumatic complaints, and pain in general [22]. These properties have been attributed to sesquiterpene lactones, polyphenols, phenolic acid derivatives, and triterpenoids [5]. Dandelion is “generally recognized as safe” (GRAS) as a food by the U.S. Food and Drug Administration. It can rarely cause allergic reactions, diarrhoea, and gastrointestinal upset [23].

Lemon balm, scientific name *Melissa officinalis*, is an edible officinal herb belonging to the mint family Lamiaceae [24] that commonly grows in the Mediterranean region and western Asia [25]. Lemon balm is mainly used for its aromatic leaves, which have a lemon-like scent and flavour. The leaves are harvested before or during flowering and can be used fresh or dried as food, food ingredients, food supplements, or medicinal agents [26,27].

High amounts of essential oils, triterpenes, and appreciable amounts of caffeic acid-derived polyphenols such as rosmarinic acid and flavonoids have been detected in Lemon balm [28]. Lemon balm is widespread as an herbal drug in extemporaneous use, including extracts and tinctures. In several countries, Lemon balm leaves are used for beverage preparation such as decoctions, infusions, and teas [24] and, therefore, employed in gastrointestinal disorders of nervous origin for treating psycho-vegetative cardiac problems and migraines [29–31]. The essential oil and its components are widely employed as flavouring agents (e.g., citral, citronellal, geraniol, linalool) and have been subject to comprehensive safety assessments by various regulatory agencies [32]. The Panel on Food Additives and Nutrient Sources Added to Food (ANS) concludes that due to the lack of an appropriate dossier supporting the use of oregano and Lemon balm extracts as additives, the safety of lemon balm extracts for the proposed uses in eight food categories and use levels of, respectively, 2.0 mg/kg bw/day for women and 2.3 mg/kg bw/day for men cannot be assessed [33]. In the United States, lemon balm is listed as “generally recognized as safe” under 21 CFR Part 182.10 and 182.20 for use as a flavouring agent, adjuvant, or flavour enhancer. The European Medicines Agency (EMA) has evaluated *Melissa officinalis* L., folium for its use as a medicinal agent and has established a traditional use based on sufficient safety data and plausible efficacy. However, there is a lack of conventional clinical safety data [27,34].

To explore their potential benefits and limitations, it is essential to comprehend the diverse roles of these plants, not only in traditional medicine but also as dietary staples or functional foods. It is noteworthy that the chemical and nutritional properties, as well as the physiological and morphological characteristics, of these plants are strongly influenced by environmental factors. In particular, soil and climatic conditions affect the metabolism and productivity of vegetable species [35]. The ability of plants to counter abiotic stress is precisely related to the production of secondary metabolites [36]. Several studies have assessed the effects of soil and climate conditions on crops, such as oilseed fruits [37], vine berries [38], mint [39], and eucalyptus [40], thus providing useful information on the impact of environmental factors and their role in determining chemical profiles.

The literature data regarding the metabolite profiles of Burdock, Lemon balm, and Dandelion have mainly focused on specific bioactive classes, namely polyphenols, terpenes,

and lactones, determined using different targeted approaches such as NMR/GC-MS [41], UPLC/QTRAP-MS [42], HPLC-MS [43], and HPLC-DAD [44].

Here, for the first time, untargeted NMR-based metabolomics was used to obtain the whole metabolite profiles of Burdock roots, Lemon balm's aerial parts, and Dandelion roots and aerial parts. This analytical method highlighted the effects of different altitudes and agronomical practices (Spontaneous vs. Organically Grown) on the chemical profile. NMR metabolomics is a recognised suitable approach for identifying and quantifying the primary and secondary metabolites of plants [45–47], as well as for the study of metabolic processes [48].

2. Materials and Methods

2.1. Sampling

Three ecotypes, namely the Land Spontaneous Ecotype (LSE), Mountain Spontaneous Ecotype (MSE), and Organic Ecotype (OE) of Dandelion (*Taraxacum officinale*), Lemon balm (*Melissa officinalis*), and Burdock (*Arctium lappa* L.), were provided by “Fibreno Officinali”, having been collected in Isola del Liri at 150 asl (41°41' N 13°34' E, Italy) and Colleparado at 800 asl (41°46' N 13°22' E, Italy; Figure S1, Table 1).

Table 1. Description of selected officinal plant ecotypes.

Plant	Harvesting Time	Ecotype	Altitude	Treatment
Burdock (root)	October	Land Spontaneous (LSE)	150 m	No treat, wild grown
		Mountain Spontaneous (MSE)	800 m	No treat, wild grown
		Organic (OE)	150 m	Remotion of other vegetable species from the soil
Dandelion (aerial part and root)	October	Land Spontaneous (LSE)	150 m	No treat, wild grown
		Mountain Spontaneous (MSE)	800 m	No treat, wild grown
		Organic (OE)	150 m	Remotion of other vegetable species from the soil
Lemon Balm (aerial part)	May	Land Spontaneous (LSE)	150 m	No treat, wild grown
		Mountain Spontaneous (MSE)	800 m	No treat, wild grown
		Organic (OE)	150 m	Remotion of other vegetable species from the soil

Dandelion and Burdock's ecotypes were harvested in autumn (2022), whereas the Lemon balm ecotype was harvested in spring (2022) at the complete maturity of the plants. The cultivation soil was composed of a mixture of clay and sand. The environmental growing conditions during the harvesting year are shown in Table S1.

Plant collection involved the gathering of specific sections: roots for Burdock, both roots and aerial parts for Dandelion, and aerial parts for Lemon balm.

Samples were thoroughly washed to remove impurities and freeze-dried (Buchi Lyovapor L-200, Flawil, Switzerland) for three days at $-55\text{ }^{\circ}\text{C}$ and $2 \times 10^4\text{ Pa}$ until complete water loss. Afterwards, each sample was blended using a knife mill and stored at $-80\text{ }^{\circ}\text{C}$ until extraction.

2.2. Chemicals

Methanol (CH_3OH , HPLC-grade), chloroform (CHCl_3 , HPLC-grade), and distilled water were obtained from Carlo Erba Reagenti (Milan, Italy). EDTA deuterated (98%) was purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). Monobasic potassium phosphate (KH_2PO_4) and dibasic potassium phosphate (K_2HPO_4) were purchased from Aldrich-Fluka-Sigma S.r.l. (Milan, Italy).

Deuterated water (D_2O) 99.97 atom% of deuterium, methanol- D_4 99.80 atom% of deuterium (CD_3OD), and chloroform- D 99.80 atom% of deuterium (CDCl_3) were purchased from Euriso-Top (Saclay, France).

3-(trimethylsilyl)-propionic-2,2,3,3- d_4 acid sodium salt (TSP) and 3,4,5-Trimethoxybenzaldehyde (TBZ) were purchased from Merck (Milan, Italy). Polyphenol standards (95%) were purchased from Aldrich-Fluka-Sigma S.r.l. (Milan, Italy).

2.3. Extraction Procedure for NMR Analysis

Extractions for NMR analysis followed the Bligh–Dyer protocol [49], albeit with modifications. In particular, 200 mg of sample was added with 3 mL of a CH₃OH/CHCl₃ mixture (2:1 *v/v*) and 0.8 mL of distilled water. The resulting system was sonicated (thermostat ultrasonic bath ARGOLAB DU-100 (Rome, Italy)) at room temperature for 10 min, before adding 1 mL of chloroform and 1 mL of distilled water. The hydroalcoholic and organic phases were finally separated after centrifugation (Eppendorf Centrifuge 5430 R (Milan, Italy)) for 15 min (25 °C, 7830 rpm). The leftover pellets were extracted twice using the same conditions as previously described. The three extractions obtained from each step were combined. The hydroalcoholic and organic fractions were dried via nitrogen flow. Each sample was prepared and analysed in triplicate.

2.4. NMR Analysis

The dried hydroalcoholic phase was dissolved in 700 µL of 100 mM phosphate buffer/D₂O, containing 0.4 mM TSP as an internal standard. The dried CHCl₃ fraction was dissolved in 700 µL of CDCl₃/CD₃OD (2:1 *v/v*) mixture.

NMR analyses were carried out on a JEOL JNM-ECZ 600R (JEOL Ltd., Tokyo, Japan) operating at a proton frequency of 600.17 MHz and equipped with a JEOL 5 mm FG/RO DIGITAL AUTOTUNE probe. Spectra processing and signal integration were performed with JEOL Delta software v5.3.1 (JEOL Ltd., Tokyo, Japan).

The ¹H spectra of the hydroalcoholic fraction, as shown in Figure S2, were carried out by using the following parameters: 128 scans, residual water signal suppression with a presaturation pulse, a 7.73 s relaxation delay, a 90° pulse of 8.3 µs, 64 k data points, and a 9000 Hz spectral width. The ¹H spectra of the apolar fraction were acquired by coadding 64 scans with a 7.73 s relaxation delay and using a 90° pulse of 8.3 µs, 64 k data points, and a spectral window width of 9000 Hz.

Homonuclear ¹H-¹H TOCSY and heteronuclear ¹H-¹³C HMBC and ¹H-¹³C HSQC experiments for both fractions were carried out following the previously reported experimental parameters [50].

Water-soluble metabolites were quantified by integrating the selected signals and normalising against the TSP methyl group signal (0.00 ppm), set to 100, and quantification was expressed as mg/100 g ± SD of the dried sample.

For organic extract metabolite quantification, the first step was based on the determination of saturated (SFA) and mono-unsaturated fatty acids (MUFA) integral area values, whose signals overlapped with those of other fatty acids. Their content was calculated by applying the following equations, modifying those previously reported for this purpose [49]:

$$I_{\text{MUFA}} = I_{\text{TOT UFA}} - 2I_{\text{DUFA}} - 1.5I_{\text{TUFA}} \quad (1)$$

$$I_{\text{TOT SFA}} = I_{\text{TOT FA}} - I_{\text{MUFA}} - I_{\text{DUFA}} - I_{\text{TUFA}} \quad (2)$$

I_{MUFA} , I_{TUFA} , I_{DUFA} , $I_{\text{TOT UFA}}$, $I_{\text{TOT SFA}}$, and $I_{\text{TOT FA}}$ are the integral values of mono-unsaturated fatty acids, tri-unsaturated fatty acids, di-unsaturated fatty acids, total unsaturated fatty acids, total saturated fatty acids, and total fatty acids, respectively. Signals in the 5.33–5.35 ppm range, corresponding to double-bound protons, were considered to integrate TOT UFA. Signals in the 2.28–2.30 ppm range corresponding to α-CH₂ groups of all fatty acids were considered to integrate TOT FA.

Finally, apolar metabolites were quantified using a 1 mM TBZ external standard. Each fatty acid category was expressed using the main molecule of the class: oleic acid for MUFA, linoleic acid for DUFA, linolenic acid for TUFA, and stearic acid for SFA. Results were expressed as mg/100 g ± SD of dried sample.

For the analysis of hydroalcoholic and organic extracts, the one-way (Burdock and Lemon balm data) and two-way (Dandelion data) ANOVA were applied, respectively, followed by Tukey's multiple comparison test, to show significant differences ($p < 0.0001$).

between the samples considered for each metabolite. GraphPad Prism 8.0.2 software was used for this purpose.

2.5. Statistical Analysis

Different subsets of data were generated for the hydroalcoholic extracts of each plant, with the main subsets consisting of the parts analysed (root and aerial part) and the ecotype of each plant (LSE, MSE, OE). A table with the metabolite concentration for each plant was reported in Excel (Office 2016) and subsequently imported into MATLAB (2023a, MathWorks®, Natick, MA, USA). Data were pre-processed with autoscaling before performing Principal Component Analysis (PCA) to assess any differences between the three ecotypes of each plant and between the plants themselves.

3. Results and Discussions

3.1. NMR Assignment of Hydroalcoholic and Organic Fractions

The assignments of the ¹H NMR spectra of Burdock roots', Dandelion roots and aerial parts', and Lemon balm aerial parts' hydroalcoholic fractions were carried out using 2D experiments (¹H-¹H TOCSY, ¹H-¹³C HSQC, ¹H-¹³C HMBC), with the addition of the reference standards and literature data relative to other vegetable matrices analysed using the same experimental conditions [45,50]. Primary and secondary metabolites identified in Bligh–Dyer hydroalcoholic extract are reported in Table 2.

Table 2. Relative assignments of ¹H and ¹³C NMR signals are of the metabolites identified in the Bligh–Dyer hydroalcoholic extracts of Burdock roots, Dandelion roots and aerial parts, and Lemon balm aerial parts (700 µL of phosphate buffer/D₂O containing 0.4 mM TSP) are reported. The exponents (^{B,D,L}) indicate signals selected for integration. The black dots mark the presence of the metabolite in the officinal plants.

Compound	Assignment	¹ H (ppm)	Multiplicity [J(Hz)]	¹³ C (ppm)	Burdock	Dandelion	Lemon Balm
<i>Sugars</i>							
α-D-Fructofuranose	C-2			105.9	•	•	•
	CH-3	4.11		82.3			
β-D-Fructofuranose	C-2			102.6	•	•	•
	CH-4	4.12		75.6			
β-D-Fructopyranose	C-2			99.3	•	•	•
	CH-3	3.80		68.6			
β-Galactose	CH-1	4.60 ^{D,L}	d [7.9]	97.3		•	•
	CH-2	3.51					
	CH-3	3.67					
	CH-4	3.95					
	CH-5	4.05					
	CH-6	3.78					
	CH-1	5.23 ^{B,D,L}	d [3.8]	93.3	•	•	•
α-Glucose	CH-2	3.54		72.6			
	CH-3	3.72		73.3			
	CH-4	3.41		70.8			
	CH-5	3.84		72.5			
	CH-1	4.66 ^{B,D,L}	d [7.9]	97.0	•	•	•
β-Glucose	CH-2	3.25		75.3			
	CH-1 (Glc)	5.44		93.9		•	•
Inulin	CH-2	3.57		72.1			
	CH-3	3.78		73.6			
	CH-4	3.48		70.2			
	CH-5	3.85		73.4			
	CH ₂ -6	3.83		61.1			
	CH ₂ -1' (Fru)	3.75; 3.89					
	CH-3'a	4.21		77.5			
	CH-3'b	4.26					
	CH-4'a	4.05		74.9			
	CH-5'	3.89		82.2			
Myo-inositol	CH-2,5	3.56				•	•
	CH-3,6	3.65					
	CH-4	3.28 ^{D,L}		75.0			

Table 2. Cont.

Compound	Assignment	¹ H (ppm)	Multiplicity [J(Hz)]	¹³ C (ppm)	Burdock	Dandelion	Lemon Balm
Sucrose	CH-1 (Glc)	5.42 ^{B,D,L}	d [3.9]	93.1	•	•	•
	CH-2	3.53		71.8			
	CH-3	3.72		73.6			
	CH-4	3.42		70.2			
	CH-5	3.84		73.5			
	C2' (Fru)			104.8			
	CH-3'	4.23		77.4			
	CH-5'	3.92		82.4			
	CH-4'	3.83		61.2			
<i>Organic acids</i>							
Acetate	CH ₃	1.92 ^{B,D}	s	24.4	•	•	•
	COO ⁻			184.4			
Citrate	α, γ-CH	2.56 ^{B,D,L}	d [16.0]	46.0	•	•	•
	α', γ'-CH	2.68		46.0			
	β-C			73.2			
	1,5-COO ⁻			180.2			
	6-COO ⁻			183.0			
Formate	HCOO ⁻	8.46 ^{B,D,L}	s		•	•	•
Fumarate	α, β-CH=CH	6.53 ^{B,D}	s	136.7	•	•	
Lactate	β-CH ₃	1.34 ^B	d [7.1]	21.4	•		
Malate	α-CH	4.31 ^{B,D,L}	dd [9.9; 3.1]	71.0	•	•	•
	β-CH	2.68	dd [15.5; 3.1]	43.5			
	β'-CH	2.41	dd [15.5; 9.9]	43.5			
	α, β-CH ₂	2.42 ^{B,D,L}	s	35.2			
Succinate	α, β-CH ₂	2.42 ^{B,D,L}	s	35.2	•	•	•
Tartrate	CH(OH)COO ⁻	4.34 ^{D,L}	s	75.3		•	•
<i>Amino acids</i>							
Alanine	α-CH	3.80	d [7.2]	51.0	•	•	•
	β-CH ₃	1.49 ^{B,D,L}		17.3			
	COO ⁻			178.6			
Arginine	α-CH	3.77	m	55.4	•	• *	
	β-CH ₂	1.91		28.0			
	γ-CH	1.67 ^{B,D}		25.3			
	γ'-CH	1.74		25.3			
	δ-CH ₃	3.24		41.5			
	α-CH	4.00		52.8			
Asparagine	β, β'-CH ₂	2.88 ^B ; 2.96 ^D	dd [7.7; 16.8] dd [4.3; 16.8]	35.6	•	•	
	COO ⁻		176.5				
	β, β'-CH ₂	2.70 ^L ; 2.81 ^D	dd [3.7; 17.5]	37.0			
α-CH	3.92	54.0					
γ-COO ⁻		177.3					
GABA	α-CH ₂	2.30 ^{D,L}	t [7.4]	35.4		•	•
	β-CH ₂	1.90	24.9				
	γ-CH ₂	3.01	40.4				
Glutamine	α-CH	3.76	t [7.5]	55.3	•	•	•
	β, β'-CH ₂	2.14	m	27.5			
	γ-CH	2.45 ^{B,D,L}	m	31.9			
Glycine	α-CH ₂	3.58	s	42.5	•		
Isoleucine	α-CH	1.97	d [7.1] d [7.4]	38.0	•	•	•
	β-CH	1.27		29.4			
	γ-CH ₃	1.01 ^{B,D,L}		15.7			
	δ-CH ₃	0.89					
Leucine	β-CH ₂	1.74	d [6.2]	41.0	•	•	•
	γ-CH	1.71					
	δ-CH ₃	0.97 ^{B,D,L}		23.1			
	δ'-CH ₃	0.96		22.1			
Phenylalanine	CH-2,6	7.33	m	130.7		•	
	CH-4	7.38					
	CH-3,5	7.43 ^D		130.6			
Proline	α-CH	4.13 ^L	m	62.5	•	•	•
	β, β'-CH ₂	2.07, 2.33		30.0			
	γ-CH ₃	2.01 ^{B,D}		25.0			
Threonine	δ, δ'-CH ₃	3.33, 3.41	d [6.7]	47.4	•	•	•
	α-CH	3.59		62.3			
	γ-CH ₃	1.33 ^{B,D,L}		20.7			

Table 2. Cont.

Compound	Assignment	¹ H (ppm)	Multiplicity [J(Hz)]	¹³ C (ppm)	Burdock	Dandelion	Lemon Balm
Tyrosine	CH-2, 6 ring	7.22	d [8.2]	132.0	•	•	
	CH-3, 5 ring	6.96 ^{B,D}	d [8.5]	117.0			
Tryptophan	CH-4 ring	7.74			•	•	
	CH-5 ring	7.20					
	CH-6 ring	7.29					
	CH-7 ring	7.53 ^{B,D}	d [8.1]				
Valine	α-CH	3.60			•	•	•
	β-CH	2.27					
	γ-CH ₃	1.10	d [7.1]	17.7			
	γ'-CH ₃	1.04 ^{B,D,L}	d [7.1]	19.0			
<i>Other compounds</i>							
Choline	+N(CH ₃) ₃	3.20 ^{B,D,L}	s	55.1	•	•	•
Ethanolamine	α, β-CH ₂	3.14 ^{B,D}	t [5.2]	42.1	•	•	
Trigonelline	CH-1	9.13 ^{B,D}	s		•	•	
	CH-3	4.42	s				
	CH-4	8.09					
Caftaric acid	CH-10	5.31 ^D	d [2.2]			•	
	CH-11	4.57	d [2.2]				
	CH-2	6.49	d [16.0]	115.4			
	CH-3	7.72	d [16.0]	148.0			
	CH-8 ring	6.96	d [8.2]	117.6			
	CH-9 ring	7.17	dd [2.0; 8.2]	124.2			
	CH-5 ring	7.25	d [2.0]	117.5			
	COO ⁻			171.4			
Chicoric acid	CH-10,10a	5.54 ^D	s	75.9		•	
	CH-2, 2a	6.49	d [16.0]	115.4			
	CH-3, 3a	7.72	d [16.0]	148.0			
	CH-8, 8a ring	6.96	d [8.2]	117.6			
	CH-9, 9a ring	7.17	dd [2.0; 8.2]	124.2			
	CH-5, 5a ring	7.25	d [2.0]	117.5			
	COO ⁻			171.4			
Chlorogenic acid	CH-2	6.22	d [16.0]	115.8			•
	CH-3	7.37	d [16.0]	146.8			
	CH ₂ -2'	1.89; 2.10	m	41.7			
	CH-4'	3.90	m	70.8			
	CH ₂ -6'	1.99; 2.06	m	38.6			
3,5-Di-caffeoylquinic acid	CH-2, 2a	6.13, 6.25 ^B	d [16.0]	115.7, 116.5	•		
	CH-3, 3a	7.40, 7.32	d [16.0]	148.0, 147.8			
	CH-3', 5'	5.335.37	m	72.3			
			m	72.9			
	CH ₂ -2', 6'	1.99, 2.02	m	40.0			
	CH-8, 8a ring	6.58, 6.66	d [8.3]	116.9, 117.0			
	CH-9, 9a ring	6.70, 6.74	dd [1.7; 8.3]	123.9, 124.0			
	CH-5, 5a ring	7.21, 7.24	d [1.7]	116.2, 117.0			
Rosmarinic acid	CH-2	6.22	d [16.0]	115.8			•
	CH-3	7.37	d [16.0]	146.8			
	CH-1a	5.01	m	77.5			
	CH ₂ -2a	2.85; 2.93	m	37.9			
Uracil	CH	7.84 ^L	d				•
	CH	5.80	d				
Uridine	CH-6	5.90 ^B	d [8.1]		•	•	
	CH-5	7.87 ^D	d [8.1]				
	CH-1'	5.89	d [4.8]				

The exponent “B” indicates selected signals used for the integration of the Burdock (*A. lappa* L.) metabolites. The exponent “D” indicates selected signals used for the integration of the Dandelion (*T. officinale*) metabolites. The exponent “L” indicates selected signals used for the integration of the Lemon balm (*M. officinalis*) metabolites. * Detected only in Dandelion’s radical part.

In the high-field NMR region (0.8–3.6 ppm), signals of methyl and methylene groups belonging to aliphatic amino acids (leucine, isoleucine, valine, threonine, alanine, arginine, proline, GABA, glutamine, aspartate, and asparagine) and organic acids (acetate, succinate, citrate, and malate) were observed. The mid-frequency region between 3.0 and 5.5 ppm was dominated by intense signals, mainly due to monosaccharides and disaccharides, namely

myo-inositol, β -galactose, glucose, and sucrose. In the low-frequency 6.0–9.0 ppm, spectral region signals of aromatic compounds, namely aromatic amino acids (phenylalanine, tryptophan, and tyrosine), formic and fumaric acids, trigonelline, uracil and uridine were identified. The assignment of polyphenols will be discussed in detail in the next section.

The ^1H NMR spectra of Bligh-Dyer organic extracts, Figure S3, showed the presence of sterols, fatty acids, lipid polar heads, and pigments, Table 3, identified by literature data [49].

Table 3. Compounds and relative selected signals (ppm) for quantitative analysis in the organic extracts of Burdock root, Dandelion root and aerial part, and Lemon balm aerial part. The black dots mark the presence of the metabolite in the officinal plants.

	ppm	Group	Compounds	Burdock	Dandelion		Lemon Balm
					Root	Leaves	
I β -Sit	0.65	CH ₃ -18	β -Sitosterol	•	•	•	•
I _{Stig}	0.67	CH ₃ -18	Stigmasterol	•	•	•	•
I _{FA}	2.30	CH ₂ -11	Totally fatty acids	•	•	•	•
I _{DUFA}	2.73	CH ₂ -11	Linoleic acid	•	•	•	•
I _{TUFA}	2.77	CH ₂ -11,14	Linolenic acid	•	•	•	•
I _{PCG}	3.23	N(CH ₃) ₃	Glycerolphosphatidylcholine	•	•	•	•
I _{DGG}	4.87	CH-1	Glyceroldigalactose			•	•
I _{PHEO}	11.15	CH-5	Pheophytin			•	•
I _{CHL}	11.13	CH-5	Chlorophyll			•	•

NMR Identification of Polyphenols

The Bligh–Dyer hydroalcoholic extracts of Burdock root were characterised by the presence of 3,5-Di-caffeoylquinic acid, as shown in Figure 1 and Table 2.

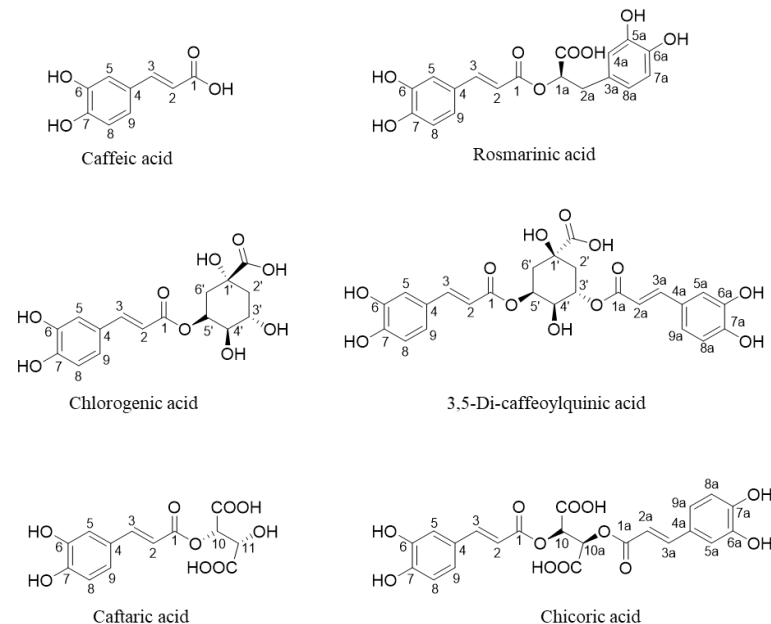


Figure 1. Molecular structures of polyphenols. To make the assignment discussion easier, the numbering of common portions is the same for the compounds.

The ^1H assignment was achieved through the olefinic proton signals H-2, H-2a, H-3, and H-3a (doublets with $J_{trans} = 16.0$ Hz) at 6.13, 6.25, 7.32, and 7.40 ppm, respectively, typical of caffeic moiety α - β unsaturated systems (^{13}C signals at 115.7, 116.5, 147.8 and 148.0 ppm, respectively). The two-dimensional ^1H - ^1H TOCSY experiment allowed for the identification of spin correlations between H-2/H-3 (6.13/7.40 ppm) and H-2a/H-3a (6.25/7.32 ppm) protons. Moreover, two aromatic systems were identified by means of

^1H and ^1H - ^1H TOCSY experiments, as well as underlying correlations between protons signals at 6.58 ppm (H-8, d, $J = 8.3$ Hz), 6.70 ppm (H-9, dd, $J = 8.3$; $J = 1.7$), and 7.21 ppm (H-5, d, $J = 1.7$ Hz) and those at 6.66 ppm (H-8a, d, $J = 8.3$ Hz), 6.74 ppm (H-9a, dd, $J = 8.3$ Hz; $J = 1.7$ Hz), and 7.24 ppm (H-5a, d, $J = 1.7$ Hz). Integrating the detected olefinic and aromatic signals revealed the same area value, thus indicating the same molar ratio for each group. This behaviour, observed in all the replicates, suggested the presence of a di-caffeoylquinic compound, whose NMR signals and correlations were in accordance with the literature data [51]. The analysis of quinic moiety in ^1H and two-dimensional experiments confirmed the relative positions of caffeic acid groups. In particular, two overlapped multiplets at 5.33 and 5.37 ppm were identified due to the quinic moiety protons CH-3'/CH-5', crossed to CH-2' and CH₂-6' (1.99 ppm; 2.02 ppm) signals in ^1H - ^1H TOCSY map. Based on the literature data [51], the observed chemical shifts and spin correlations in quinic moiety can only be attributed to 3,5-di-caffeoylquinic acid, thus confirming its presence in Burdock root.

Chicoric and caftaric acids were identified in Dandelion root and aerial part hydroalcoholic extracts. The presence of chicoric acid, as shown in Figure 1, was assessed through evidence of the spin systems formed by the α - β unsaturated system at 6.49 ppm (H-2 and H-2a, d $J_{\text{trans}} = 16.0$ Hz) and 7.72 ppm (H-3 and H-3a, d $J_{\text{trans}} = 16.0$ Hz). ^1H - ^1H TOCSY and ^1H - ^{13}C HSQC experiments also allowed us to observe the aromatic system of chicoric acid evidencing CH-5' (δ_{H} 6.96, δ_{C} 117.6), CH-6' (δ_{H} 7.17, δ_{C} 124.2), and CH-2' (δ_{H} 7.25, δ_{C} 117.6) groups. Moreover, the peculiar signal of chicoric acid, corresponding to (O)CH(COO)-10,10a groups of tartaric moiety, was identified at 5.54 ppm (singlet). The assignment of caftaric acid was carried out considering the peculiar signals of the (O)CH(COO)-10 and (OH)CH(COO)-11 groups of tartaric moiety at 5.31 ppm (d, $J = 2.2$ Hz) and 4.57 ppm (d, $J = 2.2$ Hz), respectively. ^1H - ^1H TOCSY confirmed the spin correlation between the two signals. Moreover, the α - β unsaturated system of caffeic moiety was detected at 6.41 ppm (d, $J = 16.0$ Hz) and 7.64 ppm (d, $J = 16.0$ Hz). The reported literature data confirmed the identification of both polyphenols and NMR assignments [41].

The ^1H NMR spectra of Lemon balm hydroalcoholic extracts showed the presence of chlorogenic and rosmarinic acids, as shown in Figure 1. In particular, chlorogenic acid identification was allowed starting from the typical doublets at 7.37 and 6.22 ppm ($J_{\text{trans}} = 16.0$ Hz) of caffeoyl moiety olefinic protons (CH-3 and CH-2), with short-range carbon correlations observed at 146.8 ppm and 115.8 ppm. Moreover, the presence of signals belonging to the quinic acid moiety was assessed by means of the spin correlation observed in the ^1H - ^1H TOCSY map between CH-4' (^1H 3.9 ppm, ^{13}C 70.8) and the diastereotopic protons of CH₂-2' (^1H 1.89, 2.10 ppm, ^{13}C 41.7 ppm) and CH₂-6' (^1H 1.99, 2.06 ppm, ^{13}C 41.7 ppm). Notably, the caffeoyl moiety olefinic protons identified in chlorogenic acid were also detected in rosmarinic acid at the same ppm value. Indeed, doublets at 7.37 and 6.22 ppm were broad due to the overlap of chlorogenic and rosmarinic spin systems. Anyway, rosmarinic acid assignment was based on the presence of the characteristic α proton CH-1a at 5.01 ppm (^{13}C 77.5 ppm). In the ^1H - ^1H TOCSY experiment, the signal showed a correlation with the methylene protons at 2.85 and 2.93 ppm assigned to CH₂-2a (^{13}C 37.9 ppm). These findings agreed with the literature data [52] and NMR spectra of the reference standards.

3.2. Characterisation and Comparison among the Three Ecotypes of the Same Species

Hereafter, the metabolite profile of each officinal plant will be discussed separately, and LSE, MSE, and OE will be compared.

3.2.1. Burdock—*Arctium lappa* L.

Thirty-six metabolites were identified in the three ecotypes of Burdock root, as shown in Tables 2 and 3 and quantified in Figure 2.

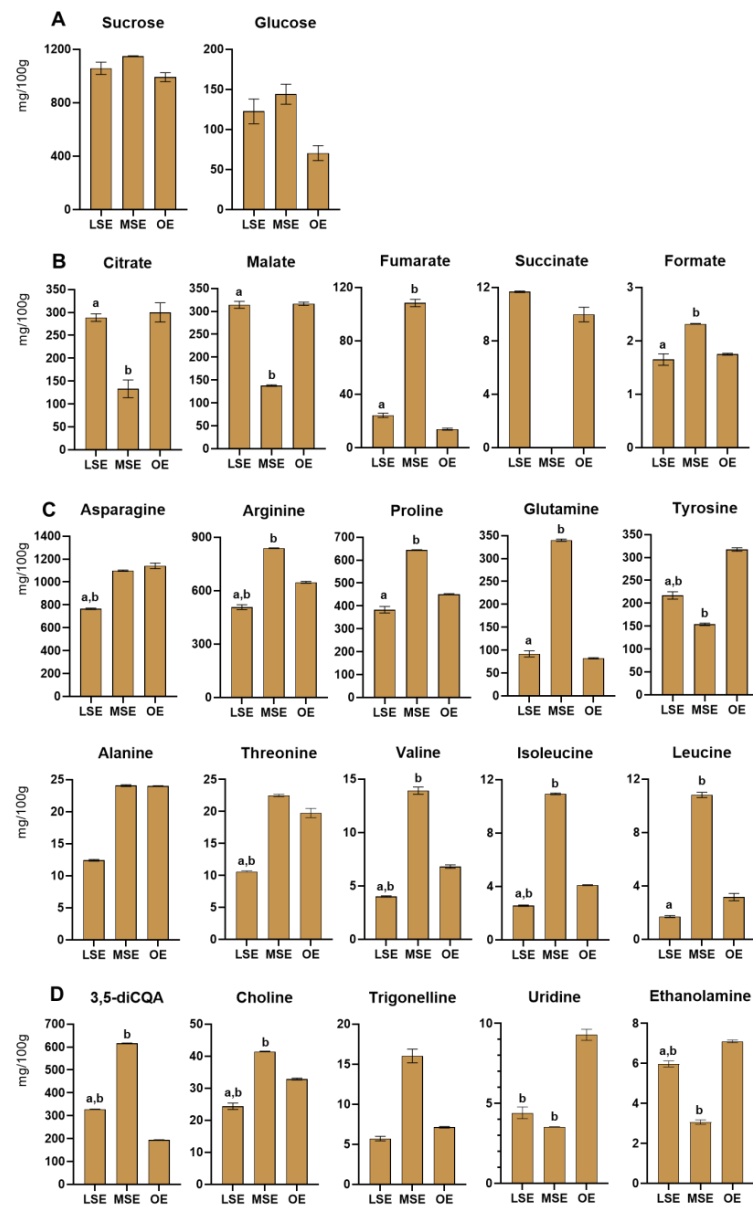


Figure 2. Histograms relative to compounds quantified (mg/100 g of dried sample $\pm$ SD) in the Bligh–Dyer hydroalcoholic extracts of Burdock in the Land Spontaneous Ecotype (LSE), Mountain Spontaneous Ecotype (MSE), and Organic Ecotype (OE). (A) Sugars, (B) organic acids, (C) amino acids, and (D) other metabolites. One-way ANOVA, followed by Tukey’s multiple comparison test, was applied to underline, among ecotypes, significant differences ($p < 0.0001$) for each metabolite: (a) vs. MSE; (b) vs. OE.

Amino acids were the most abundant metabolites among the three ecotypes, followed by carbohydrates and organic acids (Figure S4A). The total contents of sugars were similar in the three ecotypes, with sucrose being the most abundant (Figure 2A). Citrate and malate were measured in the same content range among the organic acids (Figure 2B). Interestingly, fumarate was present in high concentrations in MSE, whereas, on the contrary, succinate was not measured in this ecotype. Regarding the amino acids, MSE extracts were characterised by the highest concentrations of leucine, valine, alanine, proline, threonine, isoleucine, glutamine, and arginine, whereas LSE was characterised by the lowest ones (Figure 2C). Among other compounds, the behaviour observed for polyphenol 3,5-di-caffeoylquinic acid was interesting, with a content of 615 mg/DW in the MSE ecotype, being three times higher in MSE with respect to OE (Figure 2D).

The NMR metabolite characterisation of Burdock root was previously carried out by Jung et al. [42], in which an NMR–metabolomic approach was applied to a methanolic extract of plant root in response to copper stress. Anyway, a more complete NMR assignment concerning organic acids, amino acids, and other metabolites was obtained here. Jung et al. identified only succinate among the organic acids, whereas in the present study, acetate, citrate, formate, fumarate, lactate, and malate were also identified in the hydroalcoholic extracts of Burdock root [42]. Among the amino acids, additional glutamine, tryptophan, and glycine were identified in the ^1H NMR spectra, while phenylalanine was not detected here. Moreover, other metabolites were found, including choline, ethanolamine, trigonelline, uridine, and the phenolic compound 3,5 di-caffeoylquinic acid.

Regarding liposoluble fraction, histograms were reported in Figure S5. One-way ANOVA was performed, revealing no significant differences ($p < 0.0001$) among the samples. Notably, both Spontaneous ecotypes, with respect to OE, showed a higher level of SFA, whereas the OE extracts were characterised by the highest concentrations of UFAs. Among the latest, DUFA were the main class with respect to TUFA and MUFA. Regarding sterols, β -sitosterol and stigmasterol concentrations were quite similar in all ecotypes. The obtained data were in accordance with the literature in terms of both fatty acids and sterols' qualitative and quantitative profiles [53].

3.2.2. Dandelion—*Taraxacum officinale*

Forty-five metabolites belonging to different chemical classes were identified in both Dandelion root and aerial part extracts, as shown in Tables 2 and 3. Histograms of quantified metabolites in the hydroalcoholic phase are shown in Figure 3.

The highest total sugar content was observed for OE ecotypes in both the roots and aerial parts, whereas the lowest was in MSE (Figure S4B). Glucose and sucrose were the most abundant sugars in all ecotypes. Glucose, myo-inositol, and β -galactose were found in higher concentrations in the aerial parts of the three ecotypes, with glucose and β -galactose being three times higher than in the root. On the other hand, root extracts showed a higher concentration of sucrose compared to the aerial parts.

The MSE ecotypes showed the highest total contents of organic acids, with the aerial part extracts particularly enriched. However, few exceptions can be observed for citrate and acetate. In particular, the citrate content was three times higher and comparable in LSE and MSE root extracts. Malate was the most abundant organic acid in all ecotypes, followed by tartrate, citrate, succinate, acetate, fumarate, and formate.

According to the amino acid profile, the OE root extract showed the highest total content. All identified amino acids were present in both root and aerial part extracts, except for arginine, which was only found in the root. Additionally, glutamine was not detected in the MSE aerial part, and tryptophan was absent from the OE root. Asparagine, proline, and glutamine levels in the root were approximately three times higher than in the aerial part. This is because nitrogen-rich amino acids are used as a storage source of nitrogen in the woody parts of plants, such as the roots [54]. The highest levels of polyphenols (caftaric and chicoric acids) were observed in the MSE aerial parts, with chicoric acid content four-fold higher than in the LSE and OE samples. Choline and trigonelline were abundant metabolites in all ecotypes.

An NMR-based untargeted metabolomic analysis was previously performed on the methanolic extracts of the Dandelion aerial parts [41]. Here, a more complete assignment of the amino acids profile was achieved, quantifying asparagine, aspartate, threonine, tryptophan, and glutamine. Noteworthy carbohydrates turned out to be the most abundant class of primary metabolites, followed by organic acids and amino acids, in the three ecotypes. In contrast, in Grauso et al. [41], the organic acids were present in higher concentrations than the other classes. These differences are probably due to methodology variability (the extraction solvent's composition, type of extraction, analytical method), genetic background, and cultivation practices.

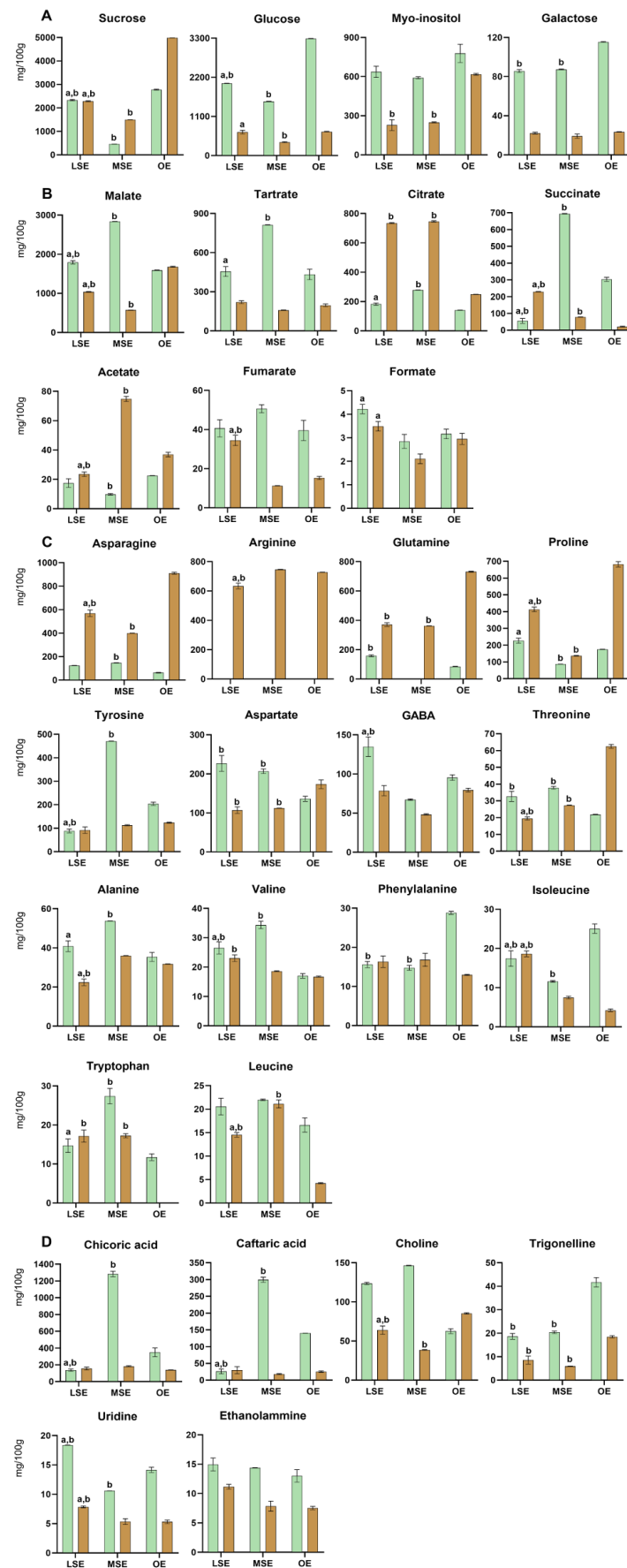


Figure 3. Histograms relative to compounds quantified (mg/100 g of dried sample $\pm$ SD) in the Blyth-Dyer hydroalcoholic extracts of Dandelion in Land Spontaneous Ecotype (LSE), Mountain Spontaneous

Ecotype (MSE) and Organic Ecotype (OE), comparing the difference between aerial part (green) and root (brown). (A) Sugars, (B) organic acids, (C) amino acids, and (D) other metabolites. Two-way ANOVA, followed by Tukey's multiple comparison test, was applied to underline, among ecotypes, significant differences ($p < 0.0001$) for each metabolite according to the same plant part: (a) vs. MSE; (b) vs. OE.

The organic Bligh–Dyer extracts of Dandelion (Figure S6) showed that the roots had high levels of SFA, while high levels of MUFA and TUFA mainly characterised the aerial parts, and no SFA was detected in them. Moreover, as expected, pheophytin and chlorophyll pigments were measured only in the aerial parts. Sterol content was quite the same in all the considered samples. Compared with the literature, similar contents of saturated fatty acids were previously measured [41]. A more comprehensive NMR assignment for organic extracts was obtained here.

3.2.3. Lemon Balm—*Melissa officinalis*

Thirty-eight metabolites were detected in Bligh–Dyer hydroalcoholic extracts of LSE, MSE, and OE (Tables 2 and 3). Histograms resulting from the quantification of water-soluble compounds are reported in Figure 4.

Lemon balm extracts were rich in organic acids, followed by carbohydrates and amino acids (Figure S4C). The MSE ecotype showed the highest sugar content, whereas the lowest level was observed in LSE. Sucrose and glucose were the most abundant sugars, with the highest concentration in MSE.

The highest organic acid total content was found in MSE and LSE. Tartrate was the most abundant in the three ecotypes, representing at least 1% of the total dried sample weight in all samples, followed by citrate, malate and succinate. Comparing the three ecotypes, the OE samples had the lowest tartrate contents but the highest citrate, malate, and succinate levels. In contrast, a similar concentration of these metabolites was found in both the Spontaneous LSE and MSE ecotypes. The total amino acid content was comparable in all samples, with the OE ecotype being slightly richer. Aspartate and glutamine were the most abundant amino acids in the three ecotypes, followed by GABA, proline, threonine, alanine, valine, isoleucine, and leucine. The content of each amino acid was similar between the Spontaneous ecotypes, whereas the OE had the highest level, except for threonine, which was not quantified. Choline, uracil, and polyphenols, namely chlorogenic and rosmarinic acids, were also identified. The choline contents in the three ecotypes were comparable, while uracil was not found in the OE. Both rosmarinic and chlorogenic acids were not quantified due to the overlapping signals.

No literature data concerning exhaustive NMR analyses of Lemon balm have previously been reported, since most studies have exclusively considered the analysis of polyphenols using other analytical techniques (HPLC-DAD, UHPLC-MS) [44,55]. A study on infusion mixtures of different officinal plants, including Lemon balm, using high-resolution ^1H NMR spectroscopy and multivariate statistical analysis, mentioned the presence of flavonoids and phenols, in particular rosmarinic and chlorogenic acid [56].

Among the liposoluble metabolites reported in Figure S7, the MSE ecotype contained the highest amount of UFAs, especially MUFAs, PC, and DGDG, while the LSE and OE had higher levels of SFAs. However, the highest concentration of TUFA and DUFA was measured in the OE. Sterol content was comparable among the three ecotypes. Pheophytin and chlorophyll were measured in concentrations lower than 3 mg/100 g, with pheophytin being mainly present in Spontaneous ecotypes. SFA and UFA distributions reported here for Lemon balm aerial parts have also been confirmed by the literature data [57].

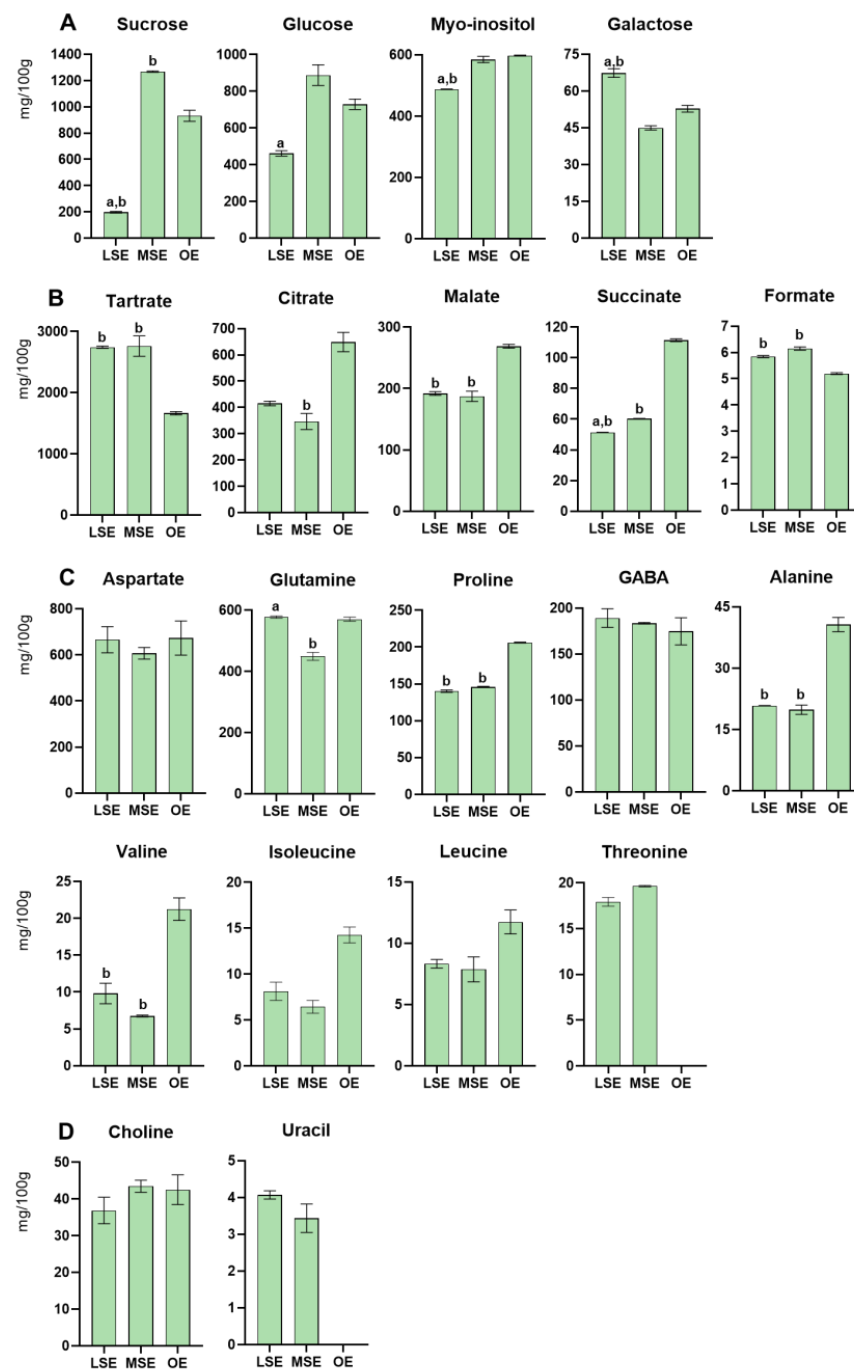


Figure 4. Histograms relative to compounds quantified (mg/100 g of dried sample $\pm$ SD) in the Bligh–Dyer hydroalcoholic extracts of Lemon balm in the Land Spontaneous Ecotype (LSE), Mountain Spontaneous Ecotype (MSE), and Organic Ecotype (OE). (A) Sugars, (B) organic acids, (C) amino acids, and (D) other metabolites. One-way ANOVA, followed by Tukey's multiple comparison test, was applied to underline, among ecotypes, significant differences ($p < 0.0001$) for each metabolite: (a) vs. MSE; (b) vs. OE.

3.3. Comparison of Burdock, Dandelion and Lemon Balm

The metabolomic profile obtained from the analysis of the selected officinal plants using NMR methodology allowed us to compare the chemical compositions of different ecotypes and tissues and further evaluate the impact of pedoclimatic conditions. The analysis revealed that the qualitative compositions of the most abundant metabolites, including sugars, amino acids, organic acids, and amines, were similar across all samples.

However, the levels of these metabolites varied significantly depending on the crop and tissue type. Comparing the total contents of the major metabolites' classes among the selected samples, the three ecotypes of Burdock roots showed the highest contents of amino acids and the lowest contents of carbohydrates and organic acids, whereas for Dandelion, both the aerial parts and roots were richest in carbohydrates, except for the MSE aerial parts characterised by the highest organic acid total content (4.6 g/100 g of DW). Lemon balm Spontaneous ecotypes were found to have the highest total organic acid contents, exceeding 3 g/100 g of DW (Figure S4).

Considering the variability in each metabolite, the three officinal plants showed similarities and differences. Branched amino acids were present in smaller amounts, whereas arginine was detected in Dandelion and Burdock roots, being a tissue-specific marker, as well as myo-inositol, galactose, DGG moiety, pheophytin, and chlorophyll, identified in the aerial parts of Lemon balm and Dandelion. Indeed, digalattosyldiacylglycerol stabilises the chloroplast membrane, maintaining its morphology and maximising photosynthesis efficiency to ensure plant survival under abiotic stresses. Pigments such as chlorophyll and pheophytin are involved as electron carrier intermediaries in photosynthesis, which takes place in the leaves. Regarding organic acids, a similar qualitative profile was observed. Tartrate and fumarate were not detected in Burdock and Lemon balm, respectively. Moreover, the comparison of the three officinal plants highlighted the presence of specific secondary metabolites. Burdock, for instance, contained 3,5 di-caffeoylquinic acid, whereas Dandelion contained caftaric and chicoric acids. On the other hand, Lemon balm contained chlorogenic and rosmarinic acids.

To evaluate the effects of pedoclimatic and genetic factors, the data obtained from the analysis of hydroalcoholic metabolites were subjected to PCA, only considering metabolites always present in the different plants. Two PCA models were made to compare the same parts of different plants, namely one PCA was carried out to compare Burdock and Dandelion roots (Figure 5A), whereas a second one was conducted to compare the aerial parts of Dandelion and Lemon balm (Figure 5B).

The first PCA model, regarding plant roots, as shown in Figure 5A, accounts for 72.4% of the variability, with separation along PC1 due to genetic basis: positive PC1 values were related to Dandelion and negative ones to Burdock. Dandelion root was shown to be mainly related to higher amounts of all the considered polar metabolites except for tyrosine, asparagine, proline, and fumarate. Moreover, within each considered plant, the obtained model underlined differences among growing conditions. Considering the Dandelion root, the organic ecotype was clearly divided from Spontaneous ones along PC2. On the contrary, in the Burdock root, the clustering occurred between the Mountain Spontaneous ecotype and the remaining ones, along with PC1 negative values. The PCA model of the aerial parts of Dandelion and Lemon balm is reported in Figure 5B, with the first two PCs accounting 84.6% of the variability. Along PC1, samples were separated according to the species, with Lemon balm, with negative PC1 values, being characterised by high levels of GABA, aspartate, and organic acids. Along PC2, a clustering among ecotypes was observed for Dandelion, with MSE being separated from the other ecotypes. For Lemon balm, no clustering among ecotypes was observed.

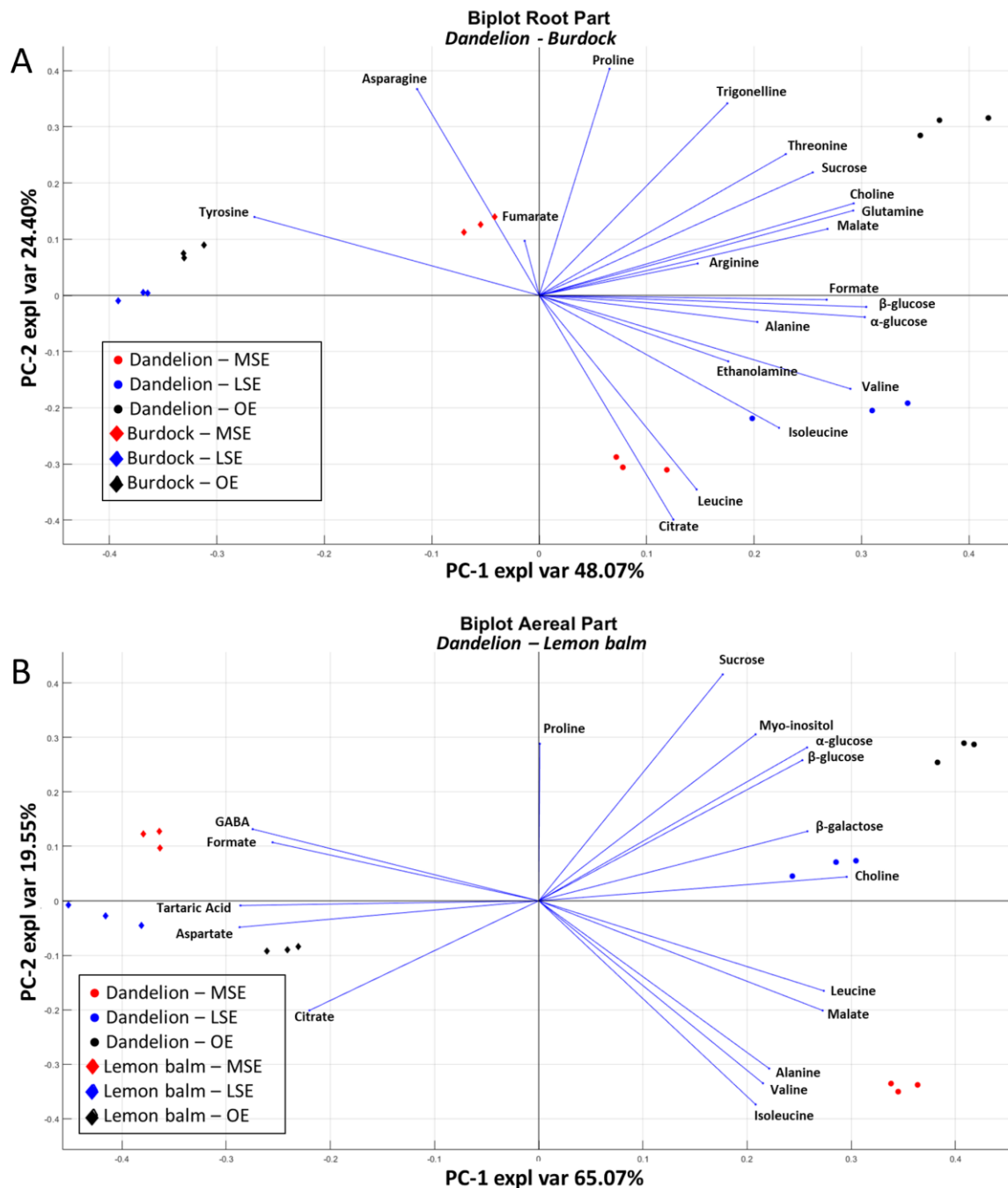


Figure 5. PCA model of (A) root parts of Burdock and Dandelion; (B) aerial parts of Lemon balm and Dandelion. The points represent the samples, while the lines represent the directions of growth of the plants' metabolites. In (A), the Dandelion samples are represented by circles and the Burdock samples are represented by diamonds. The ecotypes are differentiated by colours: red for MSE, blue for LSE, and black for OE. In (B), the Dandelion samples are represented by circles and the Lemon balm samples are represented by diamonds. The ecotypes are differentiated by colours: red for MSE, blue for LSE, and black for OE.

4. Conclusions

NMR metabolomics has allowed us to recognise distinct chemical profiles for each ecotype among the three plants. The untargeted approach demonstrated how each medicinal plant has its own, though in some cases overlapping, chemical profile responsible for its health and nutritional properties. This demonstrates that the pedoclimatic effect significantly impacts the chemical compositions of these plants and highlights the effective-

ness of NMR analysis for characterising similar matrices. However, additional sampling is required to establish a stronger correlation among the variables being considered. While PCA was able to take into account all variables in the biplot, further measures are needed to compare different plant species and ensure robustness in the results. From a holistic perspective, it is crucial to complement biological evaluation to obtain comprehensive profiles of ecotypes and plants.

The methodology used here proved its effectiveness in selecting plants with the richest phytochemical profiles. This enhances their value, promotes more conscious consumption, and encourages their use in the nutraceutical, functional food, and phytopharmaceutical industries.

Supplementary Materials: The following supporting information can be downloaded via this link: <https://www.mdpi.com/article/10.3390/foods13111642/s1>, Figure S1. Burdock root (A), Dandelion aerial part and root (B), and Lemon balm aerial part (C); Figure S2. ¹H-NMR Spectrum of the hydroalcoholic Bligh–Dyer extract in 100 mM PBS/D₂O and 0.4 mM TSP. (a) Burdock root, (b) Dandelion root, (c) Dandelion aerial part, and (d) Lemon balm aerial part; Figure S3. ¹H-NMR Spectrum of the organic Bligh–Dyer extract in CDCl₃/CD₃OD (2:1 v/v) mixture. (a) Burdock root, (b) Dandelion root, (c) Dandelion aerial parts, and (d) Lemon balm aerial part; Figure S4. Histograms resulting from the quantitative NMR analysis of the main compound's total contents of amino acids (orange), organic acids (light blue) and carbohydrates (yellow) present in the Bligh–Dyer hydroalcoholic extracts of (A) Burdock root, (B) Dandelion aerial part and root, and (C) Lemon balm aerial part in the three ecotypes: Land Spontaneous (LSE), Organic (OE), and Mountain Spontaneous (MSE). Results, expressed as mg/100 g of dried sample, refer to the mean and SD of three replicates; Figure S5. Histograms relative to compounds quantified (mg/100 g of dried sample ± SD) in Bligh–Dyer organic extracts of Burdock in Land Spontaneous (LSE), Mountain Spontaneous (MSE), and Organic (OE); Figure S6. Histograms relative to compounds (mg/100 g dried sample ± SD) in Bligh–Dyer organic extracts of Dandelion's aerial part (green) and roots (brown) in Land Spontaneous (LSE), Mountain Spontaneous (MSE), and Organic (OE). Two-way ANOVA, followed by Tukey's multiple comparison test, was applied to underline, among ecotypes, significant differences ($p < 0.0001$) for each metabolite according to the same plant part: (a) vs. MSE; (b) vs. OE; Figure S7. Histograms relative to compounds quantified (mg/100 g of dried sample ± SD) present in Bligh–Dyer organic extracts of Lemon balm in Land Spontaneous (LSE), Mountain Spontaneous (MSE), and Organic (OE). One-way ANOVA, followed by Tukey's multiple comparison test, was applied to underline, among ecotypes, significant differences ($p < 0.0001$) for each metabolite: (a) vs. MSE; (b) vs. OE; Table S1. Environmental growing conditions in Collepardo and Isola del Liri (Italy).

Author Contributions: Conceptualisation, D.A. and C.I.; methodology, D.A., F.M., E.R. and R.G.; software, R.G.; validation, C.I.; formal analysis, D.A., F.M. and E.R.; investigation, D.A. and F.M.; resources, L.M.; data curation, D.A., C.I. and M.S.; writing—original draft preparation, D.A. and F.M.; writing—review and editing, C.I. and M.S.; visualisation, L.M. and C.I.; supervision, L.M.; project administration, L.M.; funding acquisition, L.M. All authors have read and agreed to the published version of the manuscript.

Funding: This study was funded by the European Union—NextGenerationEU (the National Recovery and Resilience Plan (NRRP), Mission 4 Component 2 Investment 1.3—Call for tender No. 341 of 15 March 2022 of Italian Ministry of University and Research; Award Number: Project code PE00000003, Concession Decree No. 1550 of 11 October 2022 adopted by the Italian Ministry of University and Research, CUP D93C22000890001), Project title “ON Foods—Research and Innovation Network on Food and Nutrition Sustainability, Safety, and Security—Working ON Foods” and by the European Union — NextGenerationEU project within the Agritech National Research Center (the National Recovery and Resilience Plan (NRRP), Mission 4 Component 2 Investment 1.4—D.D. 1032 17/06/2022, CN000000022). This manuscript reflects only the authors' views and opinions; neither the European Union nor the European Commission can be considered responsible for them.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The original contributions presented in the study are included in the article and Supplementary Materials, further inquiries can be directed to the corresponding author.

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

Abbreviations

DGG	Digalactosyldiacylglycerol
DUFA	Di-unsaturated fatty acids
GABA	γ -amino-butyric acid
GC-MS	Gas Chromatography–Mass Spectroscopy
^1H - ^{13}C	HSQC Heteronuclear single quantum coherence Spectroscopy
^1H - ^{13}C	HMBC Heteronuclear Multiple Bond Correlation Spectroscopy
^1H - ^1H	TOCSY Total Correlation Spectroscopy
HPLC-DAD	High-Performance Liquid Chromatography–Diode Array Detector
HPLC-MS	High-Performance Liquid Chromatography–Mass Spectroscopy
LSE	Land Spontaneous Ecotype
MUFA	Mono-unsaturated fatty acids
MSE	Mountain Spontaneous Ecotype
NMR	Nuclear Magnetic Resonance
OE	Organic Ecotype
PCA	Principal Component Analysis
QTRAP-MS	Mass Spectroscopy
UFA	Unsaturated Fatty Acids
UHPLC-MS	Ultra-High-Performance Liquid Chromatography–Mass Spectroscopy
UPLC	Ultra-Pressure Liquid Chromatography
TBZ	3,4,5-Trimethoxybenzaldehyde
TSP	3-(trimethylsilyl)-propionic-2,2,3,3-d ₄ acid sodium salt
TUFA	Tri-unsaturated fatty acids

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Supplementary Materials

NMR Metabolomics of *Arctium lappa* L., *Taraxacum officinale* and *Melissa officinalis*: A Comparison of Spontaneous and Organic Ecotypes

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Figures

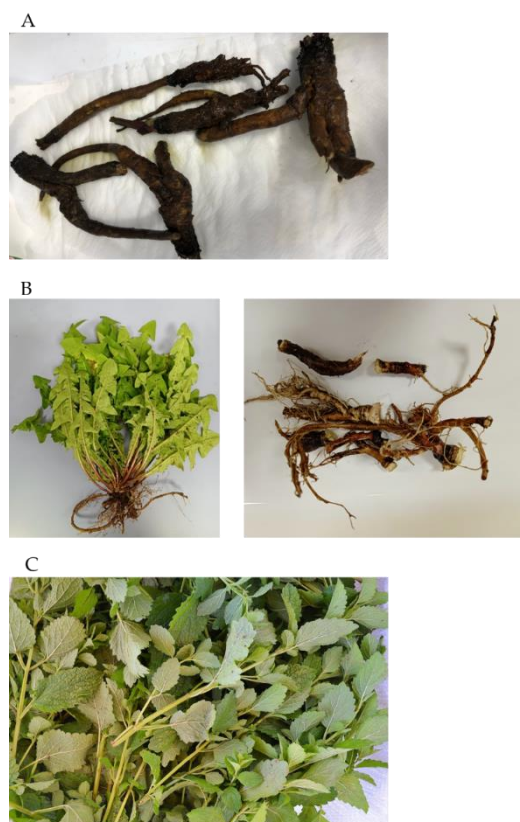


Figure S1. Burdock root (A), Dandelion aerial part and root (B), Lemon balm aerial part (C)

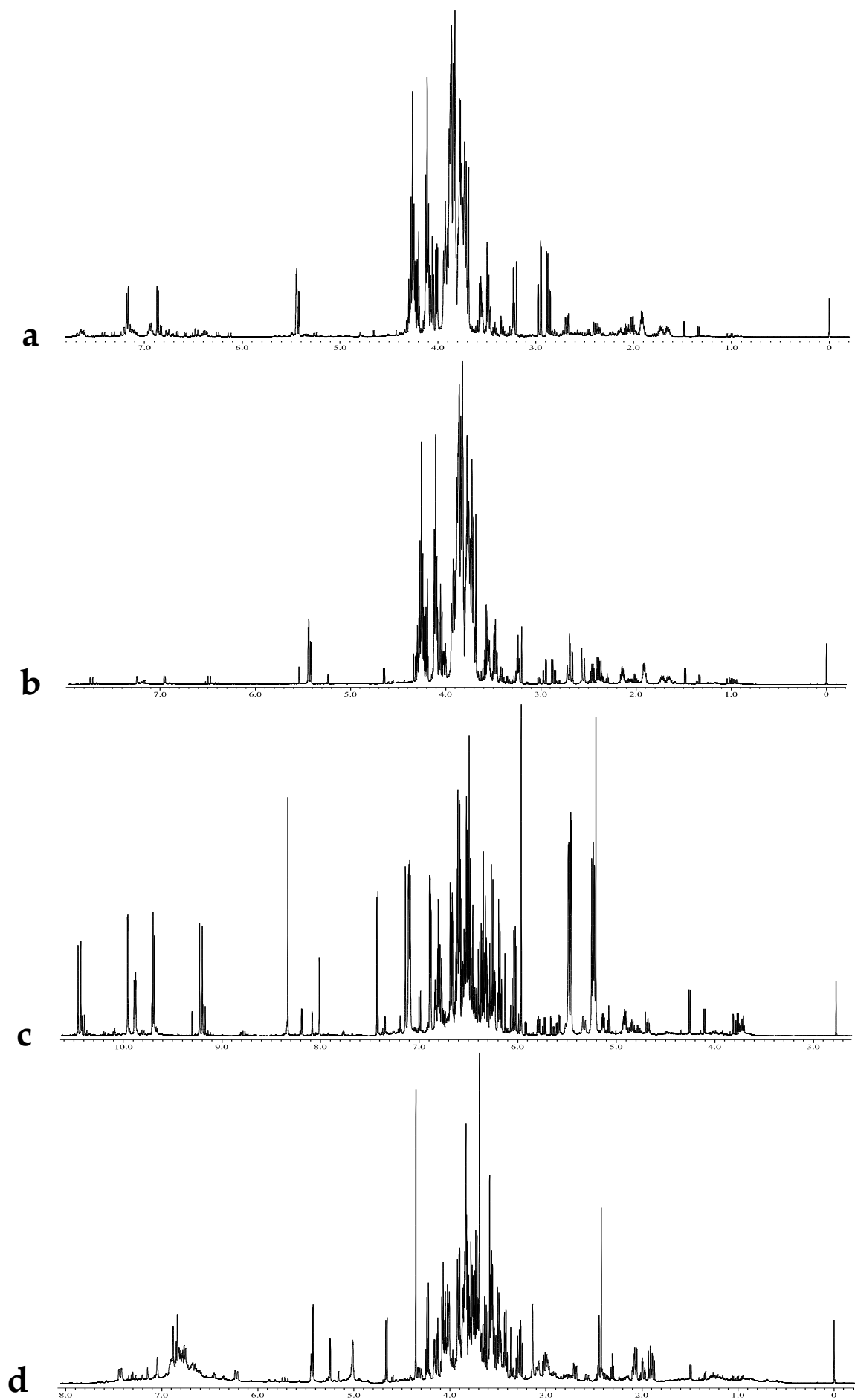


Figure S2. ¹H-NMR Spectrum of the hydroalcoholic Bligh-Dyer extract, in 100 mM PBS/D₂O, 0.4 mM TSP.
(a) Burdock root, (b) Dandelion root, (c) Dandelion aerial part, (d) Lemon balm aerial part.

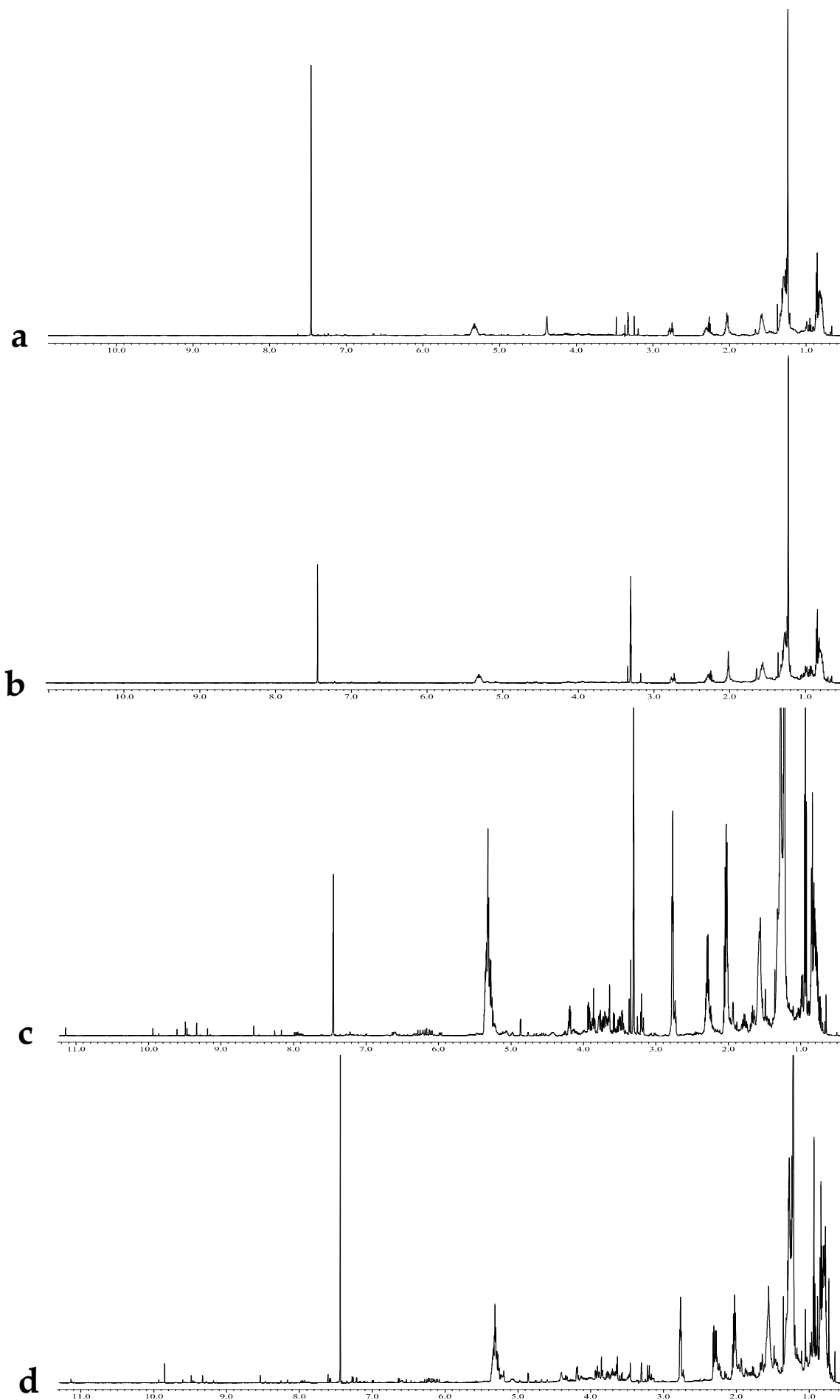


Figure S3. ^1H -NMR Spectrum of the organic Bligh-Dyer extract, in $\text{CDCl}_3/\text{CD}_3\text{OD}$ (2:1 v/v) mixture. (a) Burdock root, (b) Dandelion root, (c) Dandelion aerial part, (d) Lemon balm aerial part.

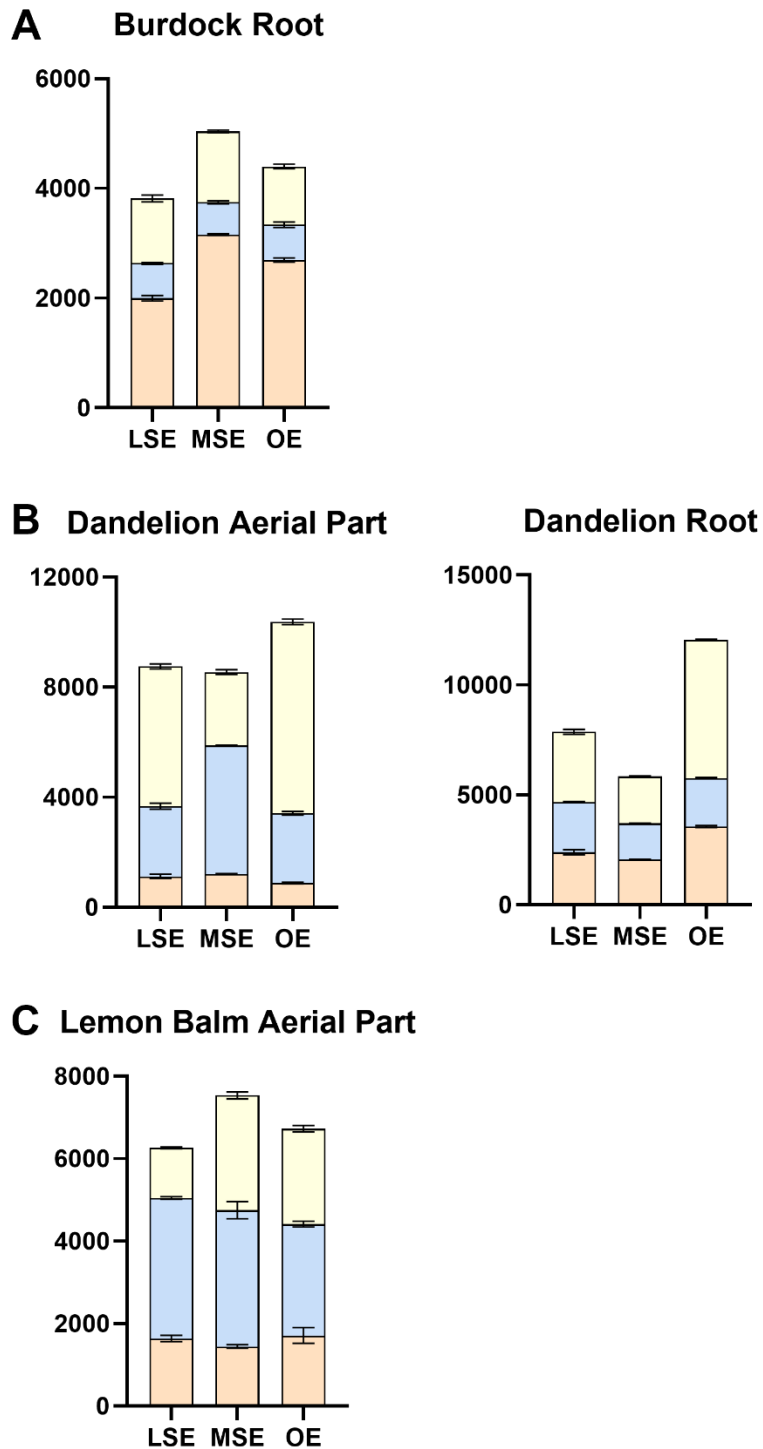


Figure S4. Histograms resulting from the quantitative NMR analysis of main compound total content amino acids (orange), organic acids (light blue) and carbohydrates (yellow) present in the Bligh-Dyer hydroalcoholic extracts of (A) Burdock root, (B) Dandelion aerial part and root, and (C) Lemon Balm in the three ecotypes Land Spontaneous (LSE), Organic (OE), Mountain Spontaneous (MSE). Results, expressed as mg/100 g dried sample, refer to the mean and SD of three replicates.

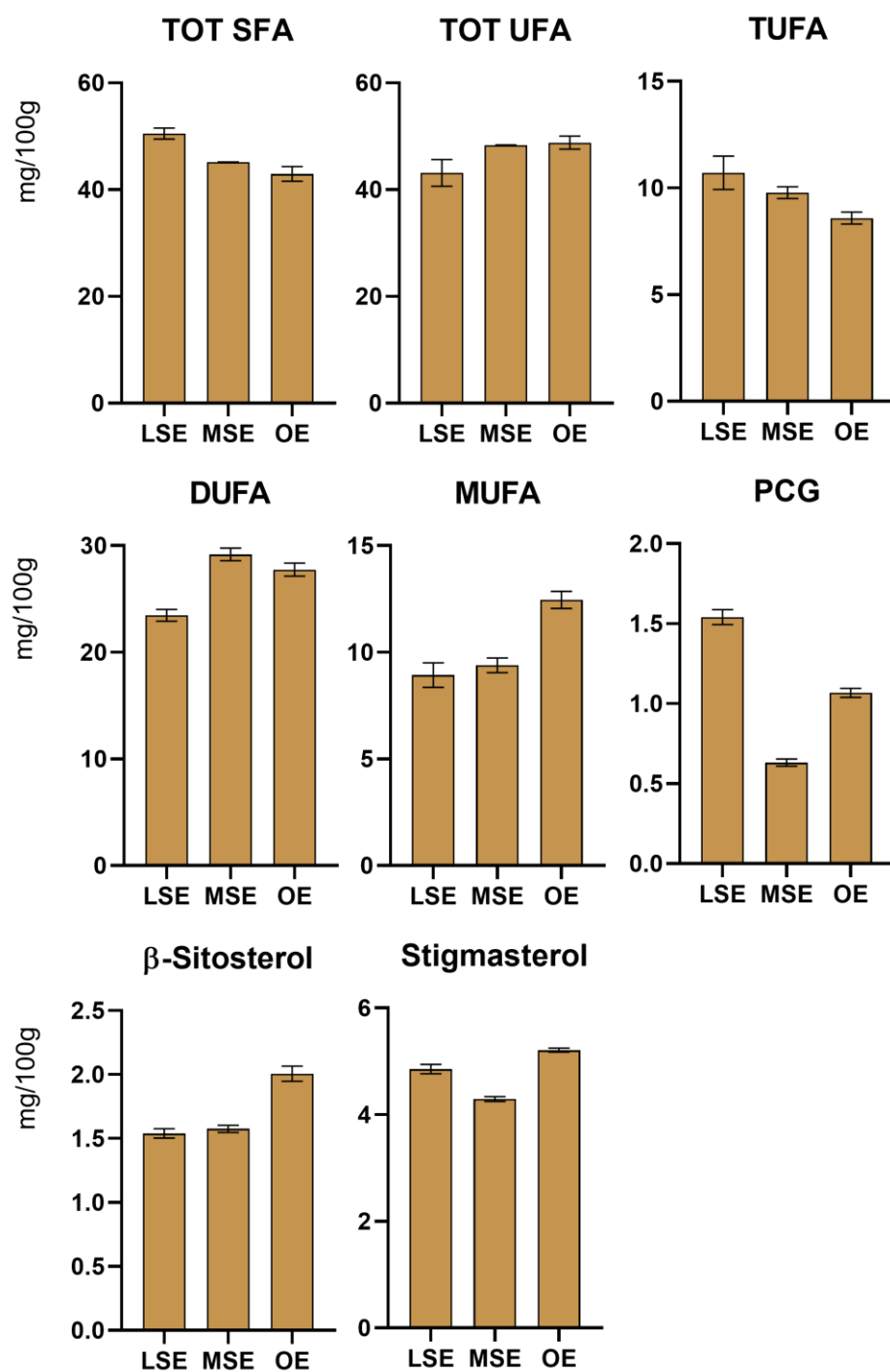


Figure S5. Histograms relative to compounds quantified (mg/100 g of dried sample $\pm$ SD) in Bligh-Dyer organic extracts of Burdock in Land Spontaneous (LSE), Mountain Spontaneous (MSE) and Organic (OE).

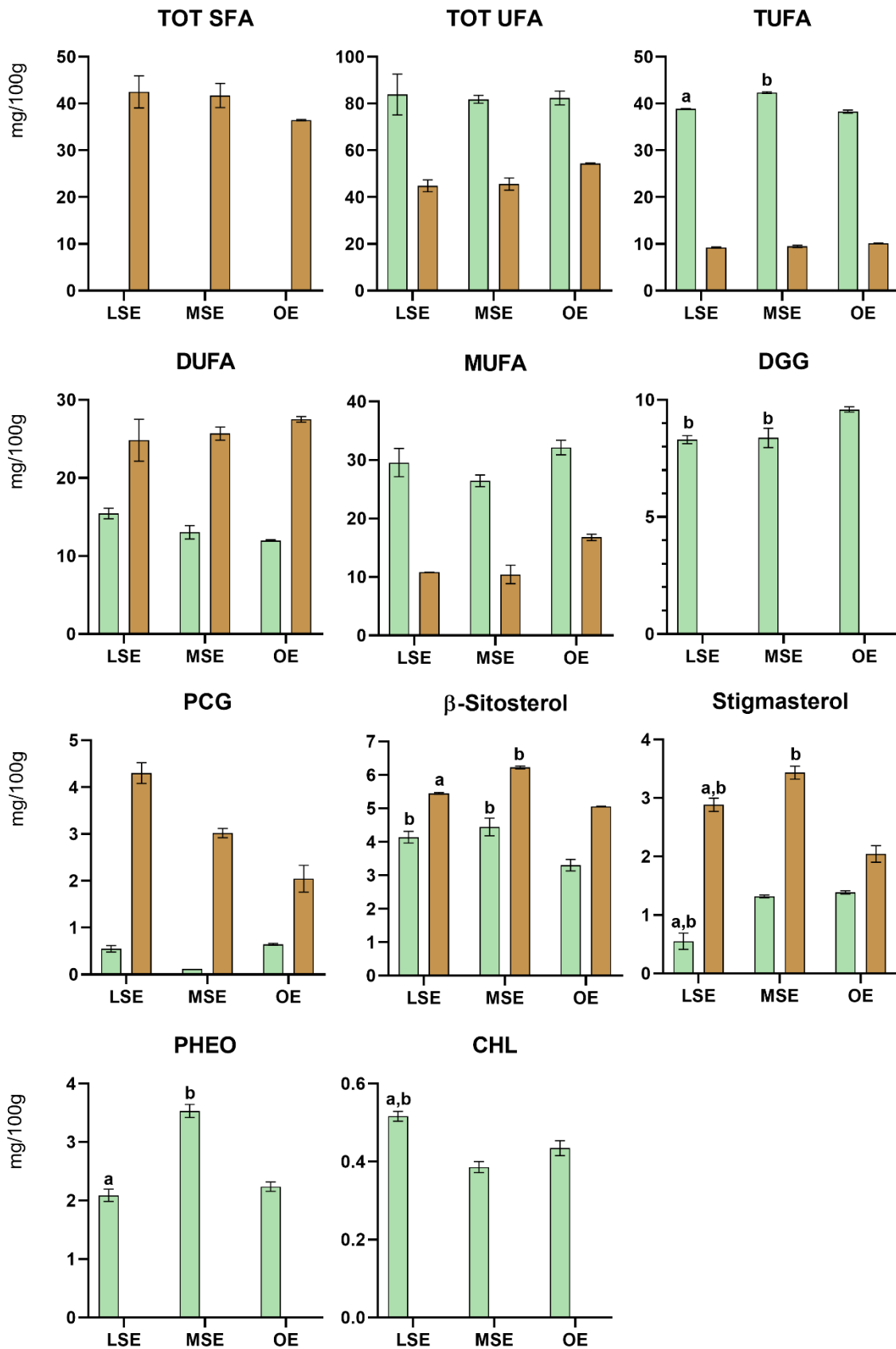


Figure S6. Histograms relative to compounds (mg/100 g dried sample $\pm$ SD) in Bligh-Dyer organic extracts of Dandelion aerial part (green) and root (brown) in Land Spontaneous (LSE), Mountain Spontaneous (MSE) and Organic (OE). Two-way ANOVA, followed by Tukey's multiple comparison test, was applied to underline, among ecotypes, significant differences ($p < 0.0001$) for each metabolite according to the same plant part: a) vs. MSE, b) vs. OE.

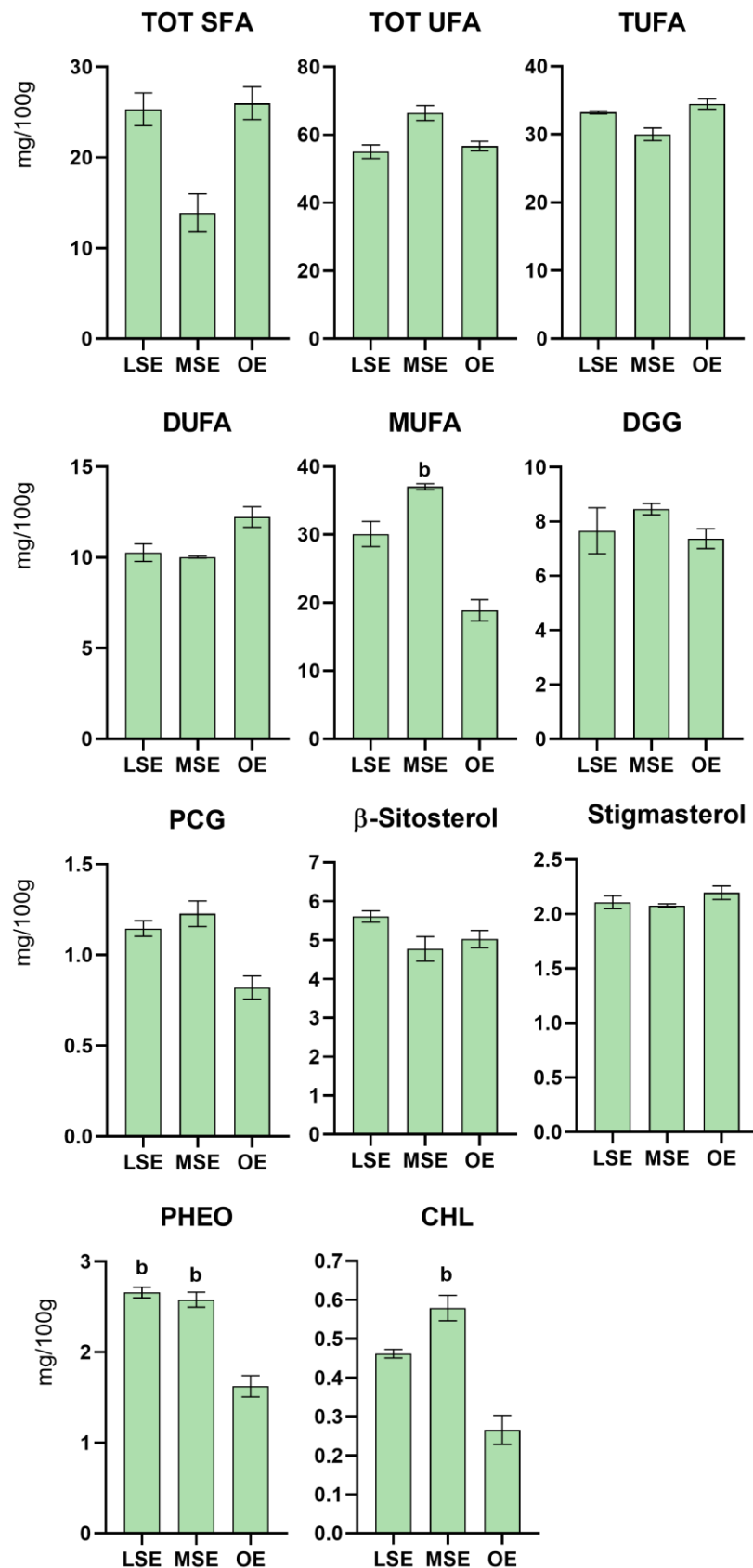


Figure S7. Histograms relative compounds quantified (mg/100 g of dried sample $\pm$ SD) present in Bligh-Dyer organic extracts of Lemon balm in Land Spontaneous (LSE), Mountain Spontaneous (MSE) and Organic (OE). One-way ANOVA, followed by Tukey's multiple comparison test, was applied to underline, among ecotypes, significant differences ($p < 0.0001$) for each metabolite: a) vs. MSE, b) vs. OE.

Tables

Table S1. Environmental growing conditions in Colleparado and Isola del Liri (Italy).

Colleparado	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Mean
Temperature ^T	3 °C	3 °C	6 °C	9 °C	14 °C	18 °C	21 °C	21 °C	17 °C	12 °C	7 °C	3 °C	11 °C
Precipitation (mm) ^P	49.3	51.4	42.2	42.7	33.2	26.0	18.8	23.3	55.2	74.2	86.9	61.1	47.0
Humidity ^H	0 %	0 %	0 %	0 %	0 %	1 %	4 %	8 %	3 %	0 %	0 %	0 %	1.3 %
Daylight hours ^{DH}	9.5	10.6	12.0	13.4	14.6	15.2	14.8	13.8	12.4	11.1	9.8	9.2	-
Sun energy (kWh) ^{SE}	2.0	2.9	4.2	5.5	6.6	7.4	7.5	6.5	4.9	3.4	2.2	1.7	4.5

Isola del Liri	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Mean
Temperature ^T	5 °C	6 °C	9 °C	12 °C	16 °C	21 °C	24 °C	24 °C	19 °C	15 °C	10 °C	6 °C	14 °C
Precipitation (mm) ^P	55.2	55.0	46.6	43.1	32.7	25.8	19.0	23.4	55.3	73.9	89.3	64.7	48.7
Humidity ^H	0 %	0 %	0 %	0 %	4 %	4 %	11 %	18 %	4 %	2 %	0 %	0 %	3.4%
Daylight hours ^{DH}	9.5	10.6	12.0	13.4	14.6	15.2	14.8	13.8	12.4	11.1	9.8	9.2	-
Sun energy (kWh) ^{SE}	2.0	2.9	4.2	5.5	6.6	7.4	7.4	6.5	4.9	3.4	2.2	1.7	4.5

^T The average temperature, considering the maximum and minimum for a month.

^P The average rainfall accumulated during 31 days.

^H Humidity is based on the comfort level at the dew point (if perspiration will evaporate from the skin, cooling the body).

^{DH} The number of hours in which the sun is visible.

^{SE} The average amount of shortwave solar energy reaching the ground per unit area.

A2.3 Case study 2

A2.3.1 Introduction

Lemon balm, also known as *Melissa officinalis*, is a perennial shrubby plant from the Lamiaceae family. While it grows worldwide, its exact origins are not well-defined, though it's believed to have originated in the Eastern Mediterranean, Western Asia, Southern Europe, Caucasus, and Northern Iran. Italy and France are the major producers of lemon balm (Sabet Teymouri and Sabet Teimouri 2019). In Europe, two main subspecies of lemon balm have been identified: *Melissa officinalis* subsp. *officinalis* and *Melissa officinalis* subsp. *altissima* (Sm.), which are distinguished by the indumentum of the leaves and the shape of the fruiting calyx (Chizzola, Lohwasser, and Franz 2018). The *Melissa officinalis* subsp. *altissima* is also referred to as *Melissa romana* MILLER or wild lemon balm (Usai, Atzei, and Marchetti 2016).

The Lamiaceae family is important as a source of medicinal, aromatic, and edible plants. Indeed, the lemon balm plant is rich in biologically active components, including terpenes, phenolic acids, and flavonoids, and has been cited for significant pharmacological activities, as antibacterial, antifungal, antiviral, anti-inflammatory, and antioxidant properties (Petrisor et al. 2022).

The growing preference for healthy lifestyle and nutrition has led to increased consumption of natural and functional food products. As a result, there has been a significant expansion in the use of plants and their derivatives in the food industry and for nutraceutical applications (Deans and Ritchie 1987). The use of lemon balm leaf drugs for mild stress symptoms, sleep aid, and mild digestive disorders has been approved by the HMPC (Committee on Herbal Medicinal Products) of the EMA (European Medicines Agency) based on traditional use. Although there aren't enough clinical studies, the efficacy of these herbal medicines is well-founded, and evidence shows their safe use in these ways for at least thirty years. Ethnopharmacological studies have reported that lemon balm is used in many countries to treat various disorders, mainly in the pharmaceutical forms of essential oil, infusion, and decoction (Shakeri, Sahebkar, and Javadi 2016).

In addition to this, there are studies in the literature discussing the potential positive effects of this medicinal plant on memory formation and neuroprotection (Eivani and Khosronezhad 2020). Through mechanisms including rosmarinic acid, its main polyphenol, lemon balm

appears to inhibit the formation of β -amyloid fibrils (A β 1-40 and A β 1-42) (Yang et al. 2005; Ono et al. 2004; Noguchi-Shinohara et al. 2020).

It is in this context that plant metabolomics, which focuses on identifying bioactive chemical compounds responsible for clinical efficacy, has gained attention (Liu et al. 2024). The vegetable metabolome consists of primary and secondary metabolites with different physicochemical properties, including some that are species-specific. Therefore, it is essential to gather as much information as possible about the composition of an aromatic plant, and thus the metabolites extracted, the characterization of the volatile component, and biological properties that are related to it.

In this study, *Melissa officinalis* subsp. *Altissima* (MOA), wild-growing species in Corsica (France) and harvested in July, was analysed using a multi-methodological analytical protocol to obtain a complete metabolomic profile. In particular, high-resolution Nuclear Magnetic Resonance (NMR) spectroscopy was applied to study the hydroalcoholic and organic metabolites obtained by Bligh-Dyer extraction. In addition, the chemical compositions of the essential oils obtained from the vegetative parts of the plant were analysed by means of the analytical technique of Gas Chromatography coupled to Mass Spectrometry (GC-MS).

The aim of this research was the determination of the phytochemical composition of extracts and essential oil in relation to antioxidant and antimicrobial activities respectively. In addition, the effects on general inflammation and neuroinflammation were evaluated by biological assays. Specifically, the biocompatibility of hydroalcoholic MOA extracts was evaluated in rat hypothalamic cells (HypoE22). The same cell line was tested to evaluate the antioxidant activity exerted by MOA extracts hydroalcoholic extracts against cell damage induced by hydrogen peroxide (H₂O₂). Then, the gene expression of inflammatory and neurotrophic mediators such as TNF- α (Tumour necrosis factor), NOS-2 (Nitric oxide synthase-2) and BDNF (Brain-derived neurotrophic factor) in brain samples from C57/BL6 mice (prefrontal cortex and hypothalamus) treated with MOA extracts hydroalcoholic was evaluated. Additionally, the cortical tissues of mice were exposed to β -amyloid to subsequently assess the expression of IL-6 (Interleukin-6) and ACHE (Acetylcholinesterase) genes, during treatment with hydroalcoholic extracts of MOA.

One approach to enhancing a specific property, such as antioxidant, antimicrobial, anti-inflammatory, or neuroprotective activity involves combining compounds with the required

properties. The synergistic effect can lead to a higher level of required activity, even at low concentrations.

A2.3.2 Materials and Methods

A2.3.2.1 Sampling

Melissa officinalis ssp. *altissima* aerial parts were harvested in central Corsica (Corte, France, 42°17'56.5" N, 9°10'13.0" E), during the flowering stage in July 2023. The botanical determination of the plants was carried out according to the characteristics summarised in Flora Corsica (Jeanmonod and Gamisans 2007).

A2.3.2.2 NMR analysis

For NMR analysis, the fresh plant leaves were freeze-dried (Lyovapor L-200, -55 °C, 0.200 mbar) and ground into powder for NMR extractions following the Bligh-Dyer protocol (BLIGH and DYER 1959; Ambroselli et al. 2024). The dried hydroalcoholic phase was dissolved in 0.7 mL of 100 mM phosphate buffer/D₂O, containing TSP (0.4 mM) as internal standard. The CHCl₃-dried fraction was dissolved in 700 µL of CDCl₃/CD₃OD mixture (2:1 v/v).

NMR analyses were conducted using a Jeol JNM-ECZ 600R instrument operating at a proton frequency of 600.17 MHz and equipped with a Jeol 5mm FG/RO DIGITAL AUTOTUNE probe. Spectra processing and signals integration were performed with JEOL Delta software v5.3.1. Additionally, two-dimensional experiments (¹H-¹H TOCSY, ¹H-¹³C HMBC, and ¹H-¹³C HSQC), and the quantifications of the hydroalcoholic and organic portions were performed following the experimental protocols previously reported in the study on officinal plants (Ambroselli et al. 2024).

A2.3.2.3 GC-MS analysis

For GC-MS analysis, the aerial parts of the plant were air-dried (200 g) and subjected to hydro-distillation (5 h) with a Clevenger-type apparatus, according to the method recommended by

the European Pharmacopoeia (Council of Europe 1997). Hydrodistillation produced a yellow essential oil (EO) with a yield of 0.04% (w/dw)

In addition, fresh aerial parts were cut into small pieces and subjected to solid-phase microextraction (HS-SPME) using a divinylbenzene/carboxene/polydimethylsiloxane (DVB/CAR/PDMS, 30 mm) coated SPME device. 700 mg of plant material in a 20 mL SPME vial was processed according to specific extraction parameters (equilibration time: 60 min; extraction time: 30 min; extraction temperature: 70°C). After sampling, the SPME fibre was desorbed in both GC and GC-MS injection ports for volatile components.

The gas chromatographic analysis was conducted using a machine (Perkin Elmer Clarus 600 of Waltham, MA, USA), equipped with a single injector, two flame ionization detectors (FID) and two fused silica capillary columns with different polarity phases. The oven temperature ranged from 60 to 230 °C, with the injector and detector temperatures maintained at 280 °C. Hydrogen was used as the carrier gas. Retention indices (RIs) were determined using a series of n-alkanes and the Van den Dool and Kratz equation.

Plant extracts were analysed with a Perkin Elmer Turbo Mass quadrupole detector directly connected to a Perkin Elmer SQ8 (Walton, MA, USA), equipped with the same two fused silica capillary columns mentioned above. Both columns were used with the same quadrupole MS detector. Analyses were conducted sequentially using one non-polar and one polar column for each sample, resulting in two reconstructed ion chromatograms (RIC) for each sample. GC conditions remained constant and MS parameters were as follows: ion source temperature at 150 °C, ionisation energy at 70 eV and electron ionisation mass spectra acquired in a mass range of 35-350 amu during a scan time of 1 second. The injection volumes were 0.1 µL.

Identification of individual components in plant extracts was based on a methodology GC retention indices and GC-MS in EI mode. The identification of individual components was based i) on the comparison of the retention indices (RIs) determined on the polar and nonpolar columns with those of authentic compounds or literature data (R. P. Adams 2007); ii) on computer matching of the mass spectra with commercial MS libraries and the mass spectra with those listed in our homemade MS library of authentic compounds or literature data ("NIST. Spectral Database for Organic Compounds" 2008; "NIST PC Version of the NIST/EPA/NIH Mass Spectral Library" 2008; R. P. Adams 2007). The relative quantification

percentage was obtained by internal normalization of the GC-FID peak area without response factors.

A2.3.2.4 Biological assays

For biological assays, 100 mg of lyophilized sample was added to 2 mL of a H₂O/EtOH mixture (30:70 % *v/v*) with a 1:20 ratio of plant matrix to extraction solvent. The obtained system was sonicated (ARGOLAB DU-100 ultrasonic thermostat bath (Rome, Italy)) at 30 °C for 10 min and then centrifuged (Eppendorf 5430 R centrifuge (Milan, Italy)) for 5 min (30 °C, 7830 rpm). The hydroalcoholic extract was separated and the residual pellet was extracted twice using the same protocol. The collected supernatants were pooled and filtered through 0.45 µm syringe filters, and then brought to dryness under constant flow of nitrogen at room temperature

The dried hydroalcoholic extract was dissolved in a DMSO/Water mixture using a total solvent volume of 6 mL to maintain the appropriate matrix/solvent ratio. In addition, to avoid the potential toxicity of MOA extract, the maximum phytochemical concentration tested was 200 µg/mL, with at less than 1% DMSO. Therefore, the stock solution for cell treatment or tissue incubation was prepared by adding a 50:50 % *v/v* DMSO/H₂O mixture to the dried hydroalcoholic extracts. The resulting system was then sonicated with an ultrasonic thermostat bath at room temperature for 10 min and then filtered through 0.22 µm syringe filters to obtain a sterile solution. As shown in **Table A2.1**, the stock solution was used to create subsequent dilutions for *in vitro* and *ex vivo* assays.

Table A2.1 Stock solution and its dilutions of dry hydroalcoholic extract of MOA. All concentrations were tested on cells for *in vitro* assays, but only 100 and 200 µg/mL were used to incubate cortical and hypothalamic tissues in *ex vivo* assays.

	Phytochemical concentration (µg/mL)	DMSO %
80 X	16 mg/mL	50
10 X	2 mg/mL	6.25
Highest tested	500 µg/mL	1.56*
Higher Int. tested	200 µg/mL	0.625
Lower Int. tested	100 µg/mL	0.312

Lowest tested	50 µg/mL	0.156
* This percentage is higher than that tolerated by the cells, which is why the effects on cell viability were evaluated in comparison to two control lines (Ctrl) in 96-well culture plates (one line was at this DMSO concentration)		

In vitro studies

Rat HypoE22 hypothalamic cells were cultured in DMEM supplemented with 10% (*v/v*) heat-inactivated fetal bovine serum and 1% (*v/v*) penicillin G/streptomycin in 75-cm² cell culture flasks. The cultured cells were maintained in a humidified incubator with 5% CO₂ at 37°C. When confluence reached 80%, a viability assay was performed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay to evaluate the basal cytotoxicity and antioxidant activity post-H₂O₂ treatment of the MOA extract under investigation. For this assay, cells were seeded (3x10³ cells/well) on 96-well flat-bottom culture plates and incubated overnight. After 24 h, the cells were treated with vehicle or MOA extracts at different concentrations (50, 100, 200 and 500 µg/mL) and incubated overnight. In a second set of experiments, the cells were subsequently treated with 300 µM pro-oxidant stimuli for 3 hours. After this time, a total of 20 µL of MTT (5 mg/mL in PBS) was added to each well and incubated for 3 hours at 37 °C. The formazan dye formed was solubilized with dimethyl sulfoxide, and absorbance was recorded at 540 nm to determine cell survival. The effects on cell viability were evaluated against the control (Ctrl: untreated cells) and expressed as a value percentage of the control culture. Each condition was run in triplicate, including untreated control and cell-free blank control.

Ex vivo studies

Adult C57/BL6 mice (n = 13) were housed in Plexiglas cages (55 cm x 33 cm x 19 cm) and maintained under standard laboratory conditions (21 ± 2 °C; 55 ± 5 % humidity) with a 14/10-hour light/dark cycle, with drinks and food ad libitum. Housing conditions and experimental procedures were strictly in accordance with European Community ethical standards (EU Directive No. 26/2014) on the care of animals for scientific research. Rodents were sacrificed

by CO₂ inhalation (100% CO₂ at a flow rate of 20% of the chamber volume per minute) for brain retrieval.

For LPS stimulus, brain samples were cut to obtain sections of prefrontal cortex (split in two, n = 16) and hypothalamus (n = 8). Next, brain tissue samples were placed in 6-well plates in RPMI buffer by adding bacterial LPS (*E. coli*) (50 µg/mL) to each well, except for control (Ctrl). The prefrontal cortex samples were treated with hydroalcoholic extracts of MOA at concentrations of 100 and 200 µg/mL, while the hypothalamic sections were treated only at the higher concentrations of 200 µg/mL.

For stimulation with β-amyloid, brain samples were cut to obtain prefrontal cortex sections divided in two with n=10. Thus, the brain tissue samples were placed in 10-well plates in RPMI buffer, two of which were used as controls (Ctrl). Prefrontal cortex samples were treated with hydroalcoholic extracts of basal MOA at concentrations of 50, 100 and 200 µg/mL, and with hydroalcoholic MOA extracts (50, 100 and 200 µg/mL) after stimulation with β-amyloid (c=0.05 mg/mL). Two prefrontal cortex samples were subjected to β-amyloid (C=0.05 mg/mL) stimulation treatment alone.

After 3 h of incubation at 37 °C, all samples were collected and stored at -80 °C until RNA extraction.

Total RNA was extracted from the brain tissues using TRI reagent (Sigma-Aldrich, St. Louis, MO, USA), according to the manufacturer's protocol, and 1 µg of total RNA from each sample in a 20 µL reaction volume was reverse transcribed using High-Capacity cDNA Reverse Transcription Kit (Thermo Fischer Scientific, Waltman, Massachusetts, USA). Gene expression of TNFα, BDNF, NOS-2, IL-6, and ACHE were evaluated by quantitative real-time PCR using TaqMan probe-based chemistry, as previously reported (Chiavaroli et al. 2024). PCR primers and TaqMan probes were acquired from Thermo Fisher Scientific Inc. GADPH (Glyceraldehyde-3-phosphate dehydrogenase) was used as the house-keeping gene. The real-time PCR was carried out in triplicate for each cDNA sample in relation to the selected genes. The elaboration of data was performed with the Sequence Detection System (SDS) software version 2.3 (Thermo Fischer Scientific). Relative quantification of gene expression was performed by the comparative 2^{-ΔΔCt} method.

A2.3.3 Results and discussion

A2.3.3.1 Assignments and metabolite content by NMR

Assignments of ^1H NMR spectra of water-soluble MOA extracts in D_2O /phosphate buffer, **Table A2.2**, were based on 2D NMR experiments (^1H - ^1H TOCSY, ^1H - ^{13}C HSQC and ^1H - ^{13}C HMBC) and a previous NMR study (Ambroselli et al. 2024)

Table A2.2. List of water-soluble metabolites identified in ^1H NMR spectra at 600 MHz of MOA extracts in D_2O /phosphate buffer.

Compound	Assignment	^1H (ppm)	Multiplicity [J(Hz)]	^{13}C (ppm)
<i>Sugars</i>				
α -D-Fructofuranose	C-2			105.9
	CH-3	4.11		82.3
β -D-Fructofuranose	C-2			102.6
	CH-4	4.12		75.6
β -D-Fructopyranose	C-2			99.3
β -Galactose	CH-1	4.60	d [7.9]	97.3
	CH-2	3.51		
	CH-3	3.67		
	CH-4	3.95		
	CH-5	4.05		
	CH-6	3.78		
α -Glucose	CH-1	5.23	d [3.8]	93.3
	CH-2	3.54		72.6
	CH-3	3.72		73.3
	CH-4	3.41		70.8
	CH-5	3.84		72.5
β -Glucose	CH-1	4.66	d [7.9]	97.0
	CH-2	3.25		75.3
Inulin	CH-1 (Glc)	5.44		93.9
	CH-2	3.57		72.1
	CH-3	3.78		73.6
	CH-4	3.48		70.2
	CH-5	3.85		73.4
	CH ₂ -6	3.83		61.1

	CH ₂ -1' (Fru)	3.75;3.89		
	CH-3'a	4.21		77.5
	CH-3'b	4.26		
	CH-4'a	4.05		74.9
	CH-5'	3.89		82.2
Myo-inositol	CH-2,5	3.56		
	CH-3,6	3.65		
	CH-4	3.28*		75.0
Sucrose	CH-1 (Glc)	5.42	d [3.9]	93.1
	CH-2	3.53		71.8
	CH-3	3.72		73.6
	CH-4	3.42		70.2
	CH-5	3.84		73.5
	C2' (Fru)			104.8
	CH-3'	4.23		77.4
	CH-5'	3.92		82.4
	CH-4'	3.83		61.2
<i>Organic Acids</i>				
Acetate	CH ₃	1.92	s	24.4
	COOH			184.4
Citrate	α , γ -CH	2.56	d [16.0]	46.0
	α' , γ' -CH	2.68		46.0
	β -C			73.2
	1,5-COOH			180.2
	6-COOH			183.0
Formate	HCOOH	8.46	s	
Lactate	β -CH ₃	1.34	d [7.1]	21.4
Malate	α -CH	4.31	dd [9.8; 3.3]	71.0
	β -CH	2.68	dd [15.5; 3.3]	43.5
	β' -CH	2.41	dd [15.5; 9.8]	43.5
Quinate	CH ₂ -2, 2'	1.89, 2.06	dd [10.8;13.4], m	41.8
	CH-3	4.16		71.5
	CH-4	3.56		76.3

	CH-5	4.03		68.0
Succinate	α , β -CH ₂	2.42	s	35.2
Tartrate	CH(OH)COOH	4.37	s	75.3
<i>Amino acids</i>				
Alanine	α -CH	3.80		51.0
	β -CH ₃	1.49	d [7.2]	17.3
	COOH			178.6
GABA	α -CH ₂	2.30	t [7.4]	35.4
	β -CH ₂	1.90		24.9
	γ -CH ₂	3.01	t [7.5]	40.4
Glutamine	α -CH	3.76		55.3
	β , β' -CH ₂	2.14	m	27.5
	γ -CH	2.45*	m	31.9
Isoleucine	α -CH	1.97		38.0
	β -CH	1.27		29.4
	γ -CH ₃	1.01	d [7.1]	15.7
Leucine	δ -CH ₃	0.89	d [7.4]	
	β -CH ₂	1.74		41.0
	γ -CH	1.71		
	δ -CH ₃	0.97	d [6.4]	23.1
	δ' -CH ₃	0.96		22.1
	CH-4	7.38		
Proline	CH-3,5	7.43	m	130.6
	α -CH	4.13		62.5
	β , β' -CH ₂	2.07, 2.33		30.0
	γ -CH ₃	2.01	m	25.0
	δ , δ' -CH ₃	3.33, 3.41		47.4
	α -CH	3.59		62.3
Threonine	γ -CH ₃	1.33	d [6.7]	20.71
	CH-2, 6 ring	7.22	d [8.2]	132.0
	CH-3, 5 ring	6.96	d [8.5]	117.0
Valine	α -CH	3.60		
	β -CH	2.27		
	γ -CH ₃	1.10	d [7.1]	17.7

	γ' -CH ₃	1.04	d [7.1]	19.0
<i>Other compounds</i>				
Caffeic acid	α -CH=CH	6.36	d [16.00]	
	β -CH=CH	7.29	d [16.00]	
Caftaric acid	CH-10	5.33	d [2.2]	
	CH-11	4.57	d [2.2]	
	CH-2	6.49	d [16.0]	115.4
	CH-3	7.72	d [16.0]	148.0
	CH-8 ring	6.96	d [8.2]	117.6
	CH-9 ring	7.17	dd [2.0; 8.2]	124.2
	CH-5 ring	7.25	d [2.0]	117.5
	COOH			171.4
Chlorogenic acid	CH-2	6.39	d [16.0]	115.8
	CH-3	7.37	d [16.0]	146.8
	CH ₂ -2'	1.89; 2.10	m	41.7
	CH-4'	3.90	m	70.8
	CH ₂ -6'	1.99; 2.06	m	38.6
Rosmarinic acid	CH-2	6.22	d [16.0]	115.8
	CH-3	7.37	d [16.0]	146.8
	CH-1a	5.01	m	77.5
	CH ₂ -2a	2.85; 2.93	m	37.9
Uracil	CH	7.84	d	
	CH	5.80	d	

Five sugars (fructose, sucrose, glucose, galactose, and inulin), a polyol (myo-inositol), seven organic acids (acetate, citrate, lactate, malate, succinate, and tartrate), nine amino acids (alanine, GABA, glutamine, isoleucine, leucine, proline, threonine, tyrosine, and valine), among other compounds, four polyphenols (caftaric, chlorogenic, caffeic, and rosmarinic acids), and uracil were identified.

To the best of our knowledge, the assignment of a ¹H NMR spectrum of a hydroalcoholic extract of *Melissa officinalis* subsp. *altissima* has not been reported in the literature. On the other hand, numerous studies have focused on the analysis of polyphenols in *Melissa officinalis* subsp. *Officinalis* (Miraj, Rafieian-Kopaei, and Kiani 2017). It has been amply demonstrated

that caffeic acid, rosmarinic acid and chlorogenic acid are the most commonly found phenolic compounds in this plant (Binello et al. 2017; Dastmalchi et al. 2008; Ordaz et al. 2018). Special extraction techniques have also been developed for the efficient extraction of the dried aerial parts of lemon balm (Sentkowska, Biesaga, and Pyrzyńska 2015; Binello et al. 2017). The plant's antioxidant activity, therefore, seems to be related to its polyphenolic content. Indeed, rosmarinic acid is known for its powerful antioxidant and anti-inflammatory properties, which help reduce oxidative stress (Guan et al. 2022). Chlorogenic acid, in turn, has shown positive effects on glucose metabolism and cardiovascular health through its ability to improve insulin sensitivity (Liang and Kitts 2015; Yan et al. 2022). Finally, caffeic acid and caftaric acid are associated with antimicrobial and antibacterial properties (Khan et al. 2021; Kępa et al. 2018; Koriem 2020; Newair et al. 2023). Furthermore, studies indicate that caftaric acid may influence lipid and glucose metabolism, suggesting a potential role in managing metabolic health (Koriem 2020).

These compounds, in synergy, give lemon balm a nutraceutical profile of great interest, making it a promising candidate for integration into health and wellness strategies.

The identification of lipophilic metabolites was based on a combination of 1D and 2D NMR experiments, comparing literature data on complex lipid mixtures (Ambroselli et al. 2024; Spano et al. 2021), and is reported in **Table A2.3**.

Table A2.3. Compounds and relative selected signals (ppm) for quantitative analysis in the organic extracts.

	ppm	Group	Compounds
I β -Sit	0.65	CH ₃ -18	β -Sitosterol
I _{Stig}	0.67	CH ₃ -18	Stigmasterol
I _{FA}	2.30	CH ₂ -11	Totally fatty acids
I _{DUFA}	2.73	CH ₂ -11	Linoleic acid
I _{TUFA}	2.77	CH ₂ -11,14	Linolenic acid
I _{PCG}	3.23	N(CH ₃) ₃	Glyceroylphosphatidylcholine
I _{DGG}	4.87	CH-1	Glyceroyldigalactose
I _{PHEO}	11.15	CH-5	Pheophytin
I _{CHL}	11.13	CH-5	Chlorophyll

A2.3.3.2 GC-MS analysis of volatile compounds

The chemical compositions of the essential oil and the volatile fraction are listed in **Table A2.4**. In the essential oil MOA, 38 components were identified, constituting 90.5% of the oil. The main components of the oil were hydrocarbon compounds (70.7%), with germacrene D (44.2%) as the dominant sesquiterpene. The oxygen compounds included 11 sesquiterpenes (17.3%), one diterpene (0.7%), one monoterpene (0.4%) and one nonterpenic compound (1.2%). Of the 54 individual compounds identified, 51 were matched using EI-mass spectra and library retention indices. The main oil components besides germacrene D were α -cadinol (7.4%), β -caryophyllene (6.9%) and τ -cadinol (5.9%).

Few studies on *Melissa officinalis* L. ssp. *altissima* were found in the literature. The chemical characterization of essential oil from this subspecies (formerly known as *M. officinalis* L. var. *romana*) has been reported by Dawson et al. (Dawson, Franich, and Meder 1988). In a study (Usai, Atzei, and Marchetti 2016), the composition of MOA samples from different stations in Sardinia (Italy) and in different growing seasons was analysed. Subsequently, Miceli et al. reported the essential oil composition of samples collected in southern Italy (Miceli, Negro, and Tommasi 2006). A comprehensive survey of essential oils extracted from *Melissa officinalis* L. ssp. *altissima* collected in Tarquinia (Italy) was carried out, showing a variability in the relative amounts of the main compounds depending on the extraction time and harvest period (Božović et al. 2018).

In particular, although some of the main compounds have already been reported, their relative quantities vary depending on when they are extracted, harvested and, most importantly, the place where the plant grows. In the first cited study (Dawson, Franich, and Meder 1988), the major compound was β -cubebene (39%) and the minority β -caryophyllene (4%), totally disagreeing with Usai et al. (Usai, Atzei, and Marchetti 2016), showing a high concentration of terpenes such as β -caryophyllene (17.3-27.5%), α -muurolene (17.5-38.8%) and caryophyllene oxide (3.3-7.9%).

The EO obtained from the flowering tops of plants, in Italy, showed the predominance of caryophyllene (15.8%), cumene (14%), β -pinene (12%) and citral (12%), whereas other constituents presented in smaller quantities were menthol (6.5%), α -pinene (5.1%), spathulenol (4.8%) and γ -terpinene (4.0%) (Miceli, Negro, and Tommasi 2006). Comparing these results with the oils from Tarquinia, in particular with the August material, significant differences in the percentages of caryophyllene, β -pinene and spathulenol are observed (Božović et al. 2018).

Table A2.4. Volatile components extracted by hydrodistillation (%EO) and by HS-SPME (% VF) of MOA.

N ^{oa}	Compounds	RIalitt ^b	RIaexp ^c	RIpexp ^d	% identification ^e		Identification ^f
					EO	VF	
1	Butyraldehyde	605	607	880	-	1.1	MS, Ref
2	2-methyl 1-propanol	605	605	1081	-	0.5	MS
3	Isovaleraldehyde	632	631	1082	-	4.3	MS
4	2-methylbutanal	644	643	909	-	3.3	MS, Ref
5	Penten-3-ol	662	662	1144	-	1	MS
6	Furan-2-ethyl	691	690	960	-	0.5	MS , Ref
7	3-Methylbutanol	719	716	1177	-	2.1	MS
8	2-Methylbutanol	727	717	1178	-	1.8	MS
9	Hexanal	777	770	1308	-	5.2	MS
10	(E) Hex-3-en-1-ol	814	810	1360	-	1.3	MS
11	(Z)-Hex-3-en-1-ol	836	831	1380	-	2.8	MS
12	α -Thujene	922	922	1405	-	1.6	MS
13	Benzaldehyde	930	929	1536	0.2	-	MS
14	α -Pinene	931	931	1023	-	0.4	MS
15	Oct-1-en-3-ol	962	959	1447	1.2	3.5	MS
16	Sabinene	973	963	1124	0.4	3	MS
17	β -Pinene	978	969	1114	0.4	6.6	MS
18	Myrcene	978	976	1159	-	0.4	MS
19	P-Cymene	1015	1010	1272	0.1	0.8	MS
20	1,8-Cineole	1020	1020	1209	-	0.1	MS
21	(Z)- β -Ocimene	1029	1023	1234	0.7	5	MS
22	(E)- β -Ocimene	1040	1035	1252	3	20	MS
23	Terpinen-4-ol	1161	1161	1599	0.4	-	MS
24	α -Copaene	1379	1376	1490	1.4	-	MS
25	β -Bourbonene	1386	1379	1516	0.1	0.7	MS
26	β -Cubebene	1388	1383	1593	0.4	-	MS
27	β -Elemene	1388	1388	1590	2.6	1	MS
28	(E)- β -Caryophyllene	1421	1419	1597	6.9	3.2	MS
29	β -Copaene	1430	1426	1571	0.5	-	MS
30	α -Humulene	1455	1450	1666	1	-	MS
31	Alloaromadendrene	1462	1458	1642	1	1.8	MS
32	γ -Muurolene	1474	1471	1681	0.8	-	MS
33	Germacrene D	1479	1480	1716	44.2	16.7	MS

34	γ -Gurjunene	1472	1480	1704	-	0.3	MS
35	Ledene	1494	1492	1695	0.5	-	MS
36	α -Muuroolene	1496	1488	1723	0.8	-	MS
37	Valencene	1494	1496	1719	0.2	-	MS
38	γ -Cadinene	1507	1507	1752	1.1	-	MS
39	Cis/trans Calamenene	1512	1510	1816	0.2	-	MS
40	δ -Cadinene	1520	1516	1748	3.9	-	MS
41	Trans-Cadina -1,4-Diene	1523	1524	1763	0.1	-	MS
42	α -Cadinene	1534	1530	1748	0.4	0.6	MS
43	E-Nerolidol	1553	1547	2038	0.5	-	MS
44	Trans-hydrate-sesquisabinene	1543	1550	1990	0.8	-	MS
45	Caryophyllenoxide	1578	1575	1975	0.4	-	MS
46	Zingiberenol	1606	1608	2108	0.7	-	MS
47	Zingiberen-2-ol	1613	1615	2192	0.4	-	MS
48	Epicubenol	1623	1622	2051	0.3	-	MS
49	τ -Cadinol	1633	1628	2185	5.9	-	MS
50	α -Cadinol	1643	1642	2227	7.4	-	MS
51	Oplopenol 1	1644	1644	2205	0.4	-	MS
	14-hydroxy-9-epi-E-						
52	Caryophyllene	1658	1657	2300	0.2	-	MS
	Eudesma-4(15)-7-dien-1- β -ol	1671	1666	2348	0.3	-	MS
53	E-Phytol	2096	2098	2605	0.7	-	MS
Total identification %					90.5	89.6	
Hydrocarbon compounds					70.7	62.1	
Oxygenated compounds					19.8	27.5	
Hydrocarbon monoterpenes					4.6	37.8	
Oxygenated sesquiterpenes					17.3	0	
Hydrocarbon sesquiterpenes					66.1	24.3	
Other oxygenated compounds					2.5	27.5	

a. Compounds are listed in order of their elution from non-polar Rtx-1 column.

b. RIalitt: Retention indices for a non-polar column (R. P. Adams 2007)

c. RIaexp: Retention indices determined experimentally on the non-polar Rtx-1 column.

d. RIpexp: Retention indices determined experimentally on the polar Rtx-wax column.

e. The contents were determined on the non-polar column Rtx-1 column.

EO: essential oil, VF: volatiles sampled by SPME

Ref: compounds identified from commercial libraries

Depending on the harvest period, chemical diversity was observed; GC/MS analysis showed that cis-caryophyllene and its oxide were the main constituents of EO. Other compounds were

predominant only in certain months, such as germacrene D in July and September, and terpinene-4-ol in August and September. However, our results are in line with those from Sardinia (Usai, Atzei, and Marchetti 2016).

Variations in the amounts of the compounds reflect regional and seasonal differences: some OE constituents are characteristic of specific phenological phases, and some are only associated with the reproductive period of the plant, as seen in the case of the samples from Italy.

In the volatile fraction analyzed by HS-SPME, described for the first time, hydrocarbon compounds are dominant, accounting for 62.1% of the total composition, with 37.8% monoterpenes. The main component identified is (E)- β -ocimene (20%), followed by germacrene D (16.7%). Also detected was the presence of oxygenated molecules (1-11), mainly aliphatic aldehydes with a relatively short linear chain, including isovaleraldehyde (4.4%), hexanal (5.2%) and 2-methylbutanal (3.3%), which were not found in the essential oil. Significant quantitative and qualitative differences were observed between OE and VF analysis for oxygenated and hydrocarbon compounds. It was difficult to establish a direct correlation between the HS-SMPE and hydrodistillation techniques, since the former is controlled by an equilibrium phase, while the latter relies on almost total extraction of plant volatiles.

Thus, germacrene D and β -ocimene were the main volatile compounds identified in MOA.

Germacrene D is a sesquiterpene, a volatile organic compound belonging to the terpene class, known to occur in numerous aromatic plants, derived from the mevalonate metabolic pathway common in terpenoid synthesis. This compound is well-known for its antimicrobial, anti-inflammatory and antioxidant properties (Noge and Becerra 2009). It also plays a role in the defense of plants against pests and herbivores due to its ability to inhibit the development of pathogenic microorganisms. In cosmetics and pharmaceuticals, it is exploited for its skin-soothing and protective properties.

β -ocimene is a monoterpene, a volatile aromatic compound found in many plants, flowers and fruits. This compound contributes to the fresh, floral fragrance of many natural essences.

β -ocimene has antifungal, antimicrobial and antioxidant properties, and is used in aromatherapy and fragrance production due to its pleasant aroma. In addition, this terpene plays an important role in plant communication, attracting pollinators and repelling harmful

insects (Farré-Armengol et al. 2017). In cosmetics and pharmaceuticals, beta-ocimene is also valued for its soothing and protective qualities.

A2.3.3.3 Antioxidant and neuroprotective activity of MOA hydroalcoholic extract

In vitro studies

In the present assay, the cytotoxic effects of hydroalcoholic extracts of MOA, in the concentration range 50-200 $\mu\text{g/mL}$, on the viability of the hypothalamic HypoE22 cell line under basal conditions were investigated. The cells were exposed to the MOA extracts under basal conditions and were tolerated up to a concentration of 500 $\mu\text{g/mL}$, demonstrating good biocompatibility, see **Figure A2.3**.

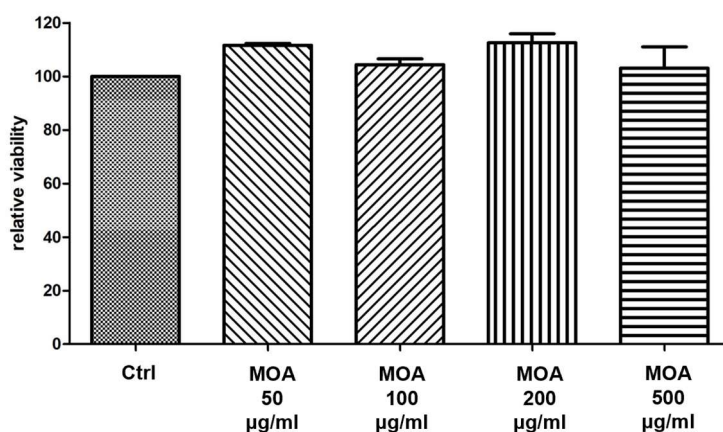


Figure A2.3. Effects of MOA extracts (50-200 $\mu\text{g/mL}$) on HypoE22 cell viability in basal condition. ANOVA, $P < 0.001$; *** $P < 0.001$ Vs. LPS.

In addition, hydroalcoholic extracts of MOA (50, 100, 200 and 500 $\mu\text{g/mL}$) were tested on the HypoE22 cell line, even in the presence of H_2O_2 , which is the reference pro-oxidant stimulus capable of reducing cell viability below the biocompatibility limit (70% viability compared to the control group), see **Figure A2.4**.

MOA extracts were effective in protecting cells from cytotoxicity induced by H_2O_2 (300 μM) from a phytochemical concentration of 200 $\mu\text{g/mL}$, demonstrating good antioxidant activity.

In the literature (Kara et al. 2024), a novel lemon balm extract (based on a phospholipid carrier) showed an enhanced neuroprotective antioxidant effect on SH-SY5Y cells against H_2O_2 -induced oxidative stress.

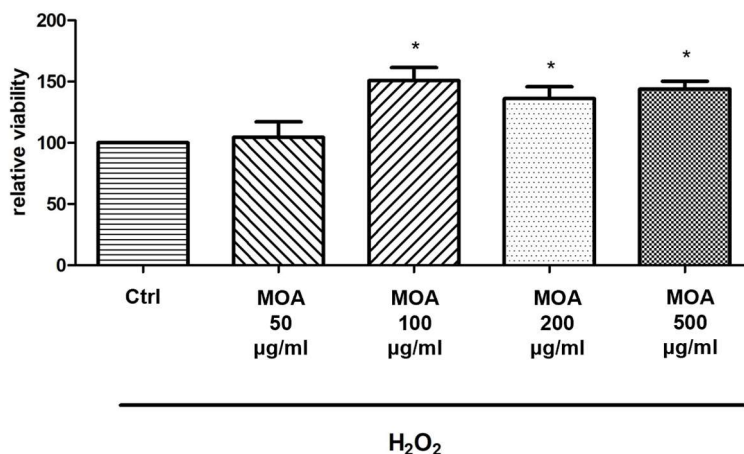


Figure A2.4. Effects of MAO extracts (50-500 µg/mL) on HypoE22 cell viability under hydrogen peroxide (H_2O_2) stimulus. ANOVA, $P < 0.001$; *** $P < 0.001$ Vs. LPS.

Ex vivo studies

MOA extracts (100 and 200 µg/mL) were tested in isolated samples of mouse cortex and hypothalamus treated with LPS from *E. coli*, chosen as a pro-inflammatory stimulus, to assess their potential protective activities exerted on neuroinflammatory pathways.

In this context, gene expression of $TNF-\alpha$ and NOS-2 was analysed and LPS stimulus (50 µg/mL) induced their up-regulation in both the cortex and hypothalamus (**Figure A2.5**).

All extracts tested proved effective in reversing the increased expression of these two genes, suggesting potential protective effects on neuroinflammation. Furthermore, $TNF-\alpha$ and NOS-2 gene expression were even lower than in the control groups after administration of MOA extract at a concentration of 200 µg/mL in cortical samples (**Figure A2.3A**). $TNF-\alpha$ and NOS-2 are highly active pleiotropic modulators of cell function that are generated in abundance during inflammation (Sun et al. 1998). These pro-inflammatory factors contribute to the accumulation of reactive oxygen species (ROS) and dysregulation of oxidative stress, which in turn leads to neurodegenerative processes (Nathan and Cunningham-Bussel 2013).

Particularly, $TNF-\alpha$ is identified as one of the most potent and early inflammatory mediators that can trigger a cascade response leading to apoptosis when overproduced. Therefore,

intervening at an earlier stage of the inflammatory processes could potentially improve the prevention of neurodegenerative and cognitive disorders (A. Frankola et al. 2011; Iova et al. 2023). The effect of this aromatic plant could be related to its phenolic compounds, which appear to possess a dose-dependent protective effect through the suppression of this proinflammatory cytokine (Draginic et al. 2021).

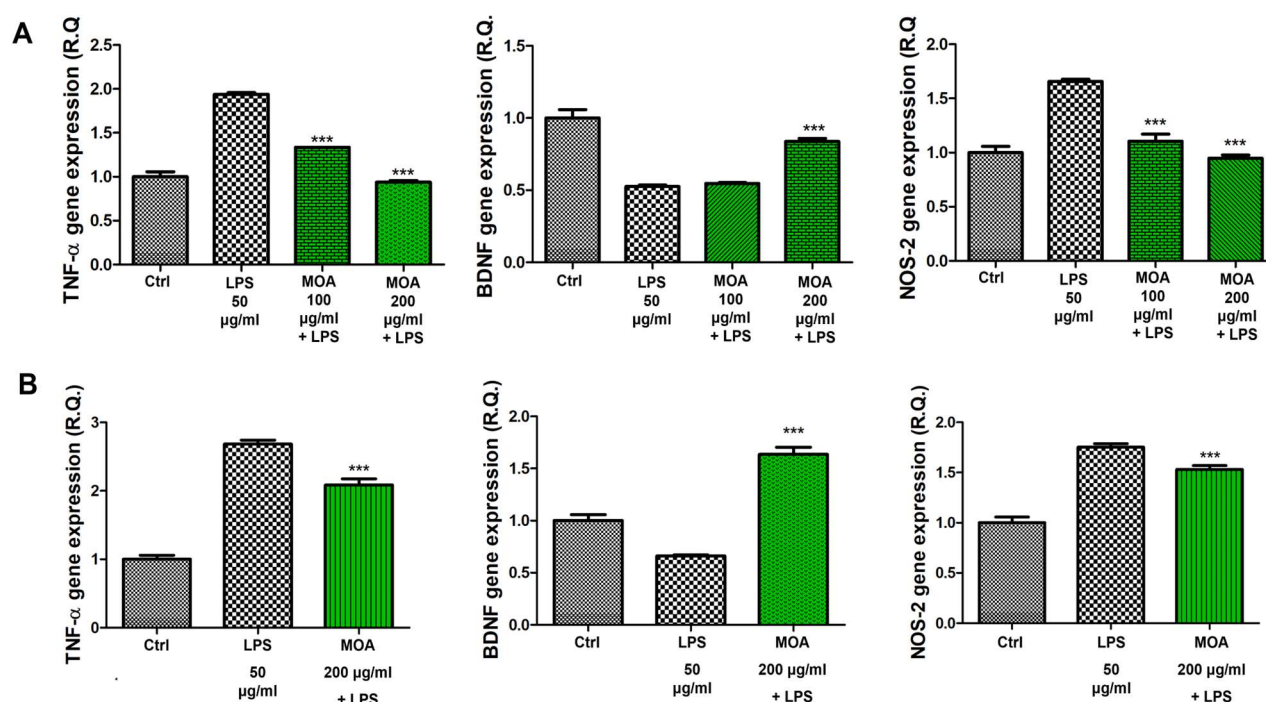


Figure A2.5. Effects of MAO hydroalcoholic extract on LPS-induced and basal TNF- α , NOS-2 and BDNF gene expression in cortical (A) and hypothalamic (B) tissues of mice. ANOVA, $P < 0.001$; *** $P < 0.001$ Vs. LPS.

Furthermore, the effects of MOA extracts on LPS-induced BDNF levels, a neurotrophic factor that is strongly reduced in proinflammatory states of brain tissue, were investigated. Treatment with the extracts was effective in both tissues tested in counteracting LPS-induced down-regulation of the BDNF gene, demonstrating a neuroprotective effect. The higher concentration of MOA extract (200 µg/mL) reversed the LPS-induced decrease in BDNF gene expression. Indeed, BDNF levels were higher after MAO treatment than in vehicle-incubated hypothalamic tissue (**Figure A2.3B**). BDNF is a major neurotrophic factor that plays a key role in neuronal development and survival, neuronal process development and synaptic plasticity. High levels of BDNF expression are closely associated with the pathophysiology of several neurological disorders, including Alzheimer's, Parkinson's and Huntington's disease (Lee et

al. 2005; Peng et al. 2005; Kara et al. 2024). The results of the present study show that MAO could potentially positively regulate BDNF expression levels in the brain and improve neuronal plasticity. A previous study investigated the effect of a hydroalcoholic extract of *Melissa officinalis* on learning and memory (Ozarowski et al. 2016). It was observed that BDNF gene expression increased and was associated with the presence of polyphenols in the hydroalcoholic extract.

At the same time, MOA extracts (50, 100 and 200 $\mu\text{g/mL}$) were tested in isolated samples of pre-frontal cortex from β -amyloid-treated mice. This pro-inflammatory stimulus was chosen to evaluate the potential ameliorative activities exerted by MOA on specific neuroinflammatory pathways involved in certain neurodegenerative disorders such as Alzheimer's disease (AD). In this context, the presence of β -amyloid stimulus (0.05 mg/mL) increased the expression of ACHE and IL-6 genes in cortical tissue (**Figure A2.6**). All extracts tested proved effective in reversing the increased expression of these two genes, suggesting potential protective effects also on specific neuroinflammation.

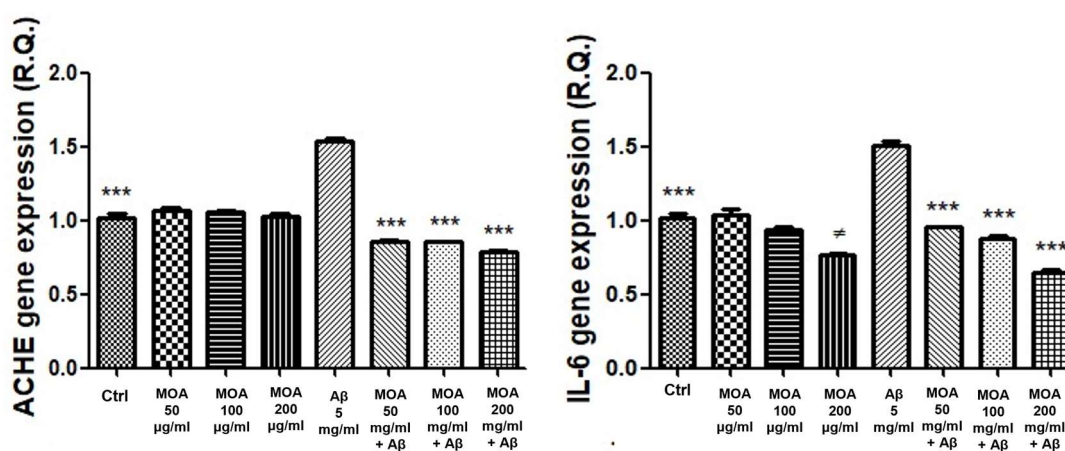


Figure A2.6 Effects of MAO hydroalcoholic extract on β -amyloid and basal ACHE, IL-6 gene expression in cortical tissues of mice. ANOVA, $P < 0.001$; $\neq P < 0.0001$ Vs. Ctrl $< ***P < 0.001$ Vs. A β .

Gene expression of ACHE, the primary target in AD, was even lower than that of the control group after administration of MOA extracts in cortical samples. A previous study had evaluated by means of the acetylcholinesterase inhibition assay that lemon balm extract was able to inhibit the enzyme in a time- and dose-dependent manner (Dastmalchi et al. 2009). In the assays of this study, the MOA extracts at concentrations of 100 and 200 $\mu\text{g/mL}$ also reversed the β -amyloid of the IL-6 gene, reaching values lower than those shown by the

vehicle-incubated cortex. Treatment with the highest concentration of MOA extract (200 µg/mL) reduced IL-6 gene expression even under basal conditions. IL-6 comprises a potential point of convergence between memory dysfunction and metabolic alterations in AD (Lyra e Silva et al. 2021). Thus, targeting pro-inflammatory IL-6 signalling may be a strategy to alleviate memory impairment and metabolic changes in the AD.

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Chapter A3

Agritech: innovation and sustainability

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A3.1 Introduction

The creation of future agricultural and rural systems that operate within safe limits has become a pressing concern for researchers and policy makers. Knowledge and innovation are the critical tools to achieve these goals. A major societal challenge is to produce safe food for a growing population without overexploiting natural resources. This is a global challenge set in a complex context of an unstable climate, increasing competition for land, water and energy, and an increasingly urbanised and globalised world. The scale and importance of this challenge requires a significant research effort that goes beyond the capabilities of any single institution. Addressing this problem effectively requires the development of an integrated, large-scale and multidisciplinary research programme. This is the background of Agritech (National Research Centre for Agricultural Technologies), which has defined a program based on pre-existing collaborative research, higher education initiatives, infrastructure networking, and sharing of large equipment.

The main feature of the Agritech research programme is its structural organisation, which reflects the high level of integration between a wide diversity of participating research institutes and companies (<https://agritechcenter.it/programme/>):

- Combine the top research expertise required to adequately address in a truly multidisciplinary context the multifaceted problems associated with sustainable agriculture;
- Integrate the research infrastructures and equipment available at each site.
- Exploit and apply the most suitable Key Enabling Technologies (KET) that can allow a profitable advance in productivity, sustainability, ecological and digital transition in the agricultural sector;
- Work with companies and farmers to co-design research efforts and exploit at the best the results to increase the resilience and economic competitiveness of agri-food supply chains;
- Develop and disseminate new models and organizational capabilities to create and implement largescale, strategic research programmes that cross discipline boundaries and industrial sectors;
- Train the next generation of Agritech scientists and managers to generate the necessary human capital and skills required;

- Support policy makers and influence public opinion to promote a social context favoring the development of stable and equitable agri-food supply chains;

Specifically, the project was to identify chemical-biological hazards in food matrices and to improve food safety and wholesomeness through a multi-methodological approach.

Based on an extensive literature search on traditional and novel plant-based foods, fruit and vegetable matrices were listed and classified.

As pilot matrices, two products with high safety risks (pesticides, contaminants) were chosen as part of Agritech: tomatoes and apples.

A3.2 Golden Delicious apple

The apple (*Malus domestica*) is widely consumed globally due to its nutritional and sensory qualitative. In 2022, the primary apple producers in Europe are Poland (34.0%), Italy (18.0% of the EU total), and France (14.2%) (<https://www.fao.org/faostat/en/#data/QCL>). Despite a recent decrease in its area, the Golden Delicious variety remains predominant at both continental and national levels (Aguzzoni et al., 2020). In northern Italy, especially in the Alpine districts of South Tyrol and Val di Non (Trentino-Alto Adige), the unique climatic and geological conditions make it an excellent area for apple cultivation, with almost two-thirds of Italian apples being produced in this region. This fruit includes phenolic compounds such as flavonols, dihydrocalcones, anthocyanidins, hydroxycinnamic and hydroxybenzoic acids, as well as vitamins and other bioactive substances. In particular, this quality of apple, Golden Delicious, is preferred by consumers over other apple varieties for its sweetness, flavour, colour and texture.

Fresh apples are increasingly appreciated by consumers for their health benefits, cleanliness and convenience. However, when apples are cut or sliced, enzymatic browning occurs, which leads to rapid deterioration during storage and negatively affects taste, smell, texture, colour and nutritional value (Luo et al., 2024). Consequently, efforts are currently being made to extend the shelf life of fresh apples while preserving their quality. For this reason, various techniques, including modified atmosphere packaging, smart packaging and edible coatings,

have been developed to slow down the metabolic processes of fresh fruit and preserve it for a long time.

Food packaging plays a crucial role in preserving fresh food by protecting it from physical, chemical, and biological contaminants (Versino et al., 2023). While petroleum-based plastics like polypropylene are commonly used due to their affordability and effectiveness, their non-biodegradable nature poses risks to both humans and the environment. As an alternative, biodegradable materials made from biopolymers such as cellulose, chitosan, starch, polylactic acid (PLA), collagen, and casein are being explored for use in the food industry. These materials must meet specific criteria, such as being non-toxic and renewable, to be viable options for food packaging.

In a recent article, the functionality of commercial biofilms in preserving freshly cut pumpkin samples was examined with the aim of obtaining information on the shelf life of minimally processed pumpkins (Di Matteo, Di Matteo, et al., 2021). In particular, non-target NMR analysis was used to obtain a complete metabolic profile of the pumpkin samples in relation to the different packaging.

Within the framework of the Agritech project, the present study aimed to evaluate the effect of two biodegradable packaging materials on the quality and safety of fresh apples during cold storage (21 days, 5 °C) compared to packaging in conventional polymer bags. Analyses to monitor the chemical profile and new and (re)emerging hazards in funnels stored at three time points (0, 14, and 21 days) were performed with non-targeted (FT-ICR MS, NMR) and targeted (HPLC-DAD, HPLC-MS/MS) methodologies. In addition, assessments of chemical, mechanical, and microbiological quality and safety of use were performed on fresh and packaged samples.

In the context of a project involving multi-methodological approaches, only the parts of interest, i.e. NMR analyses, will be discussed below.

A3.2.1 Materials and methods

Commercial biodegradable films (biofilms) of a vegetable origin (PLA – ECO), and a commercial low-density polyethylene (LDPE) film used as a reference (REF), were utilized as food bags. The biofilm compositions as stated on the package label are shown in **Table A3.1**.

Table A3.1. Composition of the three-packaging used for analysis purposes.

Biofilm	Composition
REF	Commercial low-density polyethylene (LDPE)
ECO	Corn starch, cassava and eucalyptus
PLA	Polylactic acid from corn starch

Golden Delicious' apples were supplied by Consorzio Melinda S.c.a. (Cles (TN) - Val di Non - Italy). The fruits were washed, dried and sliced. Then, approximately 100 g were packed using the two commercial biofilms mentioned above and polyethylene film (REF). Each package had a volume of approximately 1000 cm³ (**Figure A3.1**). All packed samples were then stored for 21 days (t₂₁) at 5°C and monitored at the intermediary time of 14 (t₁₄).

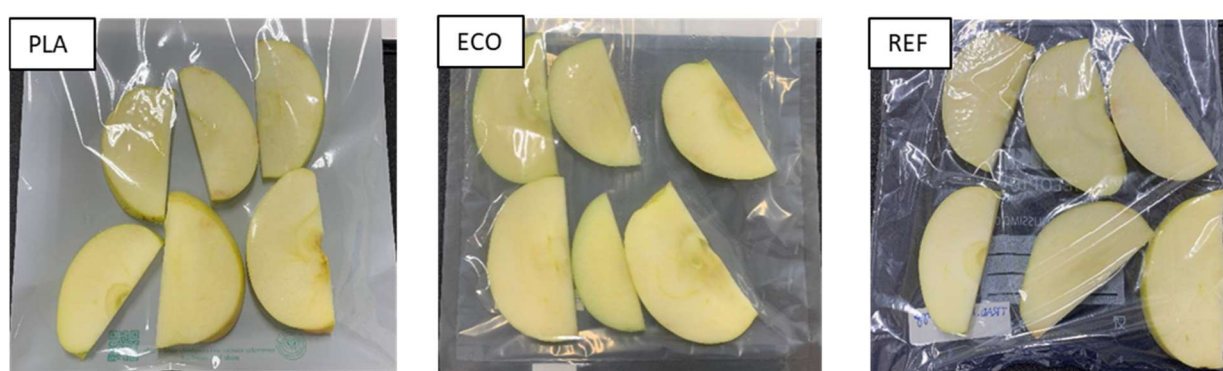


Figure A3.1. Apple slices stored in PLA, ECO and REF packaging

Extracts for NMR analysis were obtained following the previously described protocol with some modifications (Spano et al., 2021). The powder (0.2 g) was added sequentially to a 3 mL methanol/chloroform (2:1 v/v) mixture containing 1 mL of chloroform and 1.8 mL of water,

which was slightly agitated after each solvent addition. The organic and hydroalcoholic phases were separated and the residual pellet was extracted again to ensure complete extraction of the soluble metabolites. A slow flow of N₂ was used for drying.

NMR analysis was performed with a 600 MHz spectrometer (Jeol JNM-ECZ 600 R) equipped with a 5 mm FG/RO DIGITAL AUTOTUNE probe. The dried hydroalcoholic extracts were solubilized in 700 µL of 100 mM phosphate buffer/D₂O, containing TSP (3-(trimethylsilyl) propionic acid sodium salt) 0.5 mM as internal standard. The dried organic extracts were solubilized in 700 µL of a 2:1 *v/v* CDCl₃/CD₃OD mixture. One-dimensional ¹H and two-dimensional ¹H-¹H TOCSY, ¹H-¹³C HSQC, and ¹H-¹³C HMBC NMR experiments were performed using the same experimental parameters as previously described (Ambroselli et al., 2024) to analyze both hydroalcoholic and organic extracts. JEOL Delta software (v5.3.1) was used to manually correct the phase and base of the spectra and to integrate the signals used for metabolite quantification. Specifically, in the hydroalcoholic extracts, metabolite concentrations were measured against the internal standard TSP. Three replicates were performed for each sample, and the results were expressed as mg/100 g sample ± SD.

For organic extracts, the first step was based on indirect determination of the signal area of saturated and monounsaturated fatty acids superimposed on those of other fatty acids. Their content was calculated by applying the following equations:

$$\% \beta\text{-SIT} = 100(0.66I_{\beta\text{-SIT}} / I_{\text{tot}}) \quad (1)$$

$$\% \text{TUFA} = 100(0.5I_{\text{TUFA}} / I_{\text{tot}}) \quad (2)$$

$$\% \text{DUFA} = 100(I_{\text{DUFA}} / I_{\text{tot}}) \quad (3)$$

$$\% \text{MUFA} = 100(I_{\text{TOT UFA}} - 2I_{\text{DUFA}} - 1.5I_{\text{TUFA}}) / I_{\text{tot}} \quad (4)$$

$$\% \text{TOT FA} = 100(I_{\text{TOT FA}} / I_{\text{tot}}) \quad (5)$$

$$\% \text{TOT UFA} = \% \text{MUFA} + \% \text{DUFA} + \% \text{TUFA} \quad (6)$$

$$\% \text{TOT SFA} = \% \text{TOT FA} - \% \text{TOT UFA} \quad (7)$$

$$\% \text{PC} = 100(4I_{\text{PC}} / 9I_{\text{tot}}) \quad (8)$$

$$\% \text{DGDG} = 100(4I_{\text{DGDG}} / I_{\text{tot}}) \quad (9)$$

where %β-SIT, %TUFA, %DUFA, %MUFA, %TOT FA, %TOT UFA, %TOT SFA, %PC, and %DGDG, are molar % of β-sitosterol, tri-unsaturated fatty acids, di-unsaturated fatty acids, mono-unsaturated fatty acids, total fatty acids, total unsaturated fatty acids, total saturated

fatty acids, phosphatidylcholine, and digalactosyldiacylglycerol, respectively. I β -SIT, ITUFA, IDUFA, ITOT UFA, ITOT FA, IPC, and IDGDG, are integrals, whereas Itot is calculated according to the following equation:

$$\text{Itot} = \text{ITOT FA} + 0.66\text{I}\beta\text{-SIT} \quad (10)$$

A3.2.2 Results and discussion

A3.2.2.1 Metabolites assignments

The assignments of the ^1H NMR spectra of the hydroalcoholic fractions of the apple samples were carried out using 2D experiments (^1H - ^1H TOCSY, ^1H - ^{13}C HSQC, ^1H - ^{13}C HMBC), with the addition of reference standards and literature data from other matrices analyzed under the same experimental conditions (Chiavaroli et al., 2024; Di Matteo, Spano, et al., 2021). The primary and secondary metabolites identified in Bligh-Dyer's hydroalcoholic extract are shown in **Table A3.2**.

Table A3.2. Metabolites identified in the 600.17 MHz ^1H -NMR spectra of apple Bligh-Dyer hydroalcoholic extracts dissolved in 100 mM phosphate buffer/D $_2$ O containing TSP 1 mM.

Compound	Assignment	^1H (ppm)	Multiplicity [J(Hz)]	^{13}C (ppm)
<i>Sugars</i>				
α -D-Fructofuranose	C-2			105.9
	CH-3	4.11		82.3
β -D-Fructofuranose	C-2			102.6
	CH-4	4.12		75.6
β -D-Fructopyranose	C-2			99.3
α -Xylose	CH-1	5.20	d [3.6]	92.8
	CH-2	3.52		72.3
	CH-4	3.71		74.0
	CH-5,5'	3.83		
β - Xylose	CH-1	4.58	d [7.9]	96.0
	CH-2	3.23		74.9
	CH-3	3.46		76.8
	CH-4	3.62		72.4
	CH-5,5'	3.31, 3.92		

α -Glucose	CH-1	5.23	d [3.8]	93.3
	CH-2	3.54		72.6
	CH-3	3.72		73.3
	CH-4	3.41		70.8
	CH-5	3.84		72.5
β -Glucose	CH-1	4.65	d [7.9]	97.0
	CH-2	3.24	dd [8.0; 9.5]	75.3
	CH ₂ -6,6'	3.90, 3.72		61.6
Sucrose	CH-1 (Glc)	5.42	d [3.9]	92.8
	CH-2	3.53		71.8
	CH-3	3.72		73.6
	CH-4	3.42		70.2
	CH-5	3.84		73.5
	C2' (Fru)			104.8
	CH-3'	4.22		77.4
	CH-5'	3.92		82.4
	CH-4'	3.83		61.2
Rhamnitol	CH ₃	1.28*	d [6.4]	20.0
<i>Organic Acids</i>				
Acetic acid	CH ₃	1.92*	s	24.4
	COOH			184.4
Citramalic acid	CH ₃	1.34*	s	26.2
	β -CH ₂	2.45	d	47.7
	β' -CH ₂	2.74	d	47.7
Citric acid	α, γ -CH	2.56 *	d [16.0]	47.0
	α', γ' -CH	2.68		47.0
Formic acid	HCOOH	8.46 *	s	
Lactic acid	β -CH ₃	1.33*	d [7.0]	21.4
Malic acid	α -CH	4.30	dd [9.9; 3.1]	71.2
	β -CH	2.68	dd [15.5; 3.1]	43.9
	β' -CH	2.39*	dd [15.5; 9.9]	43.9
Quinic acid	CH ₂ -2, 2'	1.89*; 2.06	dd [10.8;13.4], m	
<i>Amino acids</i>				
Alanine	α -CH	3.80		51.0

Asparagine	β -CH ₃	1.48 *	d [7.2]	17.3
	β,β' -CH ₂	2.88*; 2.96	dd (7.6; 16.9); dd (4.3; 16.9)	35.6
GABA	α -CH ₂	2.30 *	t [7.4]	35.4
	β -CH ₂	1.90		24.9
<i>Miscellaneous metabolites</i>				
Chlorogenic acid	CH-2'	7.18	d[1.6]	116.0
	CH-5'	6.95	d [8.3]	117.6
	CH-6'	7.09	dd [8.3;1.6]	123.8
	CH-7'	7.67	d [16.0]	147.0
	CH-8'	6.42*	d [16.0]	115.8
Epicatechin	CH-8	6.13	d [2.0]	
	CH-6	6.11	d [2.0]	
	CH-3', CH-2'	6.94	dd [8.4]	
Phloridzin	CH-2,6	7.16	d (8.3)	130.6
	CH-3,5	6.84	d (8.3)	116.0

*Asterisks indicate signals used for the integration and quantification of metabolites.

The ¹H NMR spectra of the Bligh-Dyer organic extracts revealed the presence of sterols, fatty acids, polar lipid heads, as identified by literature data (Di Matteo, Spano, et al., 2021), see **Table A3.3**.

Table A3.3. Metabolites identified in the 600.13 MHz ¹H-NMR spectra of the Bligh-Dyer organic extracts of apple samples dissolved in CDCl₃/MeOD 2:1 *v/v* mixture. Selected signals are related to quantitative analysis (integration).

	ppm	Group	Compounds
I _{β-SIT}	0.65	CH ₃ -18	β-Sitosterol
I _{FA}	2.30	CH ₂ -11	Totally fatty acids
I _{DUFA}	2.73	CH ₂ -11	Linoleic acid
I _{TUFA}	2.77	CH ₂ -11,14	Linolenic acid
I _{PC}	3.23	N(CH ₃) ₃	Phosphatidylcholine

A3.2.2.2 Metabolites quantifications

Metabolic components, quantified in apple slices at 14- and 21-days cold storage in the three packages, included primary and secondary sugars, amino acid and organic acid metabolites, fatty acids, sterols and other compounds. In principle, cold storage delays ripening and senescence processes, in particular the reduction of catalytic activities, including enzymes involved in the respiration pathways, which influence primary and secondary metabolism. **Figure A3.2** and **Figure A3.3** show the trends of metabolites that were identified and quantified at each time (t_0 , t_{14} , t_{21}), which will be discussed below.

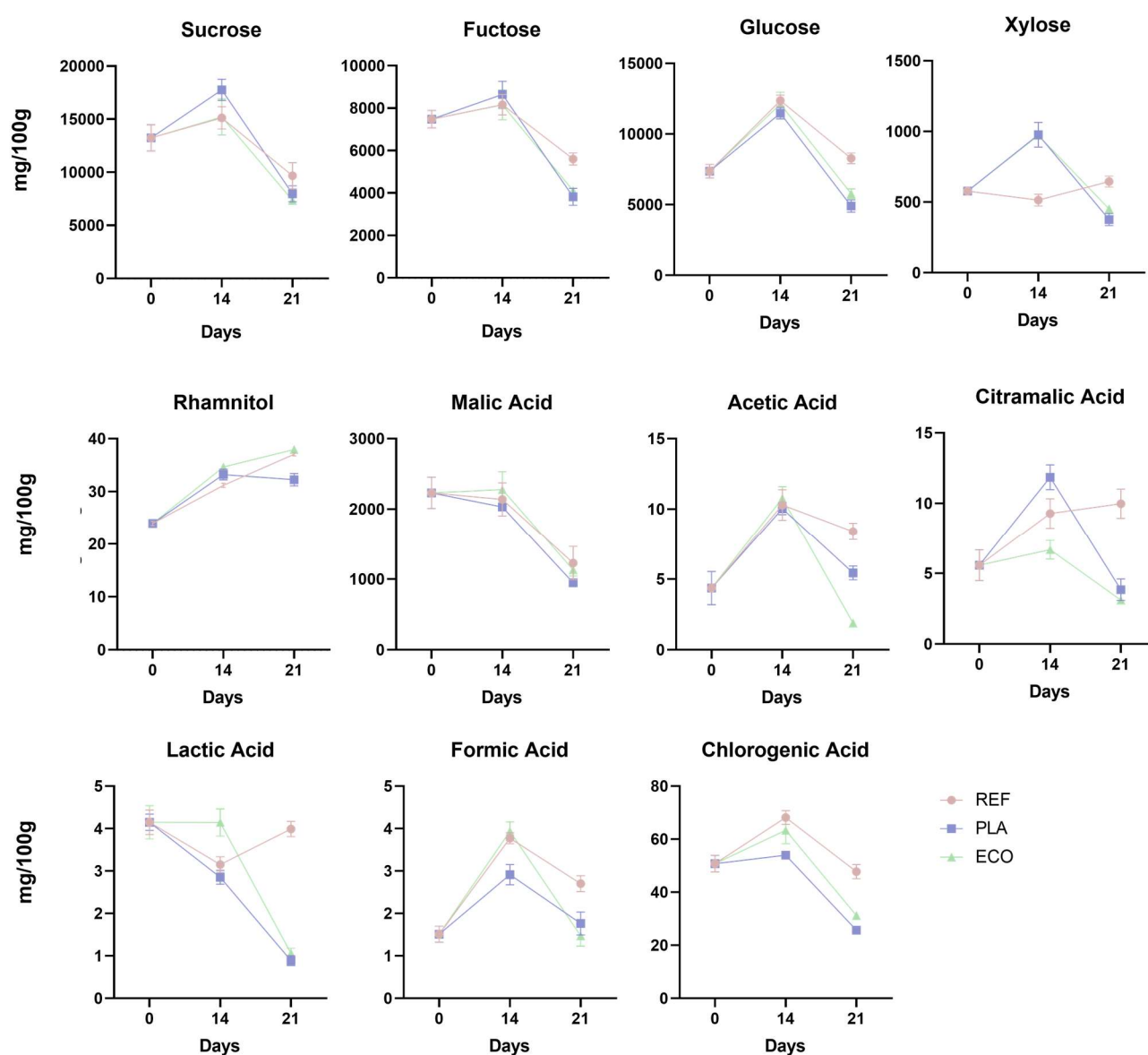


Figure A3.2. Trend over time (0, 14, 21 days) of apple samples quantified in the hydroalcoholic fraction according to respective packaging (REF, PLA, and ECO).

The main sugar and sugar alcohols identified in the NMR spectrum of the apples hydroalcoholic fraction were sucrose, glucose, fructose, xylose and rhamnitol. Signals from the carbohydrate sorbitol were not detected. Indeed, sorbitol is produced during the growth of the fruits on the tree and rapidly converted into fructose, sucrose and glucose (Aprea et al., 2017; Yamaki & Ino, 2019). The sugar fruit profiles stored in the two biodegradable packages were very similar. The general upward trend of sugar content was in line with the loss of starch during the storage period. Higher sucrose content after 14 days of storage results from the balance between degradation and biosynthesis, which may contribute to membrane stability (Brizzolara et al., 2020). The main differences compared to the apples stored in the reference package can be observed between the xylose and rhamnitol trends. Xylose is one of the predominant non-cellulosic sugars in the cell walls of ripe-stage apples (Fischer & Amadò, 1994). Its content is generally reported to increase with storage in most apple cultivars and may result from the enrichment of cell walls in xyloglucan due to the metabolism of pectin during storage. The enrichment may lead to an increase in the hemicellulose content of the cell wall. However, there is currently no evidence to suggest an association between xylogalacturonans and the wall-softening process. Compared to the control, apple samples stored in both biodegradable packages showed increased xylose content, almost double the amount at day 14, and then decreased below the starting content at day 21.

There is increasing evidence that organic acids act as intermediates in carbon metabolism and critical components in response to environmental stress factors (Brizzolara et al., 2020). In most fruits, the organic acid pool decreases during ripening, and low-temperature storage limits the titratable acidity (TA) loss rate: this is imputed to reduced metabolism, particularly respiration. Reducing organic acid concentrations in plant tissues may serve as a survival strategy by limiting the flux towards nitrogen metabolism while maintaining the production of glycolysis substrates (Aprea et al., 2017). The content of malic acid, the primary organic acid in apples that accounts for approximately 90% of the acid content (Ackermann et al., 1992), was almost 2 gr/100gr of dried weight at t_0 . The malic acid levels remained relatively stable after 14 days of storage in all three types of packaging, with minimal variation falling within the standard deviation of the measurements. However, a significant change occurred on day 21, as the malic acid content quantity is reduced by half in the apples preserved in all three types of packaging. This trend can be attributed to the concentration of oxygen and the

respiration process. Lower oxygen concentration during storage helps maintain higher malic acid content (Brizzolara et al., 2020). However, malic acid content generally decreases during the ripening and storage of apples as respiration increases since malic acid is the principal metabolic substrate, along with sugars (Ackermann et al., 1992). Citramalic, acetic and formic acids are present in lower amounts, but the trend over the 21 days of storage is similar. The apples stored in PLA exhibited a significant increase in the concentration of the three acids, more than doubling their levels before experiencing a dramatic decrease at 21 days. In contrast, the citramalate content in apples stored in ECO remained constant for 14 days before decreasing to a concentration similar to that of the apples stored in PLA. Recently the presence of a citramalate pathway in apple fruit has been demonstrated, which contributes to the formation of aroma active esters during fruit ripening (Sugimoto et al., 2021). However, the behaviour of citramalic acid during storage in apples has not been studied, thus making it difficult to establish any correlation with the current variables.

Apples are an important source of phenolic compounds, including flavonoids and chlorogenic acid (Liao et al., 2021). Here, epicatechin, phloridzin, and chlorogenic acid were identified, but only the latter was quantified in all three packaging during the storage period. The time course of chlorogenic acid was the same for the three packaging, with an increase after 14 days and a decrease after 21 days. Substantial amounts of chlorogenic acid have been shown to reduce levels of lipoperoxigenase, β -galactosidase, NADP-malic enzyme and enzyme activities of lipoperoxigenase and UDP-glucose pyrophosphorylase, all of which are known promoters of fruit ripening and senescence (Xi et al., 2016). Furthermore, it is known that the antioxidant activity of apple extracts varies between samples of different apple cultivars and depends on the composition of the controlled atmosphere (Butkeviciute et al., 2022).

With regard to the lipophilic fraction, the trend was almost variable (**Figure A3.3**). In particular, at 21 days, higher values for unsaturated and monounsaturated fatty acids were evident in all three packages. This behaviour could be related to the storage and release of aromatic components, which act as flavour precursors (Fărcaș et al., 2022). During ripening, in contrast, levels of beta sitosterol and phosphatidylcholine decreased at first and then increased. Indeed, the presence of oxygen may have influenced phospholipid metabolism (Bartley, 1985), especially for ECO packaging.

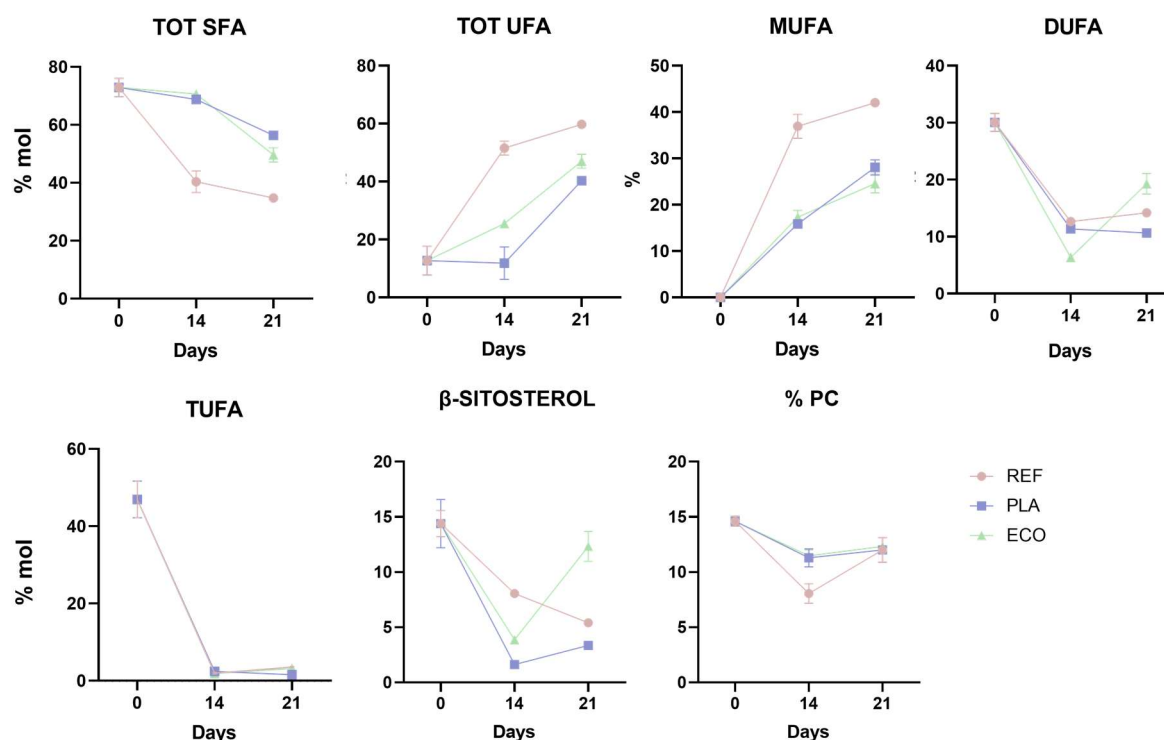


Figure A3.3. Trend over time (0, 14, 21 days) of apple samples quantified in the organic fraction according to respective packaging (REF, PLA, and ECO).

A3.3 Pomodoro Riccio ecotype

The tomato (*Solanum lycopersicum*) stands as a critically important crop, being one of the most vital, extensively cultivated, and consumed vegetables worldwide (Abreu & Fernández, 2020). The composition and nutritional value of tomatoes are gaining growing attention from both consumers and producers. Indeed, Tomatoes are vegetables/fruits that contain significant amounts of dietary nutrients, including dietary fibre, reducing sugars, vitamins, minerals, protein, essential fatty acids, phytosterols and carotenoids (Tan et al., 2021; Wang et al., 2023). Bioactive constituents exhibit antioxidant, antiproliferative, anti-diabetic, anti-inflammatory and other health-promoting activities, indicating the vast potential of tomatoes in the prevention and/or amelioration of various chronic degenerative diseases (Ali et al., 2021). However, the processing of tomatoes into various end products (paste, sauces), including mechanical and heat treatments, is considered to be one of the most significant factors that are potentially affected by anti-nutritional compounds and that cause changes in the bioavailability of antioxidants (Wang et al., 2023).

The focus on enhancing fruit quality, particularly in terms of beneficial nutrients and flavor/aroma components, is driven not only by their added economic value but also by their impact on organoleptic qualities and health benefits (Van Dyk et al., 2016). This has sparked significant research interest within the nutraceutical and horticultural sectors.

Over the centuries, tomato varieties have been selected according to specific agronomic, climatic and market requirements, resulting in a great diversity of cultivars, each of which is adapted to particular growing conditions or gastronomic uses (Collins et al., 2022). Among the best-known Italian cultivars, those destined for fresh consumption are highly valued for their organoleptic characteristics, such as flavour and texture. Among these include Cuore di Bue, San Marzano, Ciliegino, Datterino, and Roma tomato (Constantino et al., 2022; di Gioia et al., 2010; Ingallina, Maccelli, et al., 2020; Manzo et al., 2018; Piccolo et al., 2024). Cultivars destined for industrial production of sauces, preserves and processed products are selected for their ability to produce homogenous fruit suitable for mechanical processing. Some of the main cultivars in this category include: Tondo Liscio, Cento, and Heinz tomato. Certain tomato varieties are particularly suitable for preservation, such as dried or canned tomatoes. Required characteristics include good disease resistance and optimal shelf life (such as Datterino tomatoes).

However, tomatoes are a food category extremely exposed to safety risks that can be related to the presence of chemical residuals, like pesticides (Gambacorta et al., 2005), and microbial contaminants, including bacteria and fungi, as well as characterized by a significant amount of solid waste, including vascular tissue, seeds and peels (BISHOP et al., 1982; Concina et al., 2009; Hylin, 2016). In this context is the 'Pomodoro Riccio', a variety of tomato characterised by an irregular, ribbed shape, with folds and protuberances giving it a wrinkled, curly appearance. It is often large, with a thin skin and a rich, sweet flavour. It is an old cultivar particularly well adapted to the local clay soils, with low water requirements. The reduced water intake could mean a lower intake of contaminants since water has been shown to be the primary source of heavy metals and pesticide residues.

Considering all these characteristics of the cultivar of interest, the aim of the study was to metabolomic profiling and possible identification of new and (re)emerging hazards of a particular tomato ecotype. Specifically, NMR analysis was applied to delineate the metabolic profiles of the same cultivar grown in two different years (2022 and 2023). Indeed, in addition to ripeness, which may be related to the time of harvest, factors influencing the nutritional

composition of tomatoes may be variety, climate, location, and agricultural practices. Untargeted approach by NMR also allowed the comparison of different matrix variants, whole tomatoes, tomato concentrate and tomato waste.

A3.3.1 Materials and Methods

Sampling

Samples of 'Pomodoro Riccio', whole tomatoes (T), tomato paste (TP) and tomato waste (TW) were provided by the company 'La Sbecciatrice' (Caserta, Campania, <https://www.lasbecciatrice.it/index.php/it/>). In particular, samples from two years, 2022 and 2023, were subjected to a pre-treatment protocol, including freeze-drying, homogenization and storage at -80°C, up to the specific extraction procedures. The **Table A3.4** shows the growing climate conditions of tomatoes cultivation during the two years, in Piana di Monte Verna (CE, Italy - 41°10' N, 14°20' E).

Table A3.4. Environmental growing conditions in Piana di Monte Verna (CE), Italy, during the years 2022 and 2023. The values expressed are the result of the average of the days of the month considered.

	May 2022	June 2022	July 2022	August 2022	May 2023	June 2023	July 2023	August 2023
Temperature min (°C)	13.5	17.0	21.1	19.3	13.6	18.9	21.3	20.5
Temperature max (°C)	17.9	27.7	31.9	30.7	25.2	31.7	32.6	31.9
Precipitation (%)	58.0	30.0	3.2	19.4	19.4	10.0	3.2	35.5
Humidity (%)	78.1	71.4	68.8	68.9	69.1	60.6	59.0	61.8
Wind (km/h)	11.4	10.0	10.7	10.7	11.3	10.6	10.9	10.6

Extraction procedures

The Bligh-Dyer extraction method was applied to freeze-dried tomato samples. In detail, approximately 200 mg of ground lyophilised sample (whole, pulp and peel tomato) was mixed with 3 mL of methanol/chloroform mixture (2:1 *v/v*), followed by 0.8 mL of distilled water, stirring thoroughly after each addition. The resulting monophasic system was sonicated for 10 min at room temperature. Then, the extract was added sequentially with 1 mL of chloroform and 1 mL of distilled water to obtain a two-phase system. The extract was then centrifuged (7000 rpm for 15 min at 25 °C) and the upper (hydroalcoholic) and lower (chloroform) phases were carefully separated. The extraction procedure was repeated two more times on the pellet

to ensure complete extraction of the compounds; the separated fractions were then pooled. Both the hydroalcoholic and chloroform fractions were dried under a stream of N₂ until the solvent was completely evaporated. The dried phases were stored at -20 °C until further analysis. For each type of by-product, n = 3 replicates were extracted and analysed by NMR spectroscopy.

NMR samples preparation

The dried hydroalcoholic fraction was dissolved in 0.7 mL of buffered D₂O (100 mM phosphate buffer, pH = 7.0) containing the internal standard for chemical shift referencing and quantification (1 mM TSP). The solution was clarified by centrifugation and 0.7 mL of supernatant was placed in a standard 5 mm NMR tube. The chloroform-dried fraction was dissolved in 0.7 mL of CDCl₃/CD₃OD mixture (2:1 v/v).

NMR experiments

The NMR spectra of all extracts were obtained at 25°C using a JEOL JNM-ECZ 600R (JEOL Ltd., Tokyo, Japan). The spectrometer operates at a proton frequency of 600.13 MHz and is equipped with a multinuclear inverse z-gradient probe head.

The proton spectra were referenced to the TSP signal (δ = 0.00 ppm) for the hydroalcoholic fraction and to the TMS signal (δ = 0.00 ppm) for the chloroform fraction.

For the hydroalcoholic fraction, the ¹H spectra were collected using 256 transients with a recycling delay of 7 seconds, a 90° pulse of 12-15 μ s, 32k data points, and a spectral window width of 10.2 ppm. The residual H₂O signal was suppressed using a soft pulse presaturation scheme during the relaxation delay.

For the chloroform fraction, the ¹H spectra were acquired using 256 transients with a recycling delay of 5 seconds, a 90° pulse of 10-11 μ s, 32k data points, and a spectral window width of 13 ppm. All proton spectra were zeroed at 64k data points and Fourier transformed using an exponential multiplication factor of 0.3 Hz. Manual phase and baseline corrections were applied.

For metabolite quantification, selected signals were integrated, and the integrals were normalized with respect to the signal integral of the internal standard at 0.0 ppm (TSP or TMS for aqueous or organic extracts, respectively).

The 2D NMR spectra of all extracts were acquired under previously reported experimental conditions (Ingallina et al., 2023). The ^1H - ^1H TOCSY experiments were performed with a mixing time of 80 ms, the ^1H - ^{13}C HSQC experiments with a $J(\text{C-H})$ coupling constant of 150 Hz, and the ^1H - ^{13}C HMBC experiments with a 100 ms delay for long-range coupling evolution.

A3.3.2 Results and discussion

A3.3.2.1 Metabolomic characterization

The ^1H NMR spectral assignments of hydroalcoholic (Table A3.5) Bligh-Dyer extracts were carried out by means of 2D experiments and literature data regarding other vegetable matrices analyzed in the same experimental conditions (Ingallina, Maccelli, et al., 2020; Ingallina, Sobolev, et al., 2020). Three sugars, three organic acids, fifteen amino acids, choline, uridine, and trigonelline were detected in all the hydroalcoholic Bligh-Dyer extracts and their characteristic ^1H NMR signals were used for compound quantification. Among organic acid, citrate was not quantified because of its signals overlapping. Indeed, the characteristic diastereotopic protons α , γ -CH (2.56 ppm) and α' , γ' -CH (2.68 ppm) overlapped with the diastereotopic protons β -CH (2.68 ppm) and β' -CH (2.41 ppm) of malate. Therefore, the malate was integrated at 4.30 ppm for the quantification.

Table A3.5. Metabolites identified in the 600.13 MHz ^1H NMR spectra of Bligh-Dyer hydroalcoholic extracts of T22, T23, TP22, TP23, TW22, and TW23.

Compound	Assignment	^1H (ppm)	Multiplicity [J(Hz)]	^{13}C (ppm)
<i>Sugars</i>				
α -D-Fructofuranose	C-2			105.9
	CH-3	4.11		82.3
β -D-Fructofuranose	C-2			102.6
	CH-4	4.12*		75.6
β -D-Fructopyranose	C-2			99.3
α -Glucose	CH-1	5.23*	d [3.8]	93.3
	CH-2	3.54		72.6

	CH-3	3.72		73.3
	CH-4	3.41		70.8
	CH-5	3.84		72.5
β -Glucose	CH-1	4.66*	d [7.9]	97.0
	CH-2	3.25		75.3
Sucrose	CH-1 (Glc)	5.42 *	d [3.9]	93.1
	CH-2	3.53		71.8
	CH-3	3.72		73.6
	CH-4	3.42		70.2
	CH-5	3.84		73.5
	C2' (Fru)			104.8
	CH-3'	4.23		77.4
	CH-5'	3.92		82.4
	CH-4'	3.83		61.2
<i>Organic Acids</i>				
Citrate	α, γ -CH	2.56	d [16.0]	46.0
	α', γ' -CH	2.68		46.0
	β -C			73.0
	1,5-COO-			180.2
	6-COO-			180.0
Formate	HCOOH	8.46*	s	
Malate	α -CH	4.30*	dd [9.9; 3.1]	71.0
	β -CH	2.68	dd [15.5; 3.1]	43.5
	β' -CH	2.41	dd [15.5; 9.9]	43.5
<i>Amino acids</i>				
Alanine	α -CH	3.80		51.0
	β -CH ₃	1.49*	d [7.2]	17.3
	COOH			178.6
Asparagine	α -CH	4.00		52.8
	β, β' -CH ₂	2.86* ;	dd [7.7; 16.8]	35.6
		2.96	dd [4.3; 12.5]	
	COOH			176.5
Aspartate	β, β' -CH ₂	2.70 ;	dd [3.7;17.5]	37.0
		2.81*		

	α -CH	3.92		54.0
	γ -COO ⁻			177.3
GABA	α -CH ₂	2.30*	t [7.4]	35.4
	β -CH ₂	1.90		24.9
	γ -CH ₂	3.01	t [7.5]	40.4
Glutamine	α -CH	3.76		55.3
	β , β' -CH ₂	2.14	m	27.5
	γ -CH	2.46*	m	31.9
Histidine	β , β' -CH ₂	3.30; 3.24		28.2
	α -CH	4.02		55.4
	CH-2	8.13		
Isoleucine	α -CH	1.97		38.0
	β -CH	1.27		29.4
	γ -CH ₃	1.01*	d [7.1]	15.7
	δ -CH ₃	0.89	d [7.4]	
Leucine	β -CH ₂	1.74		41.0
	γ -CH	1.71		
	δ -CH ₃	0.95*	d [6.2]	23.1
	δ' -CH ₃	0.96		22.1
Lysine	α -CH	3.76		55.2
	β -CH ₂	1.92		31.0
	γ -CH ₂	1.49		22.5
	δ -CH ₂	1.74		27.4
	ε -CH ₂	3.04	t [7.6]	
Phenylalanine	CH-2,6	7.33	m	130.7
	CH-4	7.38		
	CH-3,5	7.42*	m	130.6
Proline	α -CH	4.13*		62.5
	β , β' -CH ₂	2.07, 2.33		30.0
	γ -CH ₃	2.01	m	25.0
	δ , δ' -CH ₃	3.33, 3.41		47.4
Threonine	α -CH	3.59		62.3
	γ -CH ₃	1.33*	d [6.7]	20.71
Tyrosine	CH-2, 6 ring	7.22	d [8.2]	132.0
	CH-3, 5 ring	6.96*	d [8.5]	117.0

Valine	α -CH	3.60		
	β -CH	2.27		
	γ -CH ₃	1.10	d [7.1]	17.7
	γ' -CH ₃	1.04*	d [7.1]	19.0
<i>Micellaneous</i>				
Choline	+N(CH ₃) ₃	3.20*	s	55.1
Trigonelline	CH-1	9.13*	s	
	CH-3	4.42	s	
	CH-4	8.09		
Uridine	CH-6	5.91*	d [8.1]	
	CH-5	7.87	d [8.1]	
	CH-1'	5.89	d [4.8]	

* Asterisks indicate signals used for the integration and quantification of metabolites.

The ¹H NMR spectra of Bligh-Dyer organic extracts, showed the presence of sterols, fatty acids, and lipid polar heads, identified by literature data (Di Matteo, Spano, et al., 2021; Ingallina, Sobolev, et al., 2020; Spano et al., 2021), **Table A3.6**.

Table A3.6. Metabolites identified in the 600.13 MHz ¹H NMR spectra of Bligh-Dyer organic extracts of T22, T23, TP22, TP23, TW22, and TW23.

	ppm	Group	Compounds
I _{β-Sit}	0.65	CH ₃ -18	β -Sitosterol
I _{Stig}	0.67	CH ₃ -18	Stigmasterol
I _{FA}	2.30	CH ₂ -11	Totally fatty acids
I _{DUFA}	2.73	CH ₂ -11	Linoleic acid
I _{TUFA}	2.77	CH ₂ -11,14	Linolenic acid
I _{PC}	3.23	N(CH ₃) ₃	Phosphatidylcholine
I _{DGDG}	4.87	CH-1	Digalactosyldiacylglycerol

A3.3.2.1 Metabolomic quantification

The quantitative profile of hydrophilic and lipophilic metabolites (**Figure A3.4**, **Figure A3.5**) obtained by the proposed analytical protocol is described and discussed according to classes of compounds below.

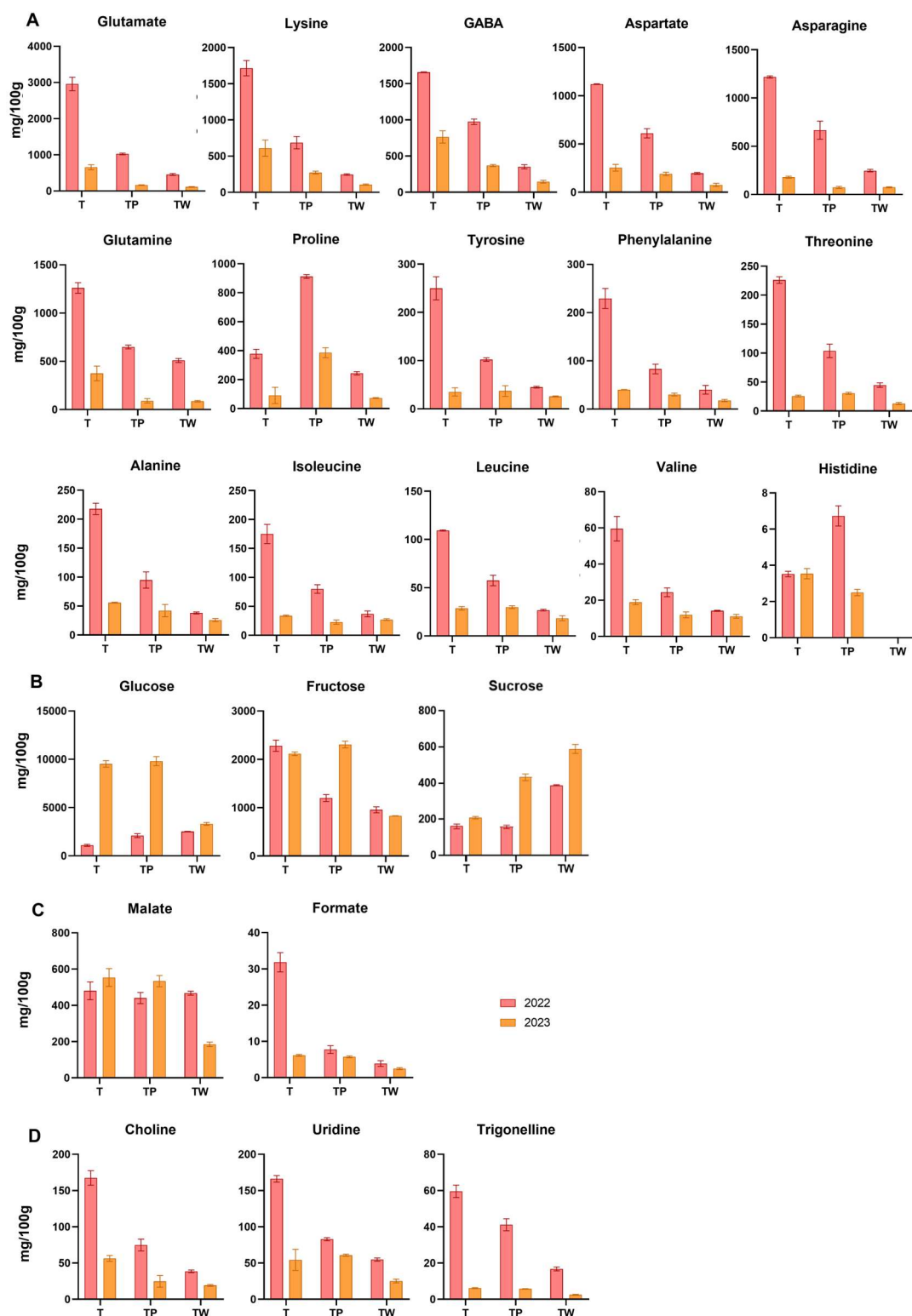


Figure A3.4. Bar graphs of identified and quantified metabolites (mg/100 g DW $\pm$ SD) in ^1H NMR spectra of hydroalcoholic extracts of T, TP, and TW samples during 2022 and 2023. (A) amino acids, (B) sugars, (C) organic acids, and (D) miscellaneous metabolites.

Amino acids

The highest amino acid concentrations were measured in T22, which was particularly rich in glutamate, and the lowest in TW23, see **Figure A3.4(A)**. Glutamate was the most abundant amino acid in all samples, whereas histidine was the least abundant.

In tomatoes, free amino acids increase dramatically during ripening (Sorrequieta et al., 2010). This is most likely due to the increase in several peptidase activities. Specifically, the glutamate level of ripe fruits seems to be mainly related to the activities of glutamate dehydrogenase and GABA transaminase, which could contribute to the increased glutamate level during the ripening transition (Sorrequieta et al., 2010) .

Although lower than T23, T22 had high amounts of glutamate, lysine, GABA, and glutamine. Considering the TP and TW samples, amino acid levels were constantly higher in the first year than in the second. In particular, TP22 was abundant in histidine and proline compared to all other samples.

Sugars

Sugars (**Figure A3.4(B)**) represented the largest fraction of water-soluble compounds in hydroalcoholic extracts, particularly richer in quantity were the 2023 samples.

Particularly rich in sugars were samples 2023, T23 and TS23, glucose levels were approximately 90 mg/g, almost four times and nearly nine times higher than in T22 and TS22, respectively. Similar amount of fructose was found in T22 and T23, and in TW22 and TW23, whereas significantly higher in TS23 relative to TS22. Sucrose concentration was higher in TW samples, reaching approximately 6 mg/g in TW23. The sweetness and overall quality of tomato fruit are closely linked to high levels of sugar (Padmanabhan et al., 2015). As tomatoes ripen, various biochemical processes take place, transforming the initially sour unripe fruit into a sweet and aromatic one. The total sugar content rises during ripening, with unripe fruits mainly containing glucose and ripe fruits having relatively higher levels of fructose (Davies & Kempton, 1975).

Organic acids

At all maturation stages, citric acid is the dominant organic acid in tomatoes, however, quantification was not possible here due to overlapping signals.

The total content of organic acids in the matrices was rather similar, except for tomato waste, see **Figure A3.4(C)**. Malate was most concentrated in T22 and TP22, while formate had the highest levels in T22. The maturing process of tomato fruits is commonly associated with the accumulation of sugars and the breakdown of organic acids. In particular, unripe tomatoes may contain a significant content of malic acid (Davies, 1966).

Miscellaneous

Among the other metabolites (**Figure A3.4(D)**), the highest levels of choline, trigonelline, and uridine were detected in T22, following by TP22 and TW22.

Fatty acids and sterols

Considering the quantifications of the lipophilic portion (**Figure A3.5**), concentrations varied according to samples and cultivation year.

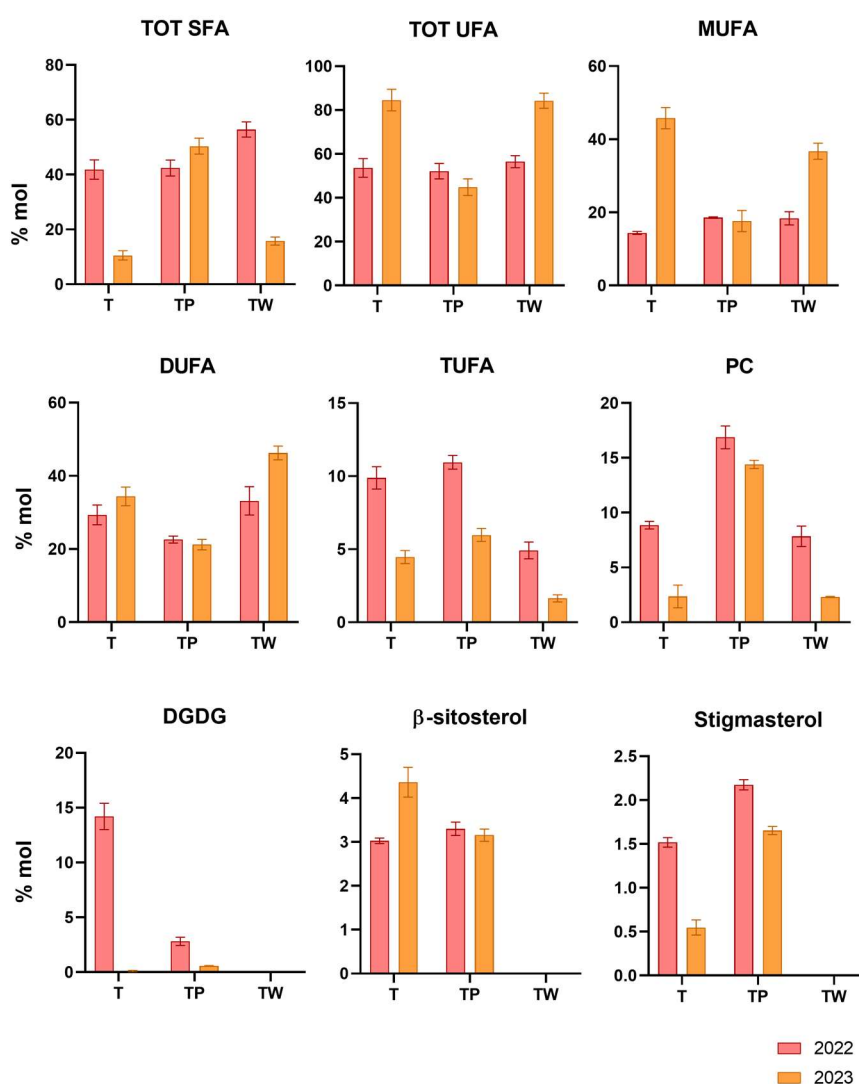


Figure A3.5. Bar graphs of identified and quantified metabolites (%mol/100 g DW $\pm$ SD) in ^1H NMR spectra of organic extracts of T, TP, and TW samples during 2022 and 2023.

TW22 and T23 were found to contain the highest and the lowest percentage SFA content, respectively. However, the SFA levels of T22 were significantly higher than in T23, which showed amounts more than half as low. Similar concentrations of SFA were detected in TS22 and TS23, while its concentrations were particularly low in TW22 with respect to TW23. Regarding the UFA content, T23 and TW23 reached significantly higher values relatively to T22 and TW22, respectively.

MUFA concentrations were comparable for T22, TS22, TP23 and TW22 (below 20%). The highest MUFA percentages were measured in T23, which had significantly higher levels than T22. With regard to DUFAs, the ratios between matrices belonging to the same product in the two years were rather similar, with a slightly higher percentage difference between TW22 and TW3, in the latter's favor. TUFA levels were higher in T22, TP22, TW22, than in T23, TP23, TW23, respectively. In T22 and TS22, TUFA concentrations were about 10% mol, whereas half that amount in TW22.

β -sitosterol and stigmasterol were detected and quantified in the organic extracts of all samples, except for TW22 and TW23 (**Figure A3.5**). β -sitosterol was found in low concentrations, relatively higher in T23 than in T22, and at similar levels in TP22 and TP23. Conversely, the stigmasterol content was higher in the 2023 samples.

Regarding polar lipids, the content of PC resulted higher in 2022 samples, with a fourfold drop from T22 to T23 and from TW22 to TW23, while a quite similar content was found in TP22 and TS23. Concerning DGDG, it was quantified in samples T22 and TP22, while turned out below the detectable limit in all other samples

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Chapter A4

Food waste: strategies to reduce waste and value food

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A3.1 From food waste by-products to bio-sustainability

A3.1.1 Introduction

In recent years, urbanization and rapid population growth have led to a significant increase in waste generation. Currently, Europe produces an estimated 250 million tonnes of rubbish, which amounts to 488 kg per person (ISTAT data 2019). Waste disposal and collection are typically carried out through incineration and landfills. However, incineration has several drawbacks such as high costs due to the need for high temperatures and the emission of dioxin- and ash-rich fumes, which harm the environment. The only advantage of incineration is the reduction in waste volume, leading to an increase in landfill lifespan. As a result, the number of incinerators in Europe has doubled from 2000 to 2016. On the other hand, landfilling, the traditional method of waste disposal, results in the release of toxic gases and leachate, which pollute the environment and alter groundwater and land. The presence of landfills leads to health problems for nearby residents, increased global warming, and the production of polluting material through waste biodegradation.

To address this issue, a shift towards a circular economy is recommended (Tamasiga et al., 2022). This system ensures that waste from one production chain becomes a resource for another, in contrast to the linear economy model of material supply, production, use, and waste. The circular economy is based on five core elements: circular input, recovery of waste materials, life cycle extension, sharing platforms, and product as a service.

Underlying the circular economy are five essential elements (Lacy et al., 2020):

1. Circular input, which consists of the supply of renewable energy, biomaterials or recycled materials.
2. Recovery of waste materials from processing, which can be exploited for other production.
3. Life cycle extension, which promotes the production of products that can be repaired or reconditioned.
4. Sharing platforms, which enable the exchange of goods to increase their utilisation rate.
5. Product as a service, which consists of the loan of an asset against a rental fee. This optimises the productivity of an asset and reduces the sale of new ones.

A3.1.2 The waste by-products

Billions of tonnes of food are produced every year in modern society to satisfy general needs. Much of this food, however, is wasted, thus causing major impacts on development, the economy and the environment.

In fact, the FAO has estimated that 1.3 billion tonnes of food are wasted during the year, which corresponds to about one third of the total food produced (FAO, 2011). The term 'food waste' refers to all parts of food that are discarded during processing, distribution and consumption, whether they are edible or not.

Parts of a food that are used for animal feed or the production of bio-based chemicals are not counted as waste.

Differences in food waste have been found between more industrialised and developing countries. Indeed, the data clearly show that in the most industrialised countries, wastage is mainly concentrated in the last stages, i.e. sale and retail consumption, whereas in developing countries, the greatest wastage occurs in the storage and transport phase due to the lack of adequate means (Coman et al., 2020).

Recent studies have attempted to estimate the environmental, financial and social impact that is created by food waste, however, it is very difficult to do so as estimates in different countries are based on data collected from different sources and methods (Laberge, 2020). Although it is not possible to have definite overall figures, in all countries the economic and environmental impact is significant. Suffice it to consider that the greenhouse gas emitted, the water consumed, the pesticides used, and the electricity required are just some of the elements required for the production of food that is largely wasted.

The majority of food waste is fruit and vegetable scraps (Lucarini et al., 2021). In Italy, waste of this kind is close to 70%. The high amount of fruit and vegetable waste is due to their quick perishability and the high percentage of non-edible (inedible) parts that make them up, such as leaves, peels, seeds and stalks.

Waste from the fruit and vegetable chain is mainly composed of water. This nature makes them more difficult to dispose of through incinerators, which will need increasingly higher temperatures for their combustion, while in landfills they will increase the amount of leachate at the bottom of the landfill (Parsafar et al., 2023).

These by-products could be reused and thus recycled, as they are rich in polyphenols, vitamins, dietary fibre and mineral salts.

Thus, due to the characteristic bioactive molecules recovered from the fruit and vegetable chain, and in order to reduce the socio-economic-environmental impact, biostimulants, biofuels or biomaterials could be derived from the by-products.

A3.1.3 Biostimulants

The European Biostimulant Industry Council (EBIC) defines biostimulants as “substances and/or microorganisms whose function, when applied to plants or the rhizosphere, is to stimulate natural processes to improve nutrient uptake, efficiency, tolerance to abiotic stress and crop quality” (Sanjuán et al., 2023)

The reason for this interest is that the use of biofertilisers is a valuable tool for the promotion and development of sustainable agriculture, which has a low environmental impact and does not cause negative effects on ecosystems.

The use of chemical fertilisers, on the other hand, not only causes problems for the environment, due to groundwater contamination and soil impoverishment, but also has major consequences on human health.

These products have a very important economic significance. Indeed, in the European Union, the economic value of biostimulants has been estimated at between 200 and 400 million euros with an annual growth rate of 10%.

To determine whether a material is suitable for the development of a biostimulant, several factors must be taken into account (Xu & Geelen, 2018):

- Absence of pesticides.

The presence of pesticides can cause problems in the production of biostimulants since it will no longer be seen as a natural product, but as an altered preparation. Moreover, pesticide contamination should be prevented because it could cause problems with legislation.

- Low cost of collection and storage.

The initial economic value of the waste material should be low since the increase in crop yield due to the application of biostimulants is usually between 5 and 10%. This limits the profitability of their sale.

- Sufficient availability.

It is essential that the biomass from which a biostimulant product is derived is available in large quantities and is ready for processing. Furthermore, these products must have the characteristic of not varying their composition over time in order not to reduce their future yield.

- Competitive use.

Biostimulants may have low yields

Biostimulants can be classified by reference to the raw materials from which they are derived: hydrolysates of plant and animal proteins, humic and fulvic substances, algae extracts, plant extracts and beneficial microorganisms (biofertilisers) (du Jardin, 2015).

I. Biostimulants derived from humic substances

Humic substances are natural constituents of soil organic matter, which are derived not only from the decomposition of plant, animal and microbial residues, but also from the metabolic activity of soil microbes utilising these substrates. Humic substances are thus the result of interactions between organic matter, microbes and plant roots. These interactions must be optimised as much as possible to obtain good biostimulant products. However, this typology of biostimulants is characterised by considerable variability. The interactions described above are not always fully exploited, as the humic compounds differ from one another. Despite this, their effectiveness is nevertheless evident; in fact, a recent study (Rose et al., 2014) showed that the application of this type of biostimulant led to an increase in overall shoot weight (22 + 4%) and root weight (21 + 6%).

Most of the biostimulatory effects of humic substances relate to an improvement in the uptake of nutrients by roots through different mechanisms. One of these mechanisms is the stimulation of the plasma membrane H-ATPase, which converts the free energy released by ATP hydrolysis into a transmembrane electrochemical potential that can be exploited for the uptake of nitrates and other nutrients. ATPase also promotes cell wall loosening that leads to cell enlargement and thus organ growth (Jindo et al., 2014). Furthermore, humic substances improve respiration and intervascular activities, providing carbon substrates, and have an antioxidant effect (Olivares et al., 2015).

II. Protein biostimulants

Protein biostimulants consist of amino acids and peptides obtained by hydrolysis from agroindustrial byproducts, both plant (crop residues) and animal (collagen and epithelial tissues) wastes (Halpern et al., 2015).

Amino acids have been seen to have an osmo-protective action, which has been attributed mainly to the amino acid proline (Chitarra et al., 2016). In fact, proline, due to its activity, can improve leaf water status and plant transpiration (Martynenko et al., 2016).

Moreover, free amino acids and small peptides also behave as signaling compounds, as they stimulate nutrient utilization, resistance to metabolic changes and oxidative stress (Luziatelli et al., 2019).

In general, protein biostimulants promote plant growth by acting on enzymes responsible for nitrogen uptake and assimilation. In particular, they regulate enzymes of the Acid Citric Cycle, contributing to the cross-talk between carbon and nitrogen metabolism.

This class of biostimulants also has chelating properties, which not only protect the plant from heavy metals but also contribute to the mobility and acquisition of micronutrients. Other features that distinguish them are their antioxidant activity and their ability to improve soil fertility when applied to soil.

III. *Biostimulants based on algae extracts*

Algae are widely used for the production of food, alginate and agar. Hence, considerable amounts of algae waste are produced each year, ending up being dumped in landfills.

One way that has been found to harness this resource is to create biostimulants based on algae extracts. Algae are rich in compounds that promote plant growth and yield, seed germination, root development, and resistance to abiotic stresses. The compounds, contained in algae, and found to be responsible for these effects are phytohormones and polysaccharides, such as alginates (Kapoore et al., 2021).

Algae actually act positively not only on plants but also on the soil, which is why they are applied to soils in order to improve performance (Calvo et al., 2014). Most of the algae used for biostimulant production belong to the *Phylum* brown algae.

IV. *Plant biostimulants*

The development of plant biostimulants from agricultural wastes or by-products is definitely an environmentally friendly solution that reduces their impact on the environment and provides innovative materials to improve crop performance and resistance to stress. Plant wastes exhibit high amounts of phenolic compounds, phytohormones, proteins, and amino acids, which can stimulate crops. In particular, the component that most contributes to the effectiveness of these biostimulants is the presence of polyphenols, which are natural antioxidants that can promote plant growth, plant water use, plant stomatal function and photosynthesis (Olthof et al., 2001).

In one study, the effectiveness of a hydrolysate extracted from apple seeds, rapeseed and rice husk residues on a kiwi plantation was evaluated (Donno et al., 2013). Analyses showed that the extract actually increased fruit weight due to its significant content in auxins, gibberellins, and cytokinins, which were mainly present in apple seeds. Rape seed and rice husk, on the other hand, exerted their stimulating effect through their high content of amino acids, proteins and minerals.

V. *Biofertilizers*

Biofertilizer is a product composed of one or more strains of microorganisms, useful for nutrient mobilization, usable as a carrier, and ready to be applied to the soil or the plant (Hezakiel et al., 2023).

So, these are preparations of live microorganisms that enable better agricultural yields.

Soil in itself already contains several beneficial microorganisms, but poor agronomic practices have led to their marked reduction. The task of these microorganisms is to colonize the rhizosphere, which represents the portion of soil surrounding the root. The direct mechanisms by which biofertilizers promote plant growth and increased soil fertility are nitrogen fixation, phosphorus solubilization and iron sequestration.

Microorganisms, such as *Rhizobium Leguminosarum*, are able to convert atmospheric nitrogen (N_2) to ammonia (NH_3) so that the plant can absorb nitrogen, which is essential for its growth.

Another way that biofertilizing microorganisms work is by converting insoluble phosphorus, which is naturally present in the soil, into soluble phosphorus (*Acetobacter* or *Agrobacterium*). Finally, the last direct mechanism by which these

bacteria interact is to sequester iron contained in the soil in such a way as to make it more accessible to the plant.

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Hydrolysates from cauliflower and artichoke industrial wastes as biostimulants on seed germination and seedling growth: a chemical and biological characterization

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Abstract

Background: Cauliflower (*Brassica oleracea* L.) and globe artichoke (*Cynara scolymus* L.) are vegetables with a high waste index mainly related to stems and leaves. In this study, enzymatic hydrolysates obtained from these wastes were proposed to be used as plant biostimulants. Life cycle assessment methodology was also applied to evaluate environmental performances related to cauliflower and artichoke byproducts.

Results: Hydrolysates (HYs) were chemically and biologically characterized. Amino acids, organic acids, amines, polyols, mineral elements, phenols, tannins, flavonoids and sulfur compounds were identified and quantified by means of NMR, inductively coupled plasma mass spectrometry and UV–visible analyses. Cauliflower leaf and flower HYs showed the highest concentration of free amino acids, whereas stems showed the highest concentration of Ca. Regarding artichoke, asparagine, glutamine and aspartic acid were exclusively detected in stems, whereas artichoke leaves showed the highest Mg and Mn levels together with the highest antioxidant activity. The HYs diluted in water were tested as biostimulants. The impacts of five concentrations of HYs (0.00, 0.28, 0.84, 2.52 and 7.56 g L⁻¹) on seed germination and early seedling growth of crimson clover, alfalfa, durum wheat and corn were investigated.

Conclusions: The application of artichoke biostimulant (0.28 g L⁻¹) positively influenced the coefficient of velocity of germination in alfalfa, crimson clover and durum wheat, whereas cauliflower biostimulant significantly improved corn germination speed.

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Supporting information may be found in the online version of this article.

Keywords: hydrolysates; biostimulants; agricultural waste; NMR analysis; antioxidant activity; LCA analysis

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INTRODUCTION

Fruit and vegetable supply chains represent some of the most important markets in the world. This sector produces significant quantities of byproducts that are often disposed of as waste rather than being reintroduced for new purposes in the food supply chain.¹ The conventional treatment of agro-food waste has shown economic, social and environmental impacts that make it a global problem. For this reason, the agriculture sector is increasingly engaged in a radical transition towards a circular, solid and resilient system based on production processes that improve agricultural activities' sustainability.² In this context, the re-use of food waste is attracting increasing interest as required by the green economy and better environmental preservation.³ In particular, the management and treatment of waste to obtain new products have become a current topic from economic and environmental points of view,² mainly considering that wastes obtained from the agricultural field are a precious source of molecules with both nutritional and bioactive activities.

Among the several applications that have been proposed for the re-use of agricultural wastes, the production of plant biostimulants (PBs)⁴ represents an interesting potential one. PBs are effective in promoting the smooth running of plant life processes and ameliorating stress-induced injury by regulating vegetal antioxidant systems⁵ or supplying essential or beneficial minerals and compounds.⁶ PB applications⁷ mainly involve foliar, soil and seed treatments, with the last one being considered a highly eco-friendly method due to the reduced treated surface area⁵: only a small PB amount per hectare is needed compared to those used for soil and foliar treatments by spraying.

Organic PBs are derived or are made from living matter, including humic substances (natural constituents of soil), seaweed extract, biofertilizer (symbiotic bacteria or fungi) and protein hydrolysates (HYs). The last type of PBs are mixtures of amino acids and peptides and their production requires the use of vegetable or animal residue extracts obtained by chemical or enzymatic processes. These formulations have a positive effect on agricultural production due to different bioactive compounds which enhance plant growth by regulating nutrient assimilation, storage, metabolism and radical scavenging.^{8,9} In particular, HYs obtained from agro-industrial byproducts are widely recognized for their positive roles in regulating plant responses to environmental stress.¹⁰ Some examples of PBs obtained from HYs include those produced using withered tomato plant,¹¹ rapeseed husks¹² and legume biomass.¹³

In the work reported in this article, HYs were produced from cauliflower (*Brassica oleracea* var. *botrytis*) and globe artichoke (*Cynara scolymus* L.), typical products of Lazio region (central Italy). These crops produce a large amount of waste and byproducts and are considered among vegetables with the highest waste index.¹⁴ To the best of our knowledge, the chemical composition and the biological activity of HYs obtained from cauliflower and artichoke waste as well as their use as biostimulants have not yet been investigated and their potential as PBs has never been evaluated.

HYs obtained from cauliflower and artichoke were investigated by using untargeted NMR,¹⁵⁻¹⁷ and targeted inductively coupled plasma mass spectrometry (ICP-MS) and UV-visible analyses,^{18,19} as well as the potential radical scavenging power being investigated through DPPH and ABTS assays. Biostimulants were developed from HYs and their effect at different concentrations was tested on seeds of crimson clover (*Trifolium incarnatum* L.), alfalfa

(*Medicago sativa* L.), durum wheat (*Triticum durum* Desf.) and corn (*Zea mays* L.).

Finally, life cycle assessment (LCA) was also applied as a globally standardized comprehensive framework for sustainability assessment.²⁰

MATERIALS AND METHODS

Sampling

Cauliflower (*Brassica oleracea* var. *botrytis*) wastes were supplied by the Azienda Agricola F.lli Calevi Alberto E Stefano SS (Viterbo, Lazio region, Italy). Artichoke (*Cynara scolymus* L.) wastes were supplied by the organic farm Azienda Agricola Sperlonga-SANVIDA (Sperlonga, Lazio region, Italy). Flavourzyme 500MG was obtained from Novozymes China Inc. (Guangzhou, China).

Sample treatment

Cauliflower stems, leaves and flowers, and artichoke stems and leaves were washed with running water to remove all dirty residue.

All matrices were freeze-dried for 3 days using a Buchi Lyovapor L-200 at -55°C and 8.0×10^{-3} Pa. The freeze-drying process promotes water removal, thereby reducing the oxidation process. Each sample was homogenized and frozen at -80°C before enzymatic hydrolysis.

Enzymatic hydrolysis

Distilled water (600 mL) was added to dried waste product (20 g) of each matrix (cauliflower stems, leaves, and flowers, and artichoke stems and leaves). The samples were heated to 90°C to inactivate endogenous enzymes and, after achieving the boiling point, the system was kept for 10 min under magnetic stirring. After cooling, the pH was adjusted to neutral value by adding 2 mol L^{-1} NaOH in the solution and then 1120 μL of protease solution (1 g matrix/56 μL proteolytic enzyme ratio) (from *Aspergillus oryzae*, Flavourzyme) was added. For enzyme activation, the temperature was maintained at 50°C for 90 min in an Eco cell oven. The hydrolysis reaction was stopped by raising the temperature to 90°C for 15 min. The inactivated homogenates were first filtered under vacuum with a Buchner funnel and a $0.45 \mu\text{m}$ paper filter, then under vacuum with a Buchner funnel and a resin filter. Liquid HY was then lyophilized with a Buchi Lyovapor L-200 at -55°C and 0.200 mbar until complete loss of water. A 1.0–1.6 g range of yield was obtained by drying 100 mL of the considered HY.

NMR analysis of HY samples

HY samples from waste matrices (cauliflower stems, leaves, and flowers, and artichoke stems and leaves) were dissolved in 750 μL of buffered D_2O (400 mmol L^{-1} phosphate buffer, $\text{pH} = 7.4$) containing 2 mmol L^{-1} 3-(trimethylsilyl)propionic-2,2,3,3- d_4 acid sodium salt (TSP) as internal standard for chemical shift referencing and quantification.²¹ The NMR spectra were recorded at 27°C with a Bruker AVANCE III HD 600 NMR spectrometer operating at the proton frequency of 600.13 MHz and equipped with a Bruker multinuclear z-gradient inverse probehead. Proton spectra were referenced to the TSP signal ($\delta = 0.00 \text{ ppm}$). The ^1H spectra were acquired by co-adding 128 transients with a recycle delay of 7 s and using a 90° pulse of 12–15 μs , 32 K data points and 12 ppm spectral window width. The residual HDO signal was suppressed using a soft pulse

presaturation scheme (Bruker pulse program zgpr) during the relaxation delay. After the acquisition, proton spectra were zero-filled to 64 K data points, and Fourier-transformed using a 0.3 Hz exponential multiplication factor. Manual phase and baseline correction were performed. For the quantification of metabolites, the selected signals were integrated and the integrals were normalized with respect to the integral of the internal standard (TSP) signal at 0.0 ppm.

ICP-MS analysis of minerals in HY samples

Mineral concentrations from artichoke leaves and stems, and from cauliflower leaves, stems and flowers were estimated by ICP-MS after the preventive digestion of each sample, according to a previously reported methodology.²²

Spectrophotometric determination of total polyphenols, tannins and flavonoids in HY samples

These analyses were performed according to the literature.²¹ The total amounts of polyphenols/tannins and flavonoids were determined from the calibration curves of gallic acid ($y = 47\,698x + 0.01084$; $R^2 = 0.99$) and quercetin ($y = 27\,495x + 0.002971$; $R^2 = 0.99$), respectively, and expressed as grams of gallic acid equivalents (GAE) and quercetin equivalents (QE) per kilogram of sample.

Spectrophotometric determination of sulfur compounds in HY samples

Sulfur compounds were determined spectrophotometrically, by exploiting their ability to be oxidized under an alkaline environment by ferricyanide, whose absorbance can be measured at 420 nm.²³ To perform the assay, the sample (50 μ L), potassium ferricyanide (12 mmol L^{-1} ; 50 μ L) and sodium hydroxide (1 N; 100 μ L) were mixed under an alkaline environment (pH = 12). Then the absorbance of potassium ferricyanide was measured at 420 nm using a microplate reader (Epoch microplate spectrophotometer, BioTek). Suitable controls, including potassium ferricyanide in the absence of the sample (negative control) and progressive concentrations of sinigrin (positive control), were included; moreover, sample in the absence of potassium ferricyanide was tested, to reveal the presence of possible interfering compounds in the extract. The total amount of glucosinolates was determined from the calibration curve of sinigrin ($y = -2.496x + 0.2517$; $R^2 = 0.98$) and expressed as grams of sinigrin equivalents (SINE) per kilogram of sample.

Radical scavenging activity of HY samples

The radical scavenging activities of the samples were determined with the DPPH and ABTS methods, carried out according to previously standardized microplate methods²¹ and calculated as percentage difference from the control. The experiments were repeated at least twice, and in each experiment each concentration was tested at least in triplicate.

Data from the antioxidant activity assays were expressed as mean $\pm$ SE of at least three experiments with at least three technical replicates ($n = 9$) and analyzed by GraphPad Prism (Version 4.00) software (GraphPad Software, Inc., San Diego, CA, USA). One-way analysis of variance (ANOVA), followed by Dunnett's multiple comparison post test, was used to highlight possible differences among the treatments, while Student's *t*-test was used to determine a significant difference between leaf and stem HYs for each matrix. The concentration–response curves were calculated using the Hill equation, according to previous studies.²¹

Biostimulant preparation from HYs

Two biostimulants, namely a cauliflower mixture (leaf, stem and flower HYs) and an artichoke mixture (leaf and stem HYs), were prepared according to the proportions reported in Table 1. The wastes ratio was maintained for each treatment in order to ensure reproducibility in crop trials. Thus, the biostimulants can be easily reconstituted adding the appropriate amounts of water.

To test the effectiveness of artichoke and cauliflower biostimulants, four different concentrations of biostimulants, namely 0.28, 0.84, 2.52 and 7.56 g L^{-1} (low dose (LD), medium-low dose (MD), high dose (HD) and very high dose (VHD), respectively), were prepared. The effectiveness of the artichoke and cauliflower biostimulants were compared with distilled water used as control (CtrlW) and a commercial biostimulant (Comm.Biost),²⁴ based on *Ascophyllum nodosum* and used by dissolving 15 mL of it in 1 L of distilled water.²⁵

Germination test on seeds of: *Trifolium incarnatum* L., *Medicago sativa* L., *Triticum durum* Desf. and *Zea mays* L

The germination test was conducted in the seed laboratory at the University of Tuscia (UNITUS), Viterbo, Italy. To evaluate the effect of HYs from artichoke and cauliflower as biostimulants for seed germination, the following crop species were tested: crimson clover (*Trifolium incarnatum* L.), alfalfa (*Medicago sativa* L.), durum wheat (*Triticum durum* Desf.) and corn (*Zea mays* L.). Seeds of the four species were taken from authorized and certified dealers. Sets of 25 seeds of corn and durum wheat and 50 seeds of alfalfa and crimson clover were used for each treatment with different doses.^{26,27} The seeds were soaked in the respective biostimulant for 24 h. Then they were placed on filter paper in sterile Petri dishes, imbibed and put in a germination chamber with automatic control of light and temperature, with photoperiod 16 h darkness/8 h light at a constant temperature of $20 \pm 2^\circ C$.²⁸ Throughout the germination period the seeds were rehydrated with additional biostimulant to prevent drying when necessary. All treatments were performed in three replicates. Seed germination was recorded daily for 7 days, leaving the seedling to develop until day 10, when measurements were made on the seedlings.^{26,28}

Seed germination and seedling growth measurements

The parameters to assess seed germination are described as follows. Percentage of germination was recorded every 24 h to assess the number of newly germinated seeds and after 7 days to calculate the total germination percentage (Gtot%). The emergence of radicle (>2 mm) was used as an indicator of germination.²⁹ Coefficient of velocity of germination (CVG)³⁰ was used to give an indication on the speed of germination as follows: $CVG = 100 \times (N_1 + N_2 + \dots N_n) / (N_1T_1 + N_2T_2 + \dots N_nT_n)$; N = number of germinated seeds per day; T = number of days from sowing corresponding for each N . The seedlings were harvested 10 days after the germination test had started. The

Table 1. HY portions (leaves, stems and flowers) used for biostimulant preparation

HY portion	Cauliflower waste (%)	Globe artichoke waste (%)
Leaves HY	42.5	50.8
Stems HY	22.0	49.2
Flowers HY	35.5	—

biomass was weighed first as fresh biomass, then after being in an oven at 70 °C for 48 h, as dry biomass. The maximum length of shoot and roots was measured with a graduated ruler to the nearest 1.0 mm and the number of roots for each seedling was recorded. Total seedling length was calculated as root length + shoot length. The seed vigor index (SVI) was calculated as Gtot% multiplied by total seedling length divided by 100.³¹

The germination test was conducted according to a randomized block with eight treatments and three replications for each crop species and biostimulant concentration. Data were subjected to one-way ANOVA. The mean values were tested for significance with Tukey's HSD test. Statistical difference was set at $P < 0.05$. Percentage data were arcsine-transformed for analysis, whereas they were presented in tables as non-transformed means. For statistical analysis, the software R version 4.2.1 was used.

Life cycle assessment

According to ISO 14040, LCA consists of four distinct phases: (i) goal and scope definition, (ii) life cycle inventory analysis and (iii) life cycle impact assessment are compulsory; (iv) interpretation of results is optional (ISO 14040, 2006). Subsequent sections elaborate on each phase. The analysis of all environmental impacts was conducted using SimaPro v 9.5.0.2 software. Details are reported in the supporting information.

RESULTS AND DISCUSSION

Hydrolysate samples

Artichoke and cauliflower wastes were subjected to enzymatic hydrolysis and analyzed using different methodologies, the results of which are here separately discussed.

Metabolite profile by ¹H NMR analysis

The NMR analysis of cauliflower and artichoke HYs was based on the methodology previously reported for crude extracts of the same byproducts.³² Amino acids, organic acids, amines and polyols were identified and quantified (Table 2). In the case of cauliflower HYs, the content of almost all free amino acids, except glutamine, was higher in flowers than in stems, with aspartic acid, glutamic acid and methionine being the most abundant. In the case of artichoke HYs, the content of amino acids in leaves was slightly higher than in stems (except for lysine, threonine and glutamic acid). Moreover, asparagine, glutamine and aspartic acid were detectable only in stems. Lactic acid was the most abundant organic acid in all HYs except in artichoke stems, followed by citric and malic acids. Unexpectedly, mannitol, not detected in crude extracts,³² was found in all cauliflower HY samples, mainly in stems. Artichoke HYs also contain inositol isomers (*scyllo*- and *chiro*-inositols), the amounts of which were similar to those found in crude extracts.³²

Inorganic elements by ICP-MS analysis

The amounts of inorganic elements in hydrolyzed artichoke leaves and stems and in hydrolyzed cauliflower leaves, stems and flowers are reported in Table S6. Data are expressed in mg kg⁻¹ DW ± SD. Thirty mineral elements were determined, spanning from nontoxic (i.e. Ba, Ca, K, Na, Fe, Mg, Mn, Zn, etc.) to potentially toxic ones (i.e. As, Cr, Ni, V, etc.). On average, Ca, K and Mn were the elements present at the highest concentration. In all samples, the levels of Hg and Cd were below the limit of instrumental detection. Levels of Cr (range: 0.000–0.036 mg kg⁻¹) and Pb (range: 0.005–0.049 mg kg⁻¹) were below the limits set by

the European Regulation³³ and/or below the limit of instrumental detection. For the other inorganic elements that are generally considered potentially toxic, the safety limits are not fixed.

Total polyphenols, tannins, flavonoids and glucosinolates by spectrophotometry

HYs obtained from the leaves of both cauliflower and artichoke contained the highest amounts of total polyphenols and tannins, which were two- to threefold higher than those of the stem and flower samples (Table 3). Polyphenols were mainly concentrated in the HYs from the leaves of both samples, whereas lower amounts were detected in those from stems of both cauliflower and artichoke, and flowers of cauliflower. Conversely, low levels of sulfur compounds were determined in the protein HYs from cauliflower leaves, stems and flowers; as expected, these compounds were lacking in the artichoke samples (Table 3).

Antioxidant activity

The ability of HYs to neutralize radical scavengers could be an important feature to favor plant development. Indeed, several environmental factors (e.g. drought, metal toxicity, pollutants, UV-B, pesticides, pathogen infection) can induce oxidative stress in plants, so affecting multiple biological processes via reactive oxygen species generation, leading to cell plant death.³⁴

Under our experimental conditions, the tested HYs were able to neutralize DPPH radicals, except for the sample obtained from cauliflower stems, for which a weak activity was revealed (Fig. 1). The HY from artichoke leaves was the most active, followed by that from cauliflower flowers; with these samples being able to induce an about 80% inhibition of DPPH at a concentration of 500 µg mL⁻¹, despite a lower than 20% inhibition being achieved with other samples. This trend was confirmed by the IC₅₀ values as presented in Table S7. Regarding HYs from cauliflower stems, the IC₅₀ value was not evaluable since at the highest tested concentration the achieved inhibition was lower than 80%. As expected, the positive control, that is, Trolox, exhibited a more potent scavenging effect, the IC₅₀ value being 133- to 500-fold lower than those of the HYs from artichoke and cauliflower leaves (Table S7).

When the antioxidant activity was assessed towards ABTS, all the tested protein HYs exhibited radical scavenging properties. HY from artichoke leaves was the most effective, being able to induce a 50% ABTS neutralization at a concentration of 200 µg mL⁻¹, followed by that from cauliflower leaves and flowers (almost 40% and 30% inhibition at 200 µg mL⁻¹, respectively) (Fig. 2). This trend was confirmed by the IC₅₀ values as displayed in Table S7. As expected, the positive control Trolox exhibited a more potent scavenging effect, the IC₅₀ value being 90- to 140-fold lower than those of the HYs from artichoke and cauliflower leaves.

It is known that small peptides, arising from the hydrolyzation process, can exhibit antioxidant activities.³⁵ Particularly, a high degree of hydrolysis is associated with marked antioxidant properties, likely due to the presence of low-molecular-weight peptides, although the correlation between molecular weight and antioxidant activity has to be clarified.^{36,37} Moreover, the time and hydrolysis conditions can strongly affect the antioxidant power.¹⁴ In line with this evidence, Yathisha et al.³⁸ reported that the peptic HY of cauliflower leaves has been endowed with ABTS radical scavenging activity, with an IC₅₀ of 0.6 mg mL⁻¹, this value being halved after pancreatic digestion. Moreover, Caliceti et al.¹⁴ have highlighted that time and hydrolysis conditions can strongly affect the antioxidant power of samples and found that 3–5 h

Table 2. Metabolite content (g kg⁻¹ DW) in HYs of cauliflower and globe artichoke by ¹H NMR analysis

	$\delta^1\text{H}$ (ppm)	Cauliflower HYs			Globe artichoke HYs	
		Leaves	Stems	Flowers	Leaves	Stems
<i>Amino acids</i>						
Alanine	1.49	2.17 ± 0.17 ^a	1.68 ± 0.08 ^b	4.62 ± 0.28 ^c	6.79 ± 0.34 ^a	1.31 ± 0.07 ^b
Arginine	3.24	2.96 ± 0.18	—	—	—	—
Asparagine	2.96	2.27 ± 0.16 ^a	1.47 ± 0.12 ^b	1.06 ± 0.07 ^c	—	2.80 ± 0.27
Aspartic acid	2.80	5.32 ± 0.27 ^a	1.98 ± 0.14 ^b	9.21 ± 0.92 ^c	—	0.99 ± 0.11
GABA	3.01	1.94 ± 0.21 ^a	1.40 ± 0.14 ^b	3.85 ± 0.31 ^c	0.90 ± 0.07 ^a	0.55 ± 0.07 ^b
Glutamine	2.46	1.87 ± 0.19 ^a	3.05 ± 0.27 ^b	5.59 ± 0.62 ^c	n.d.	3.05 ± 0.31
Glutamic acid	2.35	4.91 ± 0.34 ^a	2.42 ± 0.14 ^b	12.40 ± 0.63 ^c	2.42 ± 0.24 ^a	2.07 ± 0.17 ^a
Histidine	8.13	0.89 ± 0.07 ^a	0.23 ± 0.03 ^b	1.09 ± 0.13 ^a	n.d.	n.d.
Isoleucine	1.01	1.10 ± 0.13 ^a	0.20 ± 0.02 ^b	1.24 ± 0.10 ^a	0.56 ± 0.06 ^a	0.41 ± 0.02 ^b
Leucine	0.96	1.89 ± 0.17 ^a	0.43 ± 0.04 ^b	1.74 ± 0.16 ^a	0.92 ± 0.07 ^a	0.67 ± 0.07 ^b
Lysine	3.03	2.41 ± 0.12 ^a	0.47 ± 0.06 ^b	1.94 ± 0.19 ^a	0.28 ± 0.03 ^a	0.74 ± 0.06 ^b
S-Methyl-L-cysteine-S-oxide (methiin)	2.84	4.96 ± 0.45 ^a	3.22 ± 0.26 ^b	14.16 ± 0.99 ^c	—	—
Phenylalanine	7.43	1.96 ± 0.16 ^a	0.35 ± 0.02 ^b	1.52 ± 0.14 ^c	1.36 ± 0.11 ^a	0.57 ± 0.07 ^b
Threonine	1.34	2.13 ± 0.09 ^a	1.15 ± 0.13 ^b	1.64 ± 0.18 ^c	1.11 ± 0.10 ^a	0.89 ± 0.11 ^a
Tryptophan	7.73	0.50 ± 0.07 ^a	0.11 ± 0.01 ^b	0.24 ± 0.03 ^c	0.52 ± 0.06 ^a	0.20 ± 0.03 ^b
Tyrosine	6.91	1.36 ± 0.15 ^a	0.34 ± 0.03 ^b	1.16 ± 0.10 ^a	0.80 ± 0.12 ^a	0.32 ± 0.03 ^b
Valine	1.05	1.91 ± 0.16 ^a	0.60 ± 0.08 ^b	2.76 ± 0.29 ^c	2.27 ± 0.25 ^a	1.78 ± 0.14 ^b
<i>Organic acids</i>						
Citric acid	2.55	10.81 ± 0.54	—	n.d.	3.83 ± 0.46 ^a	3.35 ± 0.27 ^a
Formic acid	8.46	0.03 ± 0.01 ^a	—	0.064 ± 0.020 ^b	0.11 ± 0.03 ^a	0.033 ± 0.010 ^b
Fumaric acid	6.52	0.19 ± 0.04 ^a	—	0.66 ± 0.08 ^b	0.079 ± 0.02 ^a	0.16 ± 0.04 ^b
Lactic acid	1.34	41.69 ± 2.92 ^a	74.17 ± 6.70 ^b	87.81 ± 9.64 ^b	21.22 ± 2.12 ^a	0.13 ± 0.02 ^b
Malic acid	4.29	2.69 ± 0.13	—	n.d.	1.69 ± 0.14 ^a	12.34 ± 1.36 ^b
Quinic acid	1.87	—	—	—	6.81 ± 0.55 ^a	5.96 ± 0.78 ^a
Succinic acid	2.41	1.24 ± 0.14 ^a	3.45 ± 0.28 ^b	—	4.30 ± 0.43 ^a	1.18 ± 0.12 ^b
<i>Miscellaneous</i>						
Mannitol	3.68	14.25 ± 1.14 ^a	50.13 ± 2.51 ^b	16.12 ± 0.97 ^a	—	—
scyllo-Inositol	3.36	—	—	—	1.27 ± 0.10 ^a	0.39 ± 0.05 ^b
chiro-Inositol	3.58	—	—	—	4.76 ± 0.29 ^a	3.24 ± 0.26 ^b
Choline	3.21	1.37 ± 0.15 ^a	0.51 ± 0.04 ^b	2.86 ± 0.31 ^c	0.44 ± 0.05 ^a	0.60 ± 0.07 ^b

In each column, mean values (± SD) are reported together with the results of ANOVA applied to the leaves *versus* stems and flowers (for cauliflower) comparison for each crop separately. Different superscript letters (a *versus* b) indicate statistically significant ($P \leq 0.05$) differences between leaf and stem HYs, whereas the same letters indicate no significant difference.

Table 3. Total content of polyphenols, tannins, flavonoids and sulfur compounds per mg of HYs from cauliflower (*Brassica oleracea* L.) and artichoke (*Cynara scolymus* L.) leaves and stems

HY	Polyphenols (g GAE kg ⁻¹)	Tannins (g GAE kg ⁻¹)	Flavonoids (g QE kg ⁻¹)	Sulfur compounds (g SINE kg ⁻¹)
Cauliflower leaves	9.54 ± 0.14***	2.48 ± 0.17	0.90 ± 0.02	0.014 ± 0.004
Cauliflower stems	4.81 ± 0.09	1.49 ± 0.12***	bld	bld
Cauliflower flowers	2.96 ± 0.14	1.73 ± 0.14 ^s	bld	bld
Artichoke leaves	14.05 ± 0.17	5.83 ± 0.07	0.96 ± 0.03	—
Artichoke stems	5.89 ± 0.08***	2.13 ± 0.11***	bld	—

Total polyphenols and tannins were determined as gallic acid equivalents (GAE), flavonoids as quercetin equivalents (QE).

*** $P < 0.001$, denotes a significant difference of stems *versus* leaves for each crop (Student's *t*-test).

^s $P < 0.05$, denotes a significant difference of flowers *versus* leaves of cauliflower (Student's *t*-test).^a bld, below the limit of detection; —, not determined.

hydrolysis time using alcalase was the best condition to obtain remarkable antioxidant HYs from ribbon fish.

Additionally, the cauliflower and artichoke HYs are characterized by the presence of bioactive compounds that can contribute

to their antioxidant properties against DPPH and ABTS radicals. Artichoke samples were the most effective scavenging agents, although with a higher potency towards ABTS than DPPH. Such properties have been already reported for both cauliflower and

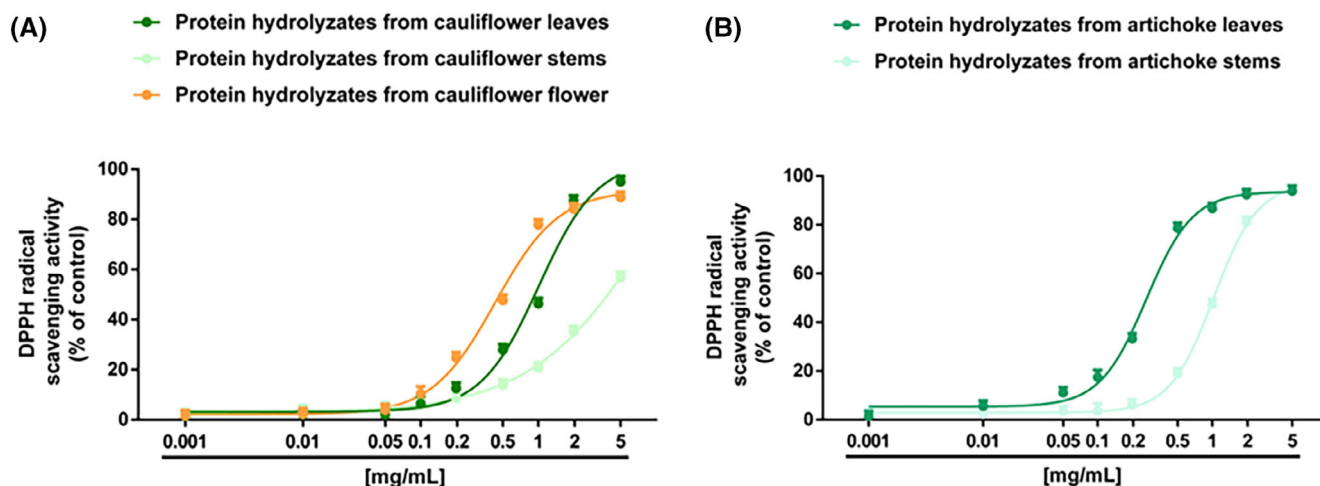


Figure 1. DPPH radical scavenging activity of cauliflower waste HYs and artichoke waste HYs. Data are displayed as mean $\pm$ SD ($n = 9$).

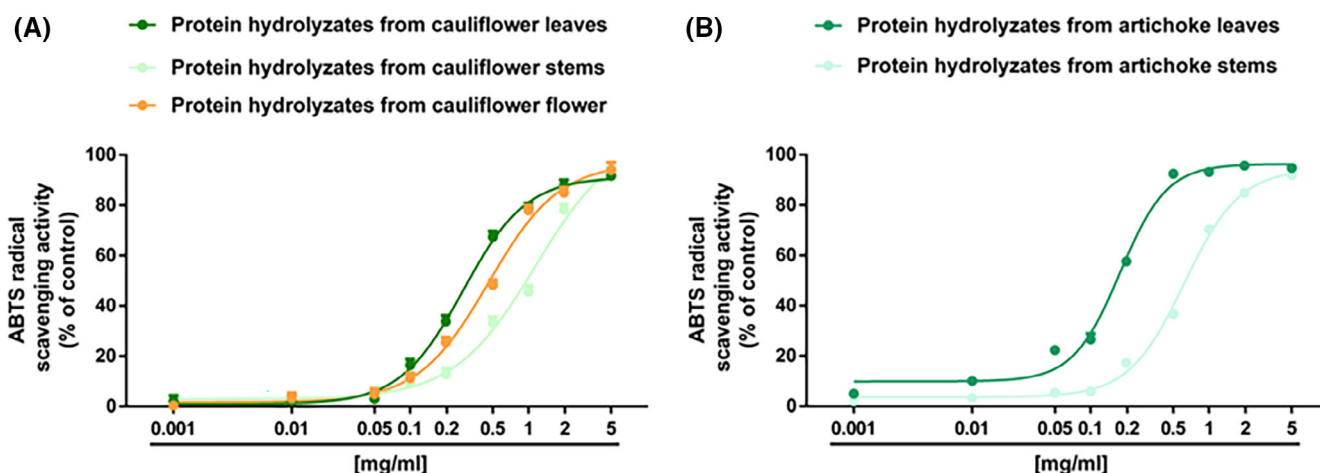


Figure 2. ABTS radical scavenging activity of cauliflower waste HYs and artichoke waste HYs. Data are displayed as mean $\pm$ SD ($n = 9$).

artichoke waste and for different classes of polyphenols and glucosinolates.³⁹⁻⁴³

Altogether the obtained results suggest an interest in the protein HYs from cauliflower and artichoke as sources of bioactive molecules useful in different application fields.

Biostimulants

Effects of biostimulants on seed germination and seedling growth

To assess the effects of cauliflower and artichoke biostimulants on seed germination and seedling growth, four different solutions (0.28, 0.84, 2.52 and 7.56 g L⁻¹) were tested on the seeds of four plant species: alfalfa, crimson clover, durum wheat and corn. The results were compared with those obtained for seeds germinated in distilled water or using a commercial biostimulant. The main parameters of seed germination and seedling growth for alfalfa, crimson clover, durum wheat and corn after the application of different biostimulant concentrations are presented in Tables 4–7, respectively.

Generally, the highest concentrated cauliflower biostimulant (7.56 g L⁻¹) caused a decrease of G_{tot}, with respect to control and commercial biostimulant, except in corn seeds where no statistical differences were detected (Table 7). However, at the

highest concentration, both cauliflower and artichoke biostimulants decreased G_{tot}, especially in alfalfa and durum wheat (Tables 4 and 6), whereas, with respect to control, corn seeds were negatively affected by the treatment with the artichoke biostimulant with the highest concentration (Table 7). On the contrary, the speed of germination, indicated by the CVG, was moderately affected by the treatments: many treatments showed CVG values statically similar to those of the control and the commercial biostimulant. However, a low concentration of artichoke biostimulant seems to exert a positive effect on the CVG in alfalfa, crimson clover and durum wheat, whereas a low concentration of cauliflower biostimulant tended to improve the germination speed in corn with respect to control. Similarly to germination parameters, the seedling growth characteristics were negatively affected by cauliflower biostimulant at the highest concentration. It was associated with the lowest values of root length, sprout length, total length and biomass of the seedlings in most of the plant species tested. With respect to control, the treatment with 7.56 g L⁻¹ cauliflower biostimulant significantly decreased the biomass (dry weight) by 30%, 79%, 54% and 48% and the seedling total length by 50%, 19%, 26% and 29% in alfalfa, crimson clover, durum wheat and corn, respectively. On the contrary, the treatment with the lowest concentration of artichoke biostimulant (0.28 g L⁻¹)

Table 4. Effect of cauliflower and artichoke biostimulants on alfalfa seed germination and seedling growth

Treatment	Biostimulant concentration (g L ⁻¹)	Gtot (%)	CVG	Seedling root length (cm)	Seedling sprout length (cm)	Seedling total length (cm)	Seedling biomass dry weight (mg)	SVI
CtrlW	0.00	86.7 ± 2.9 a	48.80 ± 2.08 ab	2.77 ± 0.18 a	1.60 ± 0.04 ab	4.38 ± 0.20 ab	1.59 ± 0.03 a	3.80 ± 0.29 ab
Comm.Biost	—	86.3 ± 1.9 a	45.63 ± 4.53 abc	2.98 ± 0.16 a	1.63 ± 0.02 a	4.61 ± 0.15 a	1.64 ± 0.05 a	3.99 ± 0.18 a
Artichoke	0.28	87.3 ± 1.2 a	56.42 ± 3.57 a	3.07 ± 0.05 a	1.74 ± 0.01 a	4.81 ± 0.06 a	1.59 ± 0.05 a	4.21 ± 0.11 a
Artichoke	0.84	82.7 ± 0.7 a	55.04 ± 4.01 a	2.51 ± 0.13 ab	1.57 ± 0.05 ab	4.08 ± 0.13 ab	1.55 ± 0.04 ab	3.37 ± 0.12 abc
Artichoke	2.52	82.0 ± 1.2 a	44.39 ± 7.32 abc	2.31 ± 0.50 ab	1.52 ± 0.02 ab	3.84 ± 0.52 abc	1.50 ± 0.05 ab	3.14 ± 0.38 abc
Artichoke	7.56	78.0 ± 1.2 a	44.90 ± 4.46 abc	1.25 ± 0.21 bc	1.48 ± 0.08 ab	2.73 ± 0.29 bc	1.40 ± 0.12 abc	2.13 ± 0.24 bc
Cauliflower	0.28	85.3 ± 1.3 a	42.07 ± 5.83 abc	2.50 ± 0.03 ab	1.51 ± 0.01 ab	4.01 ± 0.03 ab	1.42 ± 0.11 abc	3.42 ± 0.04 abc
Cauliflower	0.84	82.7 ± 1.3 a	31.30 ± 2.90 bc	2.24 ± 0.06 abc	1.45 ± 0.06 ab	3.69 ± 0.11 abc	1.31 ± 0.05 abc	3.05 ± 0.05 abc
Cauliflower	2.52	77.3 ± 3.5 a	26.78 ± 1.15 bc	1.97 ± 0.33 bc	1.37 ± 0.09 ab	3.34 ± 0.32 abc	1.22 ± 0.04 bc	2.56 ± 0.17 bc
Cauliflower	7.56	46.7 ± 8.2 b	22.78 ± 6.49 c	0.99 ± 0.63 c	1.22 ± 0.20 b	2.21 ± 0.82 c	1.12 ± 0.08 c	1.17 ± 0.60 d

In each column, mean values (± SD) followed by different letters indicate statistically significant ($P \leq 0.05$) differences (Tukey's test). Effects of control and commercial biostimulant are also reported.

Table 5. Effect of cauliflower and artichoke biostimulants on crimson clover seed germination and seedling growth

Treatment	Biostimulant concentration (g L ⁻¹)	Gtot (%)	CVG	Seedling root length (cm)	Seedling sprout length (cm)	Seedling total length (cm)	Seedling biomass dry weight (mg)	SVI
CtrlW	0.00	88.7 ± 1.8 a	26.92 ± 1.81 ab	1.13 ± 0.08 ab	1.87 ± 0.09 bc	3.01 ± 0.15 bc	1.72 ± 0.04 a	2.66 ± 0.09 bc
Comm.Biost	—	87.3 ± 1.3 a	24.65 ± 3.15 ab	1.35 ± 0.05 ab	2.24 ± 0.19 abc	3.59 ± 0.24 ab	1.75 ± 0.05 a	3.13 ± 0.16 ab
Artichoke	0.28	86.7 ± 0.9 a	29.97 ± 1.40 ab	1.60 ± 0.08 a	2.70 ± 0.23 a	4.28 ± 0.29 a	1.83 ± 0.09 a	3.71 ± 0.25 a
Artichoke	0.84	90.7 ± 4.4 a	30.82 ± 2.15 a	1.28 ± 0.16 ab	2.12 ± 0.08 abc	3.40 ± 0.24 abc	1.81 ± 0.06 a	3.08 ± 0.25 ab
Artichoke	2.52	86.3 ± 3.2 a	29.02 ± 1.09 ab	1.27 ± 0.26 ab	1.67 ± 0.21 bc	2.94 ± 0.47 bc	1.57 ± 0.06 ab	2.53 ± 0.38 bc
Artichoke	7.56	84.0 ± 1.2 ab	25.65 ± 0.65 ab	1.06 ± 0.06 ab	1.94 ± 0.02 bc	3.00 ± 0.07 bc	1.68 ± 0.02 ab	2.52 ± 0.05 bc
Cauliflower	0.28	89.0 ± 1.0 a	25.43 ± 0.48 ab	1.33 ± 0.02 ab	2.27 ± 0.13 ab	3.60 ± 0.14 ab	1.68 ± 0.03 ab	3.20 ± 0.16 ab
Cauliflower	0.84	86.3 ± 1.2 a	21.00 ± 2.47 b	1.42 ± 0.09 ab	1.99 ± 0.06 bc	3.40 ± 0.09 abc	1.64 ± 0.02 ab	2.94 ± 0.09 ab
Cauliflower	2.52	85.3 ± 0.7 ab	31.31 ± 2.35 a	1.24 ± 0.06 ab	1.79 ± 0.02 bc	3.03 ± 0.06 bc	1.40 ± 0.09 b	2.58 ± 0.04 bc
Cauliflower	7.56	76.7 ± 1.8 b	28.94 ± 1.85 ab	0.86 ± 0.05 b	1.58 ± 0.14 c	2.44 ± 0.10 c	0.36 ± 0.07 c	1.87 ± 0.11 c

In each column, mean values (± SD) followed by different letters indicate statistically significant ($P \leq 0.05$) differences (Tukey's test). Effects of control and commercial biostimulant are also reported.

Table 6. Effect of cauliflower and artichoke biostimulants on durum wheat seed germination and seedling growth

Treatment	Biostimulant concentration (g L ⁻¹)	Seed germination	CVG	Seedling root length (cm)	Seedling sprout length (cm)	Seedling total length (cm)	Seedling biomass dry weight (mg)	SVI
CtrlW	0.00	92.0 ± 2.0 ab	13.46 ± 1.41 ab	10.87 ± 1.40 ab	12.64 ± 0.56 a	23.51 ± 1.91 ab	25.76 ± 0.68 a	21.11 ± 1.42 ab
Comm.Biost	—	96.0 ± 2.3 a	15.59 ± 0.47 a	11.53 ± 0.25 ab	13.34 ± 0.50 a	24.87 ± 0.35 a	26.85 ± 1.24 a	23.88 ± 0.68 a
Artichoke	0.28	92.7 ± 1.8 ab	15.70 ± 1.03 a	11.87 ± 0.58 a	11.21 ± 0.23 a	23.08 ± 0.78 ab	27.30 ± 0.21 a	21.42 ± 1.13 ab
Artichoke	0.84	82.7 ± 0.7 bc	12.56 ± 1.20 ab	9.27 ± 0.58 ab	12.68 ± 0.75 a	21.96 ± 1.26 ab	23.53 ± 1.77 ab	18.16 ± 1.13 b
Artichoke	2.52	80.0 ± 4.6 cd	11.52 ± 1.98 ab	9.07 ± 0.50 ab	11.70 ± 0.65 a	20.77 ± 0.50 ab	24.14 ± 2.51 ab	16.63 ± 1.20 bc
Artichoke	7.56	76.0 ± 2.3 d	10.62 ± 0.85 bc	9.36 ± 0.73 ab	12.17 ± 0.58 a	21.53 ± 1.28 ab	24.11 ± 1.74 ab	16.32 ± 0.76 bc
Cauliflower	0.28	90.0 ± 2.0 ab	15.66 ± 0.75 a	10.87 ± 1.38 ab	12.36 ± 0.48 a	23.23 ± 1.10 ab	23.73 ± 1.14 ab	20.94 ± 1.39 ab
Cauliflower	0.84	74.3 ± 1.2 d	9.02 ± 0.97 bc	10.13 ± 0.28	12.06 ± 0.58 a	22.19 ± 0.68 ab	20.87 ± 0.82 ab	16.49 ± 0.32 bc
Cauliflower	2.52	58.0 ± 3.5 e	8.15 ± 0.94 bc	8.57 ± 0.33 ab	10.58 ± 1.52 a	19.16 ± 1.19 ab	17.37 ± 1.06 bc	11.15 ± 1.11 cd
Cauliflower	7.56	60.0 ± 2.0 e	5.92 ± 0.64 c	7.65 ± 0.72 b	9.81 ± 1.71 a	17.46 ± 2.16 b	11.93 ± 1.50 c	10.56 ± 1.60 d

In each column, mean values (± SD) followed by different letters indicate statistically significant ($P \leq 0.05$) differences (Tukey's test). Effects of control and commercial biostimulant are also reported.

tended to positively affect the development of seedlings, providing an increase with respect to control and to commercial biostimulant for root and sprout length in alfalfa and crimson clover,

and for dry biomass in durum wheat and crimson clover even though the values did not differ significantly from each other. Instead, the solution of cauliflower biostimulant with the lowest

Table 7. Effect of cauliflower and artichoke biostimulants on corn seed germination and seedling growth

Treatment	Biostimulant concentration (g L ⁻¹)	Gtot (%)	CVG	Seedling root length (cm)	Seedling sprout length (cm)	Seedling total length (cm)	Seedling biomass dry weight (mg)	SVI
CtrlW	0.00	93.3 ± 3.5 a	8.97 ± 0.74 ab	15.90 ± 1.21 ab	12.97 ± 0.65 ab	28.87 ± 1.79 abc	67.52 ± 0.61 abc	26.92 ± 1.80 b
Comm.Biost	—	81.3 ± 1.3 bc	5.90 ± 0.33 b	16.47 ± 1.07 ab	14.20 ± 0.10 a	30.67 ± 1.02 ab	73.42 ± 1.15 a	24.97 ± 1.25 b
Artichoke	0.28	94.7 ± 1.3 a	8.37 ± 0.53 ab	15.20 ± 0.65 ab	12.67 ± 0.35 ab	27.87 ± 0.98 abc	59.09 ± 3.70 bcd	26.36 ± 0.63 b
Artichoke	0.84	90.7 ± 1.3 ab	8.31 ± 0.33 ab	12.00 ± 1.22 b	9.60 ± 0.40 b	21.60 ± 1.48 bc	45.97 ± 3.53 cd	19.54 ± 1.05 c
Artichoke	2.52	89.3 ± 1.3 abc	8.66 ± 0.87 ab	11.30 ± 2.15 b	9.26 ± 1.31 b	20.57 ± 3.43 c	45.75 ± 6.75 cd	18.34 ± 2.94 c
Artichoke	7.56	79.3 ± 1.2 c	10.65 ± 0.34 a	11.55 ± 0.63 b	9.43 ± 0.49 b	20.97 ± 1.11 c	44.26 ± 1.68 cd	16.67 ± 0.12 c
Cauliflower	0.28	93.3 ± 2.7 a	9.91 ± 0.85 a	20.47 ± 2.35 a	13.97 ± 0.77 a	34.43 ± 3.09 a	71.26 ± 5.47 ab	32.17 ± 3.26 a
Cauliflower	0.84	93.3 ± 2.7 a	11.42 ± 0.98 a	17.73 ± 0.49 ab	12.53 ± 0.78 ab	30.26 ± 0.89 ab	66.14 ± 2.30 abc	28.23 ± 0.96 ab
Cauliflower	2.52	89.3 ± 3.5 abc	9.50 ± 1.14 ab	12.47 ± 1.79 ab	10.10 ± 1.17 b	22.57 ± 2.90 bc	50.10 ± 9.17 bcd	20.32 ± 3.20 c
Cauliflower	7.56	90.3 ± 1.5 abc	7.52 ± 1.17 ab	11.15 ± 0.71 b	9.44 ± 0.80 b	20.59 ± 1.17 bc	35.32 ± 1.97 e	18.62 ± 1.27 c

In each column, mean values (± SD) followed by different letters indicate statistically significant ($P \leq 0.05$) differences (Tukey's test). Effects of control and commercial biostimulant are also reported.

concentration (0.28 g L⁻¹) seemed to positively affect the root length, sprout length and biomass in corn. The SVI, which combines the percentage of germination and the seedling total length, provided an indication of the overall effect of the biostimulant on the germination phase. This indicator confirmed that the cauliflower biostimulant with the highest concentration (7.56 g L⁻¹) negatively affected the seed germination in all plant species. With respect to control, it significantly decreased SVI by 69%, 50% and 31% for alfalfa, durum wheat and corn, respectively, whereas in the case of crimson clover, a 30% SVI reduction was not statistically significant (Table 5). On the contrary, the treatment with the most diluted solution of cauliflower biostimulant (0.28 g L⁻¹) positively affected the SVI in corn providing an increase with respect to control and biostimulant by 20% and 29% (Table 7). Instead, the lowest concentration of artichoke biostimulant (0.28 g L⁻¹) tended to positively affect the SVI in crimson clover, providing an increase compared to control by 39% and to commercial biostimulant by 19%, although significant differences were observed only in the case of control (Table 5).

From an agronomic point of view, these two biostimulants seem to have a positive effect on seed germination and seedling growth only at low concentrations, showing an inhibitory effect at higher ones. This detrimental effect can be due to a large amount of phenolic compounds present in cauliflower and artichoke waste; in fact indeed inhibitory effects of high concentrations of various protein HYs on plant growth are already reported in the literature^{44,45} and have been observed also by other authors. Muscolo and Sidari⁴⁶ have reported that phenolic compounds are potential inhibitors of nitrogen uptake and their toxicity may have interfered with the biostimulating effect in the presence of high concentrations. It is noteworthy that the commercial biostimulant did not show any significant effect, although several authors have reported that extract of *Ascophyllum nodosum* can have a positive effect increasing the germination and seed vigor of annual crops (herbaceous) such as barley, tomato, pepper and eggplant.^{47,48} According to Ali et al.⁴⁹ the positive effects of the seaweed products are dependent on the type of the seaweed resource, quality and composition of the extract, as well as method, concentration and frequency of application. Therefore, this study confirms that no biostimulant has a generalizable effect which is clearly species-specific and product-specific; what we know about one biostimulant or about one plant does not directly transfer to another plant species. Moreover, since

seaweed extracts are complex composts, made from natural raw materials, they cannot have a standardized composition and this could lead to different effects over time. Nevertheless, it has been shown that higher dosage of amino acids, humic acid or salicylic acid could have adverse effects on many leafy vegetable crops,^{3,6,50,51} thus confirming that plant sensitivity to biostimulants is variable on a case-to-case basis.

Life cycle impact assessment

A sustainability assessment of cauliflower and artichoke production allocated to industrial waste was carried out through the application of LCA, using the ReCiPe Midpoint 2016 method. Results for all impact categories considered are detailed in the supporting information (Tables S4 and S5; Figs S1 and S2) showing the main impact categories involved in the crop life cycle; in the case of cauliflower, water used for irrigation and fuel consumption turned out to have a relevant impact on the production process whereas in the case of artichoke production, polyester mulching film and diesel burned by agricultural machinery as well the use of synthetic fertilizers were found to be the most impacting inputs.

For both crop cycles, it is noteworthy that industrial waste is responsible for an average of 57% of total greenhouse gas emissions for cauliflower production and 36% for artichoke production. In these regards, the possibility of recycling these industrial wastes was considered for a scenario analysis, thus evaluating possible mitigating strategies during the crop life cycle.

Two recycling scenarios of crop industry, namely biostimulant formulation and composting substrates, Figs 3 and 4, were investigated. In three of the five most affected categories, biostimulant formulation from waste is of advantage, mitigating the impacts associated with global warming (−143 029 kg CO₂ eq), fossil resource scarcity (−1.43 × 10⁸ kg oil eq) and ozone formation, human health (−2.30 × 10² kg NO_x eq). Nevertheless, even though the formulation of biostimulants particularly affects the impact categories TA and MRS (7.72 × 10³ kg SO₂ eq and 7.24 × 10⁵ kg Cu eq, respectively), it is still an advantageous recycling scenario for agro-industrial artichoke and cauliflower waste compared with the composting scenario, which negatively impacted three of five categories. In particular, the TA and MRS impact could be related to the production of electricity and the related combustion of fossil fuels required for the formulation of biostimulants on a laboratory scale. In these regards, it is worth

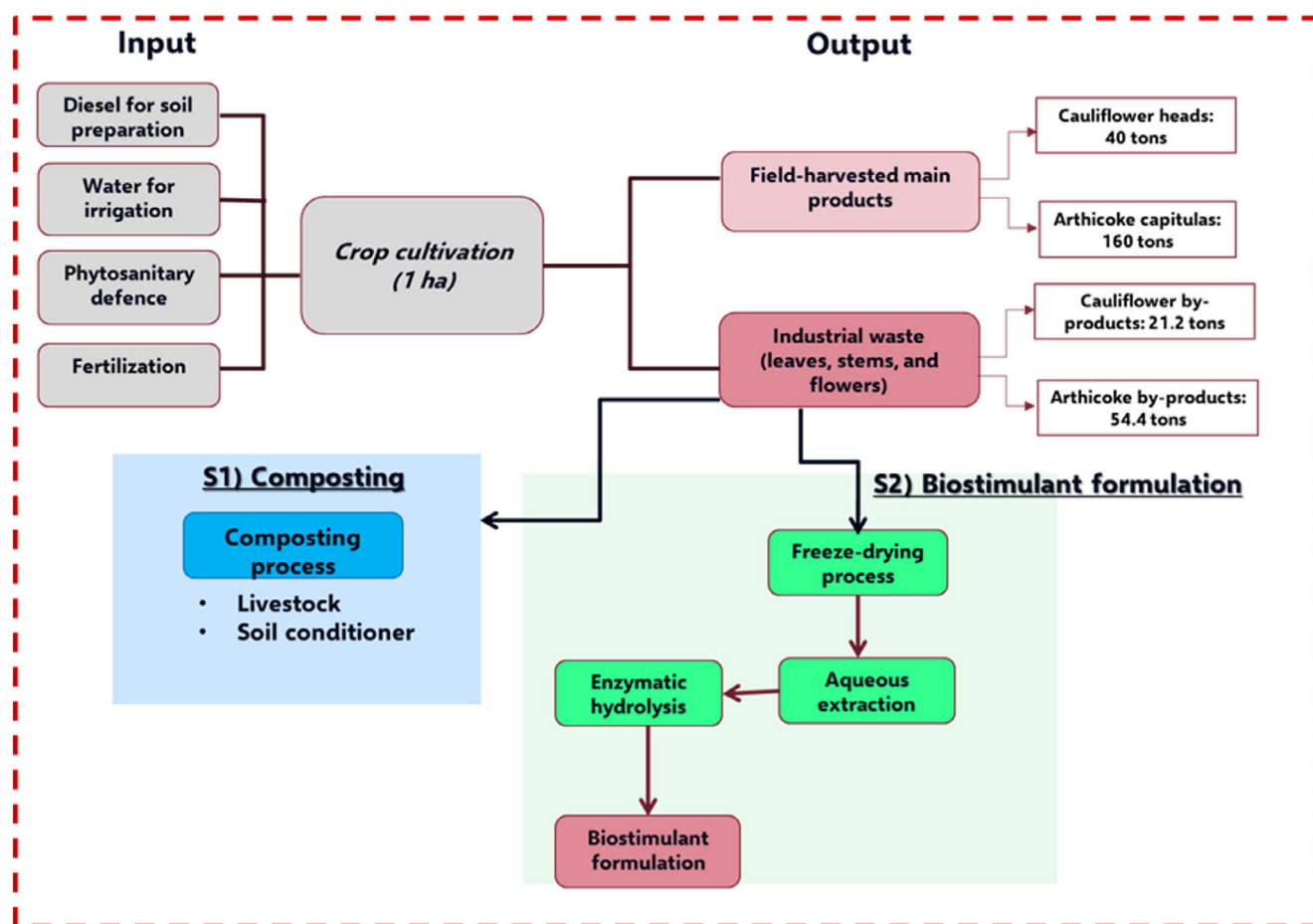


Figure 3. System boundaries of the analyzed crop cycles considering (S1) crop byproducts as composting substrates and (S2) crop byproducts for biostimulant formulation.

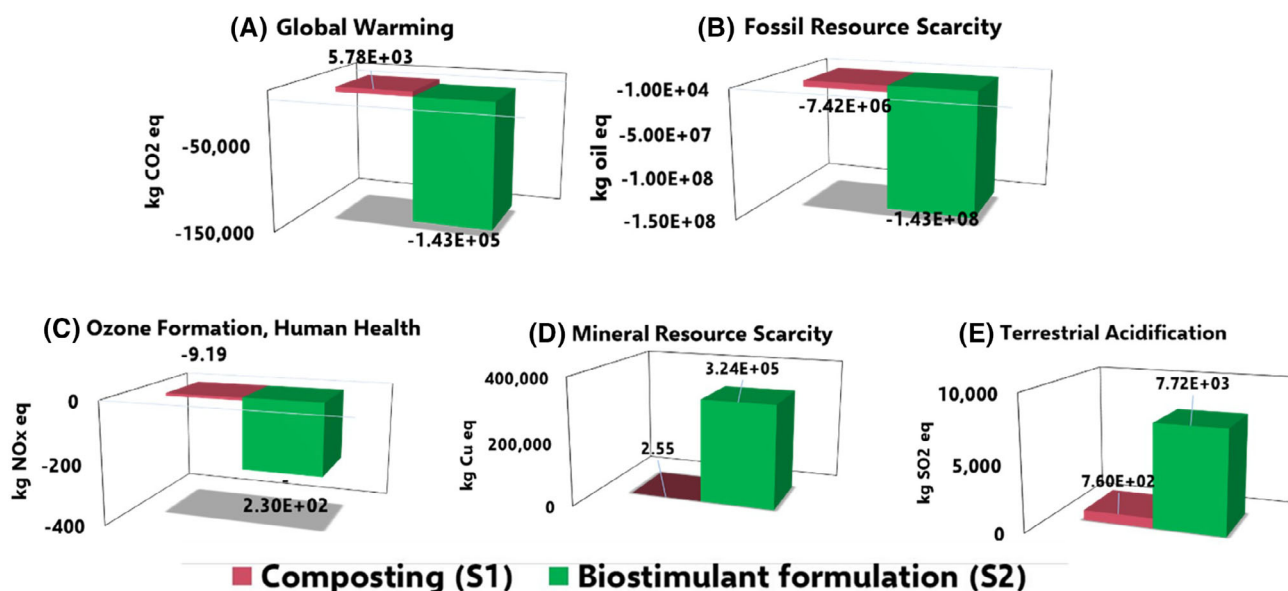


Figure 4. Recycling scenarios for industrial waste: composting (S1) and biostimulant formulation (S2). Global warming (A), fossil resource scarcity (B), ozone formation, human health (C), mineral resource scarcity (D) and terrestrial acidification (E) impact categories.

highlighting the opportunity of valorizing agricultural waste, through its conversion into biostimulants, thus representing a mitigating strategy for the whole product life cycle as well as it being able to provide nutrient-rich compost for soil health, cost savings and sustainable agriculture.⁵²

CONCLUSIONS

The obtained results highlighted the presence of several compounds involved in plant development in the protein HYs from leaves, flowers and stems of cauliflower (*Brassica oleracea* L.) and from leaves and stems of artichoke (*Cynara scolymus* L.). These properties led to a consideration of cauliflower and artichoke byproducts and their HYs as eligible raw matrices for several applications, not only as plant biostimulants but also as potential matrices to isolate and concentrate bioactive compounds. Their use as biostimulants appears to have a positive impact on seed germination and seedling growth only at low concentrations, whereas at higher concentrations, they exhibit an inhibitory effect. This detrimental effect can be due to large amounts of compounds present in cauliflower and artichoke waste, mainly amino acids. However, this study provided evidence that the effect of plant-based biostimulants on the seed germination process is species specific, and therefore further studies are necessary to explain the mechanism of action involved in seed germination and seedling growth.

Finally, LCA results show the critical points of the two life cycles giving the inspiration to find applicable and concrete solutions, since biostimulant formulations from wastes are advantageous to mitigate the impacts associated with global warming, fossil resource scarcity, ozone formation and human health.

ACKNOWLEDGMENT

This research was funded by 'Mediterranean Diet for Human Health Laboratory' project (MeDiHealthLab) – No. T5-AN-07 (POS 5), CUP: B83C22005580006. Open access publishing facilitated by Università degli Studi di Roma La Sapienza, as part of the Wiley – CRUI-CARE agreement.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

CONFLICT OF INTEREST

The authors declare no conflict of interest for the article.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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Supplementary Information

Life Cycle Assessment materials and methods

Regarding the goal and scope definition phase, the study aimed to assess the environmental impacts of cauliflower and artichoke production considering industrial waste, and possible recycling scenarios thus identifying possible hotspots in the life cycle. In these regards a *cradle-to-farmgate* approach was performed, thus considering the entire crop cycle, from soil preparation for seeding, fertilization, and pest management to in-field harvesting of products. Two functional unit (FU) were selected:

- For agricultural production: 1 hectare (ha) of cultivated land for one complete crop cycle, which for cauliflower lasts between 60 and 240 days; while for artichokes, it covers 4 years. For both crops, the first year of the crop cycle was taken as a reference. Specifically, primary data for the upstream cauliflower and artichoke crop cycle are detailed in Table S1 and Table S2. These data consist of site-specific data collected through the administration of questionnaires and face-to-face interviews with the farmers and employees in the farming company, located in Lazio, supported by technical data sheets of various machinery and treatments used during the crop cycle. The primary data were subsequently combined with secondary data referred to the background production flows (i.e., fertilizers, diesel oil burned by agricultural machines, etc.) were obtained from databases Agribalyse v3.0.1, Ecoinvent v3.8, and World Food LCA Database (WFLDB), provided by SimaPro 9.5.0.2. software. Direct emissions from field operations, such as emissions from fossil fuel consumption by agricultural machinery, were estimated based on the characterization factors proposed by “Ente Nazionale per la Meccanizzazione Agricola”.
- For End-of-Life Scenario (EoL) Analysis, consisting of the first scenario (S1), where the industrial waste is allocated for composting and used for livestock and soil conditioner applications; and second scenario (S2), where the cauliflower and artichoke waste are recycled for the biostimulant formulation in the laboratory settings. For this EoL analysis, the

identified FU was 4 g of industrial waste (considering leaves and stems of both cauliflower and artichoke crops), as representing the minimum quantity required for 1 L of biostimulant formulation in the laboratory settings. In these regards, inputs encompassed the freeze-drying process, aqueous extraction, and enzymatic hydrolysis conducted on cauliflower and artichoke industrial waste, following laboratory operating conditions outlined in Table S3.

- For LCIA, the ReCiPe 2016 Midpoint method was used thus considering 18 different impact categories, grouped into four macro areas: *i.* Atmospheric Effects: i.e., Global Warming (GW); Ionizing radiation (IR); Ozone Formation, Human Health (OFHH); Fine Particulate Matter Formation (FPMP); *ii.* Eutrophication: Freshwater Eutrophication Potential (FEP) and Marine Eutrophication Potential (MEP), *iii.* Toxicity: Freshwater Ecotoxicity (FEC); Marine Ecotoxicity (MEC); Human Non-Carcinogenic Toxicity (HNCT), *iv.* Abiotic Resources: Land Use (LU); Mineral Resources Scarcity (MRS); Fossil Resources Scarcity (FRS), Water Consumption (WC).
- The LCIA results of the biostimulant formulation were normalized and weighed by assuming an average of waste used (both artichoke and cauliflower) for the recycling of 21.2 tons.
- The way multifunctionality is addressed affects significantly the results of LCA studies related to the agri-food sector, and it is strictly related to allocation procedures (Dominguez Aldama et al., 2023). For both crop cycles, it is worth-mentioning that industrial waste obtained after processing and before distribution and marketing, are considered. Depending on the morphology and specific plant characteristics, for artichokes, non-edible industrial waste (including stem and leaves) is 34% to 45%; for cauliflowers (Khedkar MA, Nimbalkar PR, Chavan PV, Chendake YJ, Bankar SB. Cauliflower waste utilization for sustainable biobutanol production: revelation of drying kinetics and bioprocess development. *Bioprocess Biosyst Eng.* 2017, 40(10):1493-1506. doi: 10.1007/s00449-017-1806-y) industrial waste (mainly green leaves, flowers and stems) is between 48% and 58% (Cavini, S.; Guzzetti, L.;

Givoia, F.; Regonesi, M.E.; Di Gennaro, P.; Magoni, C.; Campone, L.; Labra, M.; Bruni, I. Artichoke (*Cynara Cardunculus Var. Scolymus* L.) by-Products as a Source of Inulin: How to Valorise an Agricultural Supply Chain Extracting an Added-Value Compound. *Nat. Prod. Res.* 2020). In these regards, to evaluate sustainability performance of crop by-products, a mass allocation procedure was applied to the cauliflower and artichoke system production. According to the data obtained in the LCI, mass allocation factors were calculated based on the mass rates of the main product, which for cauliflower heads is 0.45, and industrial waste (0.55), while for artichoke capitula corresponds to 0.66, and industrial waste (0.34) to allocate the potential impacts relative to the two production systems.

Table S1. LCI data for cauliflower crop cycle

INPUT	n. Intervention	Unità (Kg/ha)	Data Source
Soil preparation			
Tillage (diesel oil)	1	132.65	Ecoinvent v3.8
Ripping (diesel oil)	1	79.91	
Harrowing (diesel oil)	2	52.74	
Transplantation (diesel oil)	1	60.44	
Fertilisation			
Ammonium nitrate (26% N)	1	156	Agribalyse v3.0.1
N-P-K Organo-mineral fertilizer	1	600	
Manure from cattle	1	12,500	WFLDB
Diesel oil	3	50.76	Ecoinvent v3.8
Phytosanitary defense			
Herbicides (Devrinol, Rapsan)	1	2.7	Ecoinvent v3.8
Fungicide (Agrizole)	1	0.5	
Insecticide (Altacor, Decis Evo)	1	1.2	
Diesel oil	3	39.87	
Irrigation			
Tap water	6	1,800,000	Ecoinvent v3.8
In-field harvesting			
Diesel oil	1	241.78	Ecoinvent v3.8
OUTPUT			
Field-harvested cauliflowers		40,000	
Industrial waste (leaves, stems and flowers)		21,200	

Atmospherical Emissions			
CO ₂	Kg CO ₂ eq	2.18 x 10 ³	IPCC, 2006
CH ₄		277.92	
N ₂ O		353	
SF ₆		1.27	
Emission to water (freshwater)			
Phosphate	Kg P eq	5.77 x 10 ⁻²	IPCC, 2006
Phosphorus		3.81 x 10 ⁻²	
Emission to water (marine)			
Ammonia	Kg N eq	1.74 x 10 ⁻⁴	IPCC, 2006
Ammonium, ion		1.65 x 10 ⁻²	
Nitrate		3.79 x 10 ⁻²	
Nitrite		1.25 x 10 ⁻⁵	
Nitrogen		1.23 x 10 ⁻²	

Table S2. LCI for the artichoke crop cycle

INPUT	n. Intervention	Unità (Kg/ha)	Data Source
Soil preparation			
Molding (diesel oil)	1	71.81	Ecoinvent v3.8
Tillage (diesel oil)	2	66.80	
Transplantation (manual)	-	-	
Mulching film (polyester)	1	1,000	
Fertilisation			
Ca(NO ₃) ₂ fertilizer	1	10	Agribalyse v3.0.1
Ca(NO ₃) ₂ +16% N fertilizer	10	15	
(NH ₄) ₂ SO ₄ fertilizer	1	20	
Manure from cattle	1	40,000	WFLDB
Solustar (30% P ₂ O ₅ + K ₂ O 5%)	10	61.6	Agribalyse
Azofol (16% N)	2	4.67	
Diesel oil	25	417.5	Ecoinvent v3.8
Phytosanitary defense			
Insecticide (Metarex, a.p. <i>metaldehyde</i>)	2	5	Ecoinvent v3.8
Insecticide (Sluxx, a.p. <i>ferric phosphate</i>)	2	7	
Diesel oil	5	100.2	
Irrigation			
Tap water	21	1,470,000	Ecoinvent v3.8
Diesel oil	21	9,644.25	
In-field harvesting			
Diesel oil	1	50.1	Ecoinvent v3.8
OUTPUT			
Field-harvested artichokes (capitula)		160,000	
Industrial waste (leaves and stems)		54,400	
Atmospherical Emissions			
CO ₂	Kg CO ₂ eq	132	
CH ₄		27.3	
N ₂ O		298	

SF ₆		4.14	IPCC, 2006
Emission to water (freshwater)			
Phosphate	Kg P eq	0.103	
Phosphorus		7.15 x 10 ⁻³	IPCC, 2006
Emission to water (marine)			
Ammonia		6.93 x 10 ⁻⁴	
Ammonium, ion		1.18 x 10 ⁻²	
Nitrate	Kg N eq	3.59 x 10 ⁻²	IPCC, 2006
Nitrite		1.72 x 10 ⁻⁴	
Nitrogen		1.29 x 10 ⁻³	

Table S3. LCI of recycling scenario for biostimulant formulation.

Inputs			
Biostimulant formulation			
Crop by-products		4 g	
Phase 1 (Freeze-drying process)			
Electricity		23.28 kWh	
Phase 2 (Aqueous extraction)			
Ultrapure Water		0.1 g	EcoInvent v3.8
Electricity		0.12 kWh	
Phase 3 (Enzymatic hydrolysis)			
Protease <i>Aspergillus oryzae</i>		9,352 µL	
Electricity		94.1 kWh	EcoInvent v3.8
Outputs			
Biostimulant		1 L	

Table S4. Life cycle impact assessment midpoint results for cauliflower production and related industrial waste.

Impact category	Unit	Cauliflower production	Industrial waste
Global warming	Kg CO ₂ eq	1,225.59	649.57
Stratospheric ozone depletion	Kg CFC ₁₁ eq	0.02	0.01
Ionizing radiation	kBq Co-60 eq	505.51	267.92
Ozone formation, Human health	Kg NO _x eq	3.01	1.59
Fine particulate matter formation	Kg PM _{2.5} eq	3.93	2.08
Ozone formation, Terrestrial ecosystems	Kg NO _x eq	3.01	1.60
Terrestrial acidification	Kg SO ₂ eq	10.36	5.49
Freshwater eutrophication	Kg P eq	200.45	100.24
Marine eutrophication	Kg N eq	0.09	0.05
Terrestrial ecotoxicity	Kg 1,4-DCB	657.71	348.59
Freshwater ecotoxicity	Kg 1,4-DCB	2.10	1.11
Marine ecotoxicity	Kg 1,4-DCB	10,201.99	5,407.05
Human carcinogenic toxicity	Kg 1,4-DCB	31.22	16.55
Human non-carcinogenic toxicity	Kg 1,4-DCB	2,206.92	1,169.67
Land use	m ² a crop eq	449.96	238.48
Mineral resource scarcity	Kg Cu eq	132.65	70.30
Fossil resource scarcity	Kg oil eq	1,015.57	538.25
Water consumption	m ³	1,826.06	967.81

Table S5. Life cycle impact assessment midpoint results for artichoke production and related industrial waste

Impact category	Unit	Artichoke production	Industrial waste
Global warming	Kg CO ₂ eq	3,232.98	1,099.21
Stratospheric ozone depletion	Kg CFC ₁₁ eq	0.02	0.01
Ionizing radiation	kBq Co-60 eq	739.74	251.51
Ozone formation, Human health	Kg NO _x eq	8.12	2.76
Fine particulate matter formation	Kg PM _{2.5} eq	9.60	3.26
Ozone formation, Terrestrial ecosystems	Kg NO _x eq	8.14	2.77
Terrestrial acidification	Kg SO ₂ eq	22.02	7.49
Freshwater eutrophication	Kg P eq	311.05	167.36
Marine eutrophication	Kg N eq	0.11	0.04
Terrestrial ecotoxicity	Kg 1,4-DCB	833.68	283.45
Freshwater ecotoxicity	Kg 1,4-DCB	4.26	1.45
Marine ecotoxicity	Kg 1,4-DCB	21,997.84	7,479.27
Human carcinogenic toxicity	Kg 1,4-DCB	0.67	0.23
Human non-carcinogenic toxicity	Kg 1,4-DCB	1,577.02	536.19
Land use	m ² a crop eq	692.18	235.34
Mineral resource scarcity	Kg Cu eq	21.14	7.19
Fossil resource scarcity	Kg oil eq	2,794.79	950.23
Water consumption	m ³	1,511.20	513.81

Table S6. Major and trace essential elements and heavy metals present in hydrolysates from artichoke and cauliflower hydrolysates detected by ICP-MS. The levels of major and trace essential elements are expressed as mg/Kg DW±SD.

	Hydrolysate artichoke leaves	Hydrolysate artichoke stems	Hydrolysate cauliflower leaves	Hydrolysate cauliflower stems	Hydrolysate cauliflower flowers
Ca (mg/Kg)	1756.54±36.78	1900.71±44.33	875.85±21.93	9475±51.82	5240±47.92
P (mg/Kg)	404.496±12.02	370.256±15.82	409.459±20.71	252.742±7.09	576.16±23.45
Mg (mg/Kg)	3076.26±38.51	1793.17±29.85	1205.3±34.90	1004.39±31.02	2040.43±34.71
Na (mg/Kg)	633.243±11.00	514.22±7.44	401.9±17.69	436.631±7.23	800.17±31.09
K (mg/Kg)	2274.36±38.75	1801.11±40.66	2137.67±45.82	2070.35±44.83	3339.31±38.96
Zn (mg/Kg)	6.9±1.21	0.532±0.05	2.514±0.72	4.91±0.51	5.50±1.4
Fe (mg/Kg)	501.05±14.82	365.53±10.78	327±10.81	633±13.28	408±16.83
Cu (mg/Kg)	0.66±0.03	0.158±0.08	0.514±0.1	0.325±0.04	0.83±0.3
Ti (mg/Kg)	n/d	0,004±0,002	n/d	n/d	n/d
Li (mg/Kg)	n/d	n/d	n/d	n/d	n/d

Be (mg/Kg)	n/d	n/d	n/d	n/d	n/d
B (mg/Kg)	1243.53±23.94	1044±27.00	344±18.00	416±5.61	586±11.50
Al (mg/Kg)	4.62±0.93	2.37±0.97	6.22±0.88	4.22±1.11	7.82±3.81
Ti (mg/Kg)	0.03±0.006	0.021±0.003	0.07±0.008	0.052±0.002	n/d
Mn (mg/Kg)	3669.01±43.05	791.87±4.61	2221±37.40	1002±22.45	801±5.71
Co (mg/Kg)	n/d	n/d	n/d	n/d	n/d
Se (mg/Kg)	n/d	0.002±0.001	n/d	n/d	n/d
Sr (mg/Kg)	n/d	n/d	n/d	n/d	n/d
Mo (mg/Kg)	0.259±0.02	0.084±0.003	0.092±0.007	0.175±0.07	0.42±0.03
Ag (mg/Kg)	n/d	n/d	n/d	n/d	n/d
Sb (mg/Kg)	n/d	n/d	n/d	n/d	n/d
Ba (mg/Kg)	0.024±0.006	0.012±0.001	0.02±0.001	0.022±0.001	0.07±0.05
Bi (mg/Kg)	n/d	n/d	n/d	n/d	n/d
V (mg/Kg)	0.057±0.003	0.018±0.001	0.093±0.02	0.012±0.004	0.12±0.03
Ni (mg/Kg)	0.041±0.007	n/d	0.027±0.005	n/d	0,08±0.004
Cd (mg/Kg)	n/d	n/d	n/d	n/d	n/d
Cr (mg/Kg)	0.036±0.04	n/d	n/d	n/d	n/d
As (mg/Kg)	n/d	n/d	n/d	n/d	n/d
Pb (mg/Kg)	0.049±0.001	0.005±0.001	0.034±0.002	0.009±0.02	0.020±0.02
Hg (mg/Kg)	n/d	n/d	n/d	n/d	n/d

n/d = not detected

Table S7. IC₅₀ values of hydrolysates from cauliflower (*Brassica oleracea* L.) and artichoke (*Cynara scolymus* L.) leaves and stems in DPPH and ABTS radical scavenger assays.

HY	DPPH scavenger activity	ABTS scavenger activity
IC ₅₀ (Confidence limits) [µg/mL]		
Cauliflower leaves	1011.0 (678.4-1485.6)	279.8 (238.3-328.6)
Cauliflower stem	-	1147.0 (679.9-1935.0)
Cauliflower flower	430.6 (338.5-547.6)	460.7 (359.6-590.1)

Artichoke leaves	266.2 (218.0-325.2)	177.3 (137.7-228.1)
Artichoke stem	1029.0 (966.7-1095.8)	619.0 (517.0-741.8)
Trolox	9.0 (2.5-14.3)	2.0 (0.3-7.2)

-, not determined.

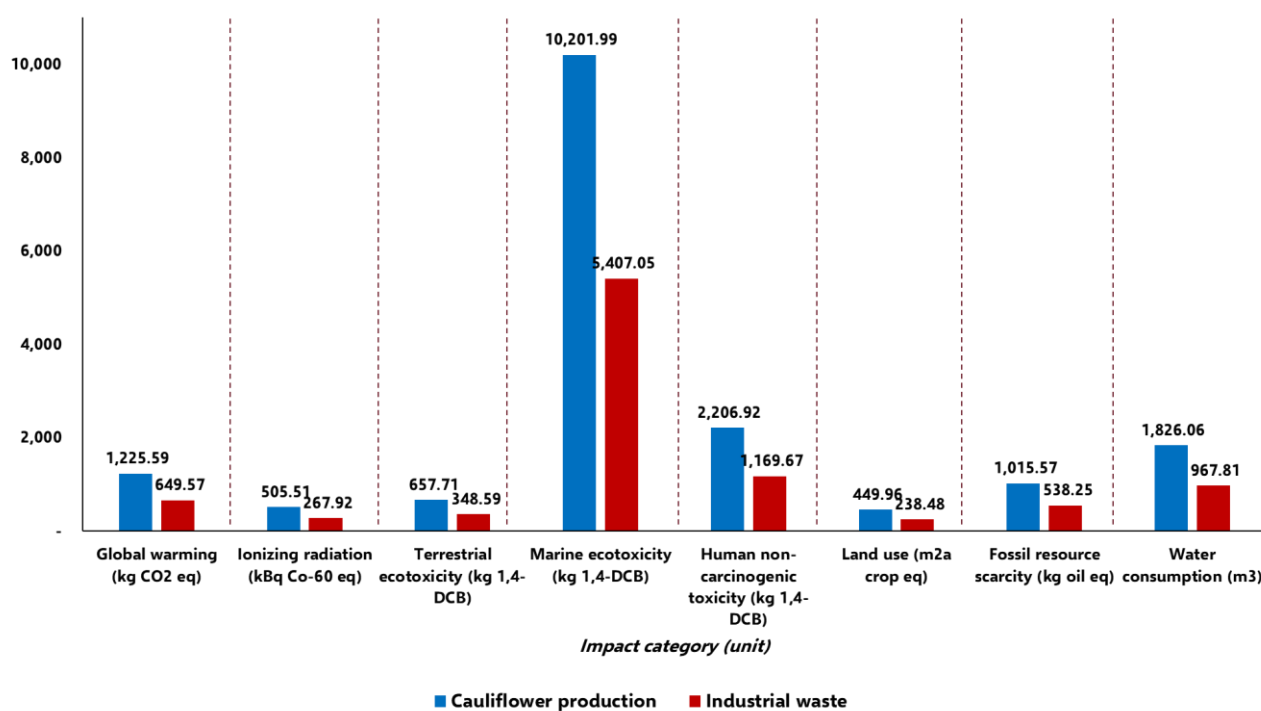


Figure S1. LCIA midpoint results for cauliflower production and industrial waste.

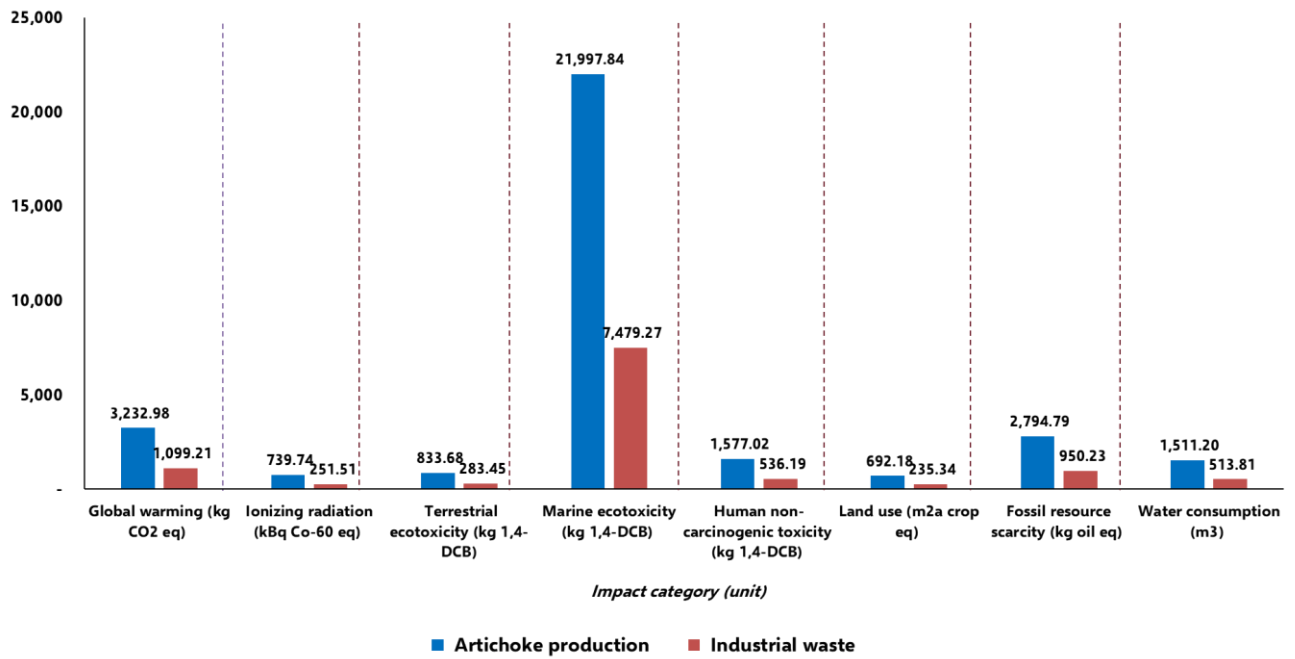


Figure S2. LCIA midpoint results for artichokes production and industrial waste.

PART B

Summary Part B

Cultural heritage (CH) monuments in outdoor environments are highly susceptible to degradation by microorganisms that can cause aesthetic and physicochemical damage to stone surfaces. Lichens, among the most common biodeterioration agents, thrive on a wide range of substrates in different climates. They consist of a microbial community composed mainly of a mycobiont (a fungus) and a photobiont (green algae or cyanobacteria). This study focuses on the Italian archaeological site of Motya Island (Sicily, Italy). This island is vulnerable to climate change, with projections indicating significant sea level rise by 2100, which could lead to flooding of key archaeological areas. The study aimed to simulate the potential impact of future sea level rise on Motya's archaeological ruins. Preliminary metabolomic analyses using nuclear magnetic resonance (NMR) provide insights into the role of lichens in biodeterioration and highlight broader environmental threats to the preservation of archaeological remains. Indeed, it was possible to determine the quantification of metabolites present on lichen rocks sampled directly from Motya Island.

These were experimentally immersed in sterile water (L1), artificial salt water (L2), and Stagnone water (L3) taken directly near the island and monitored at seven (t_1), thirty (t_2), and sixty days (t_3). The results showed a significant reduction in amino acids, sugars, organic acids, and other metabolites from time t_0 to t_3 .

Chapter B1

Lichen communities

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B1.1 Lichens: complex ecosystems

B1.1.1 Origin and classification of lichens

Lichens are complex organisms formed by the symbiosis between a fungus (mycobiont) and an alga or cyanobacterium (photobiont). In the past, they were misclassified as mosses or algae because of their appearance (Lakshnarayan Kumar Bhagarathi et al. 2022). The mycobiont is the dominant partner, as it provides structure and nutrition to the lichen, while the photobiont performs photosynthesis, generating nutrients that feed the fungus. This mutual exchange allows both partners to thrive. They are classified primarily in the kingdom Fungi and can be found in various fungal groups, including *Ascomycetes* and *Basidiomycetes*.

Their anatomy is complex and adapted to their ecological role (Supriati and Helmiyetti 2021), see **Figure B1.1**.

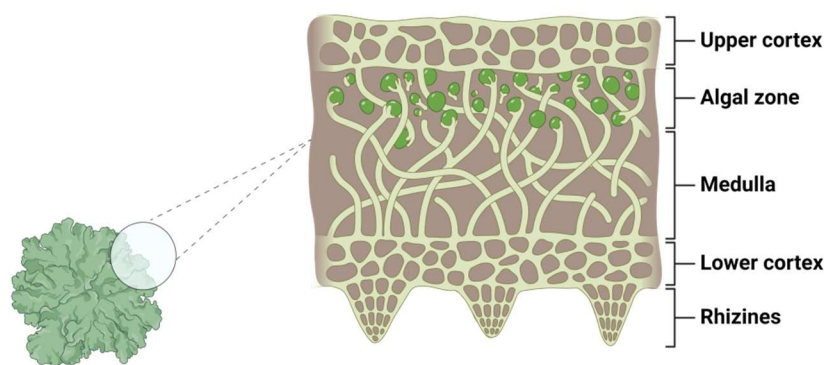


Figure B1.1. Cross-section of the lichen to identify its characteristic layers.

A typical lichen consists of three main layers:

- Upper cortex. This outermost layer consists of tightly packed fungal hyphae that provide protection against environmental stressors such as desiccation and UV radiation. The upper cortex may contain pigments that can help protect the underlying photosynthetic cells.
- Photobiont layer. Below the upper cortex is the photobiont layer, where the photosynthetic partners reside. This layer typically contains green algae and/or

cyanobacteria, which convert sunlight into energy through photosynthesis, providing essential nutrients to the fungal partner.

- Medulla. The innermost layer, known as the medulla, consists of loosely arranged fungal hyphae. This layer acts as a storage area for nutrients and water, facilitating the exchange of materials between the fungal and algal partners.

Resistant to extreme conditions, lichens can live in environments as variable as deserts, arctic tundra, rocky beaches and even toxic habitats. They are considered pioneer species, able to colonise places where other life forms could not survive. It is estimated that there are between 13,500 and 20,000 species of lichens in the world, divided into growth forms such as crustose (crust-like), foliaceous (leaf-like) and fruticose (shrub-like). These organisms can grow on different substrates, such as plant barks, mosses, rocks, soil and wood.

Lichens are ecologically significant as they collaborate with other organisms to maintain the functionality of ecosystems. They are also important biological indicators for monitoring environmental conditions, such as air pollution and acidification. Although their medical use is limited, they have historically been used for food and to extract dyes and antibacterial agents. In addition, lichens are essential to many species of organisms, such as arthropods, birds and mammals, which depend on them for survival.

B1.1.2 Classification and categories

Lichens are classified into different categories according to their morphology, habitat and growth substrate (Lakshnarayan Kumar Bhagarathi et al. 2022). The main types of lichens are outlined below.

- Crustose lichens present an adherent, crust-like growth on the surface of the substrate, which may be rocky, cortical or terrestrial. These lichens cannot be easily removed without damaging the substrate itself. They are often grey, green or black in colour and form a thin film. Common examples include *Rhizocarpon* and *Lecidea*.
- Foliose lichens have a more distinctive, leaf-like structure with well-defined overlapping lobes. They can detach easily from the substrate and are generally thicker

than crustose lichens. Foliate lichens can be further classified according to their shape, such as flat-leaved or curled-leaved. Examples include *Parmelia* and *Hypogymnia*.

- *Fruvous lichens* look like small shrubs or filaments, with a branched structure. These lichens can grow upright or pendulous and are often more voluminous. They can be found on tree trunks or rocks. Known examples include *Usnea* and *Cladonia*.
- *Foliicolous lichens* specialise in growing on the leaves of vascular plants. These lichens are generally small and are found in tropical and subtropical environments. They can contribute to the health of host plants, acting as ecological indicators of environmental quality.
- *Muscicolous lichens* grow on mosses and other low-growing plants. These lichens are often associated with wetland ecosystems and can be used to monitor the health of the habitats in which they are found.
- *Saxicolous lichens* live on rocky surfaces and are well adapted to withstand extreme conditions. These lichens are often colourful and can form distinctive aggregates on rock faces or pebbles.
- *Terricolous lichens* are found directly in the soil and are often associated with arid or sandy ecosystems. These lichens play an important role in soil stabilisation and erosion prevention.

B1.2 The role of secondary metabolites in lichens

Lichens have a unique symbiotic lifestyle, broad genetic diversity and interactions with various environmental factors. Consequently, they produce distinct lichen profiles containing primary and secondary metabolites, known as lichen substances, with interesting physicochemical properties, such as lipophilicity and UV filtration (Nguyen et al. 2013), and demonstrate significant biological activities, including antioxidant, antimicrobial, anti-inflammatory and antiproliferative properties (Boustie, Tomasi, and Grube 2011).

In recent years, there has been a significant increase in the number of scientific articles concerning the discovery, biosynthesis and bioactivity of lichen compounds, indicating a growing interest in this area of research. However, in addition to this interest, the lack of structural clarification and variable results regarding bioactivity in some studies can complicate the interpretation of the bioactivities of lichen compounds.

Among the secondary metabolites of lichens, licheniacides (Goga et al. 2020) are the phenolic compounds are among the most studied. Examples include usnic acid, known for its antimicrobial and anti-inflammatory properties, and protocetraric acid, which has demonstrated antioxidant activity. Lichenic acids are often responsible for the colouration of lichens and may contribute to protection against ultraviolet radiation (Poulsen-Silva et al. 2023). Polysaccharide compounds, produced in varying quantities in lichens, not only provide structure and support, but may also have applications in food and cosmetics due to their thickening and stabilising properties (Olafsdottir and Ingólfssdottir 2001). In addition, some lichens produce terpenes (Elkhateeb and Daba 2020), which can have significant biological activity. Terpenes are known for their antimicrobial properties and can influence ecological interactions, such as defence against pathogens.

Aromatic compounds, a variety of phenols and ketones, may also contribute to the characteristic aromas of some lichens, influencing interactions with animals and insects.

The secondary metabolites present in lichens originate from fungi (Kosanić and Ranković 2019). These substances have a crystalline form and are deposited on the surface of the hyphae of the mycobiont. They have poor solubility in water, so their isolation typically requires the use of organic solvents. The production of these metabolites in lichens is influenced by environmental factors such as light, UV exposure, altitude, temperature fluctuations and seasonality (Kosanić and Ranković 2019). Furthermore, the age of lichens significantly influences the production of lichen compounds and their distribution within the thallus.

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Chapter B2

Black lichens in cultural heritage

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B2.1 Metabolomic analysis of lichens from the Italian archaeological sites of Motya island

B2.1.1 Introduction

Historical-artistic monuments, and more broadly speaking, cultural heritage (CH) located in outdoor environments, are particularly vulnerable to degradation caused by various microorganisms ("Microorganisms in the Deterioration and Preservation of Cultural Heritage," 2021). In general, rock-dwelling organisms, also known as lithobionts, can grow on stone CH and cause aesthetic and physicochemical deterioration of the stone substrates within a relatively short time frame (Favero-Longo et al., 2022; Favero-Longo & Viles, 2020; Pinna, 2017; Upreti et al., 2015). Furthermore, the growing issue of climate change, including rising temperatures, increased precipitation, and violent weather events, may further exacerbate the irreversible degradation of these artworks (Ravanelli et al., 2019; Sesana et al., 2021). Among lithobionts, lichens are the most common agents of biodeterioration, largely due to their ability to grow on a wide range of substrates across various climatic regions (Favero-Longo et al., 2022; Grimm et al., 2021). Lichens are classically defined as a mutualistic relationship between a heterotrophic mycobiont (usually a fungus, often an ascomycete) and an autotrophic photobiont. Most described lichen species are associated with green algae as their photobiont, while a smaller proportion is associated with cyanobacteria. Interestingly, 2 to 4 percent of lichenized fungi can associate with both types of phototrophs (Grimm et al., 2021). In addition to cyanobacteria, studies have identified over 800 species of bacteria in lichens using both culturing methods and, more recently, culture-independent sequencing approaches and omics technologies (Bates et al., 2011; Grimm et al., 2021). These studies have demonstrated not only that the lichens host a complex bacterial microbiome, but also that these microbial communities play active roles in maintaining the entire lichen system. Given the co-existence of various microorganisms within the same system, the analysis of metabolites produced by lichens offers valuable insights into the mechanisms involved in the biodeterioration of CH (Komar et al., 2022; Kosanić et al., 2014). While this approach holds great potential, analysing the metabolome (i.e., the profile of metabolites at a specific time point under defined environmental conditions) in such systems is highly complex. Moreover, attributing specific metabolites to specific taxa is often difficult due to the absence of taxon-specific metabolic signatures. Few studies have focused on the metabolomic analysis of lichens using NMR, and

even fewer have examined the metabolomics of lichens involved in the biodeterioration of lithic CH (Komar et al., 2022; Kosanić et al., 2014; Torres-Benítez et al., 2017). At the same time, lichens can have a protective effect on stone CH. In some cases, lichens form a protective layer on stone surfaces, reducing direct exposure to environmental factors such as wind, rain, and UV radiation, thereby slowing down physical weathering processes. The fungal component of lichens can produce extracellular polysaccharides, which may contribute to the consolidation of stone surfaces and reduce erosion (Chen et al., 2000; Grimm et al., 2021). Indeed, also in this case, the analysis of metabolites could be interested to better understand the mechanisms involved. The case of study here reported is Motya island, one of the most prosperous Phoenician colonies in western Mediterranean. The island was already inhabited before the arrival of the Phoenicians at the beginnings of the eighth century BC, is in the province of Trapani (Sicily, Italy), in a lagoon area called the “Stagnone di Marsala” characterized by a very shallow seafloor where salt ponds are active since historical times. The small island has a surface of 0.4 km² and a maximum elevation of about 6 m above the sea level. Since 2002, the Archaeological Expedition of Motya of the Sapienza University of Rome, directed by Prof. L. Nigro, is leading systematic research and excavations in the island, under the umbrella of the ‘Whitaker Foundation’ and in agreement with the Superintendence of Trapani (Ravanelli et al., 2019). The walls of the entire archaeological sites are characterized by a massive presence of black lichens on the surfaces of the limestone rocks which worried the archologist for their conservation (**Figure B2.1**).



Figure B2.1. Calcarenite walls covered by black lichens (A-C) Motya Island

Furthermore, due to the low elevated topography, Motya island and the archaeological ruins are very sensitive to the sea level changes, in particular to the ongoing sea level rise due to climatic change. Ravanelli and co-workers have assessed the expected marine flooding scenario for 2100 for Motya and the related impacts on the archaeological zone. Taking into account several parameters, they predicted a maximum sea-level rise of 89 cm, which is crucial for some archaeological areas in particular the Kothon (in the South-East part of the island) and North-West coast of the island (Ravanelli et al., 2019), see **Figure B2.2**.

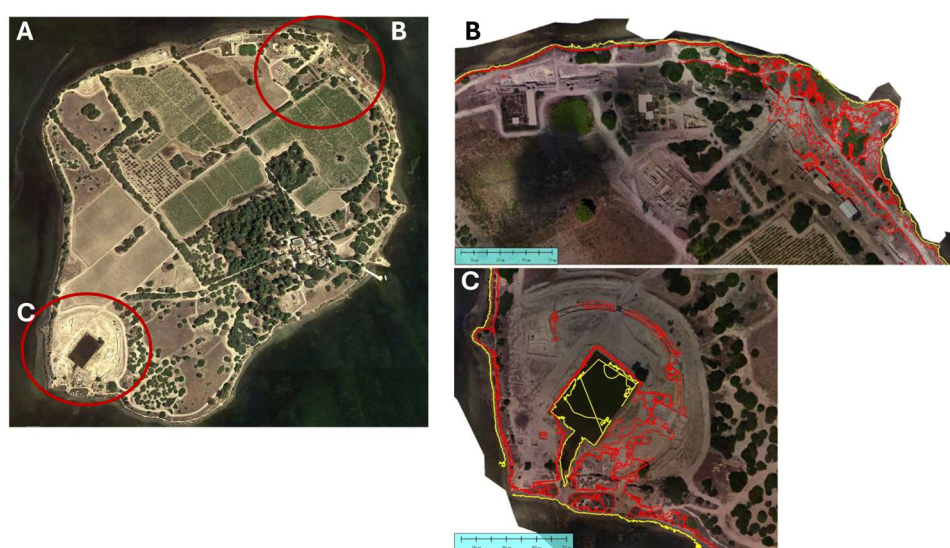


Figure B2.2. Sea level rise in Motya island. (A) Motya island; (B-C) 2100 A.D. coastline projection for the RCP 8.5 + VLM scenario including the maximum tide (30 cm), in yellow (continuous line) the current coastline, in red the coastline projection (continuous line) with its minus/plus uncertainty (dashed lines); (B) detail of the North-West area of Motya; (C) detail of the Kothon area (Ravanelli et al., 2019).

The aim of this study was to simulate in the laboratory a possible future scenario for the archaeological remains on the island of Motya due to rising sea levels. The results here reported give new insights for the analysis of the lichens from a different point of view and new information about the possible consequences of sea level rise on archaeological walls. To persuade these aims, besides to an *in vitro* growth, metabolomic analysis by NMR on the same sample has been performed.

In a study, metabolite profiles were determined from raw extracts of Alpine and Ecuadorian lichens collected from their natural habitats (Mittermeier et al., 2015). Here, for the first time,

NMR-based metabolomics was used to evaluate the power and limitations of non-targeted methodology in the analysis of black lichens attached to the walls of archaeological sites.

B1.1.2 Material and methods

B1.1.2.1 Simulation of sea level rise in Motya island

To simulate a hypothetical flooding future scenario for Motya island, predicted by Ravanelli and co-workers, a piece of rock colonized by lichens from a detached part close to the archaeological walls has been sampled. The rock was divided into four pieces of almost the same dimension, named L₀, L₁, L₂ and L₃ (**Figure B2.3**).

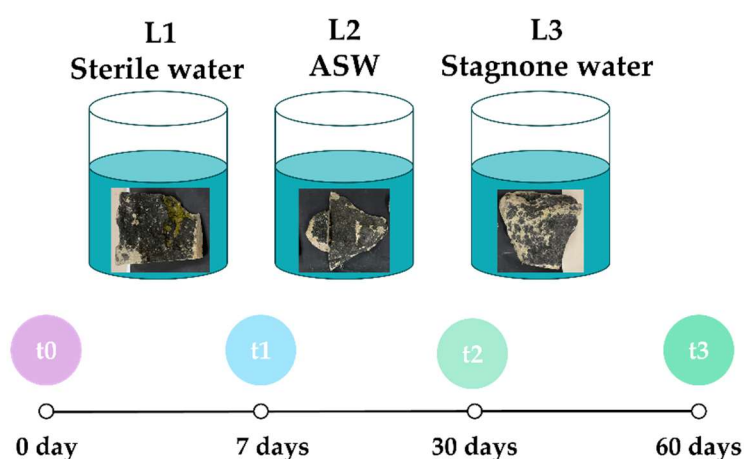


Figure B2.3. Sample of calcarenite rocks from Motya island.

Each piece underwent to four different treatments. L₁, L₂ and L₃ were immersed into sterile water, artificial sea water (ASW) and Stagnone water directly sampled from the sea around the island, respectively (**Figure B2.4**). Sample L₀, was used as control thus was kept for the entire time of the experiment into a dry environment. A small portion of lichens has been removed by sterile scalpel from each sample at three different time. At each time-point, at the beginning of the experiment (t₀, i.e. before the immersion), after one week (t₁), 30 days (t₂), and 60 days (t₃), the lichens were sampled (three biological replica), avoiding as much as possible the removing of the stone substrate, and analysed by NMR. At the same time each sampling point for NMR analysis, a part of the lichens was scratched and plated on three different media.

ASW was prepared to reproduce as much as possible the composition of the sea water which is estimated to have a salinity around 35 ‰. 28.32 g/L of Sodium Chloride (NaCl), 5.48 g/L of

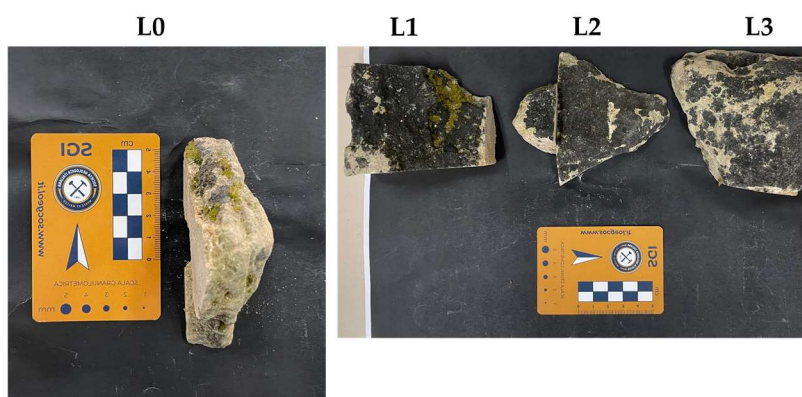


Figure B2.4. Schematic representation of the experiment carried out.

Magnesium chloride hexahydrate ($\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$), 3.60 g/L of Magnesium sulphate (MgSO_4), 1.11 g/L of Calcium chloride dihydrate ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$), 0.77 g/L Potassium chloride (KCl) and 0.2 g/L of Sodium bicarbonate (NaHCO_3) were added to 500 mL of distilled water. The pH was adjust 8-8.4 and then sterilize by autoclaving at 121°C for 15 minutes (Strickland et al., 2004). LB (Luria Bertani - 1 % (*w/v*) Bacto-triptone, 0.5% di yeast extract, 0.5% NaCl and 0.1% of NaOH 1 N, 2.1% of agar), PDA strepto/amp 100 (Potato Dextrose Agar, Formedium added with a solution of streptomycin and ampicillin $100 \mu\text{g/mL}$) and YPD (Yeast Extract–Peptone–Dextrose - 1 % (*w/v*) bacto-peptone, 1 % yeast extract, 2 % glucose, 4 % urea and 2.5 % CaCl_2 and 2.1 % of agar) plates were used to growth *in vitro* bacterial, fungal and yeast strains respectively. The plates were grown for 7 days at 28°C . After each sampling, the plates were photographed and compared with the different time-points.

B2.1.2.2 NMR analysis

Approximately 0.2 g of scraped lichen was dried and extracted according to a modified Bligh-Dyer protocol (BLIGH & DYER, 1959) . Briefly, a mixture of chloroform (2 mL), methanol (2 mL) and water (1.8 mL) was added to each sample. The samples were shaken and centrifuged at a speed of 7000 rpm. The upper hydrophilic phase was carefully separated from the lower

organic phase and dried under nitrogen flow. The hydrophilic phase was resuspended in 0.7 mL of D₂O containing sodium salt of 3-(trimethylsilyl)-propionic acid-2,2,3,3-d₄ (TSP, 0.4 mM) as an internal standard of chemical shift and concentration.

NMR experiments were conducted at 298 K using a JNM-ECZ 600R spectrometer (JEOL Ltd., Tokyo, Japan) operating at a proton frequency of 600 MHz and equipped with a multinuclear inverse z-gradient probe head. One-dimensional ¹H NMR experiments were carried out for quantitative analysis. Water suppression presaturation pulses with a duration of 2 s, a spectral width of 9.03 KHz, and 64 k data points (corresponding to an acquisition time of 5.81 s) were employed. The pulse length with a flip angle of 90° was set to 8.3 µs, while the recycling delay was set to 5.72 s. For resonance assignment, two-dimensional ¹H-¹H TOCSY and ¹H-¹³C HSQC experiments were performed based on the method described previously, along with data from the literature (Ambroselli et al., 2024; Spano et al., 2021). The quantities were expressed in mg/100 g by comparing relative integrals with the reference concentration and normalizing to the number of protons (TSP: 9 protons) and the initial fresh weight of the sample.

B2.1.3 Results

B2.1.3.1 Culturable strains from lichens submerged in sea water

At *t*₀, the samples were characterised by the presence of black fungi especially when the lichens were grown on PDA medium. After a week of submersion, black fungi didn't grow from the samples.

Furthermore, PDA and YPD plates of sample L₂ at *t*₃ were characterised by the proliferation of new whitish fungal species. L₂ is the only sample with fungal colonies. The immersion of lichens in liquid medium allows the proliferation of bacterial strains, characterised by the production of orange/yellow pigments in all samples. Thereby, in sample L₂ the bacterial community has changed after a week and remains almost the same along the three months of treatments.



Figure B2.5. In vitro growth of lichens community. LB, YPD and PDA plates of each sample sampling at four different time.

Noteworthy is the isolation and growth of different bacterial strains during the immersion period, **Figure B2.6**. Already after 30 days (t₂) fluorescent bacteria have been isolated from sample L₂. Besides them, a quite big number of bacteria able to produce pigmented colonies have been isolated alongside many strains belonging to the genera *Streptomyces* sp.

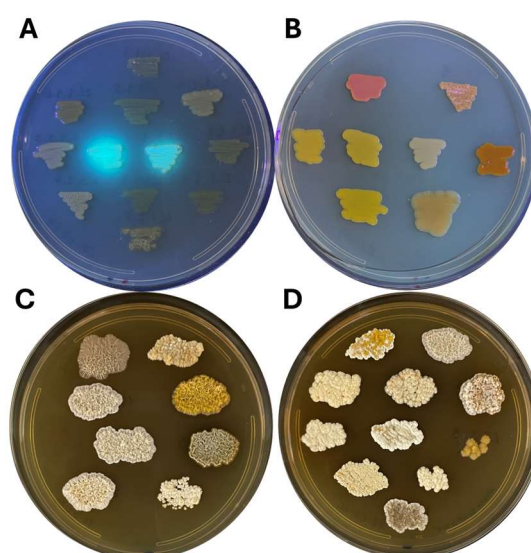


Figure B2.6 Isolated bacterial strains. (A) Fluorescent bacteria; (B) Coloured colonies; (C-D) *Streptomyces* sp. strains.

B2.1.3.2 NMR assignment

Metabolite signals in the ^1H spectra of the hydroalcoholic extract obtained from samples L₀ were assigned using one-dimensional and two-dimensional experiments, standard addition and literature data from fungi and algae (Caprara et al., 2023; Goppa et al., 2023) or plant matrices analysed under similar experimental conditions (Ambroselli et al., 2024). The use of an untargeted NMR-based approach for the chemical characterisation of these symbiont organisms, whose metabolic profile needed, to be further explored, proved to be an effective strategy, allowing metabolites belonging to different classes to be identified. In particular, amino acids and derivatives (leucine, isoleucine, valine, threonine, alanine, arginine, glutamate, glutamine, aspartate, and asparagine), sugars (threulose, mannitol, glucose, and maltose), organic acids (acetate, citrate, and formate), and miscellaneous (choline, phosphorylcholine, and betaine) were identified and quantified, see **Table B2.1**.

Table B2.1. Metabolites and related ^1H NMR signals selected for quantitative analysis in hydroalcoholic extracts.

Metabolite	Chemical shift [ppm]	Multiplicity [J(Hz)]
Leucine	0.97	d [6.2]
Isoleucine	1.02	d [7.1]
Valine	1.05	d [7.0]
Threonine	1.34	d [6.7]
Alanine	1.48	d [7.2]
Arginine	1.69	m
Acetate	1.92	s
GABA	2.30	t [7.4]
Glutamate	2.36	m
Glutamine	2.46	m
Citrate	2.55	d [15.5]
Aspartate	2.81	dd [17.4; 3.8]
Asparagine	2.95	d [16.9; 4.5]
Choline	3.21	s
Phosphorylcholine	3.23	s

Metabolite	Chemical shift [ppm]	Multiplicity [J(Hz)]
Betaine	3.27	s
Threalose	3.45	m
Mannitol	3.80	d [8.5]
β - Glucose	4.65	d [7.9]
α - Glucose	5.20	d [3.8]
Maltose	5.42	d [4.0]
Formate	8.46	s

The one-dimensional proton hydroalcoholic spectra of the samples were overlapped to get an overall view of the metabolic profiling of the lichens (L₁, L₂, L₃) at the respective sampling times (t₀, t₁, t₂, t₃), see **Figure B2.7**.

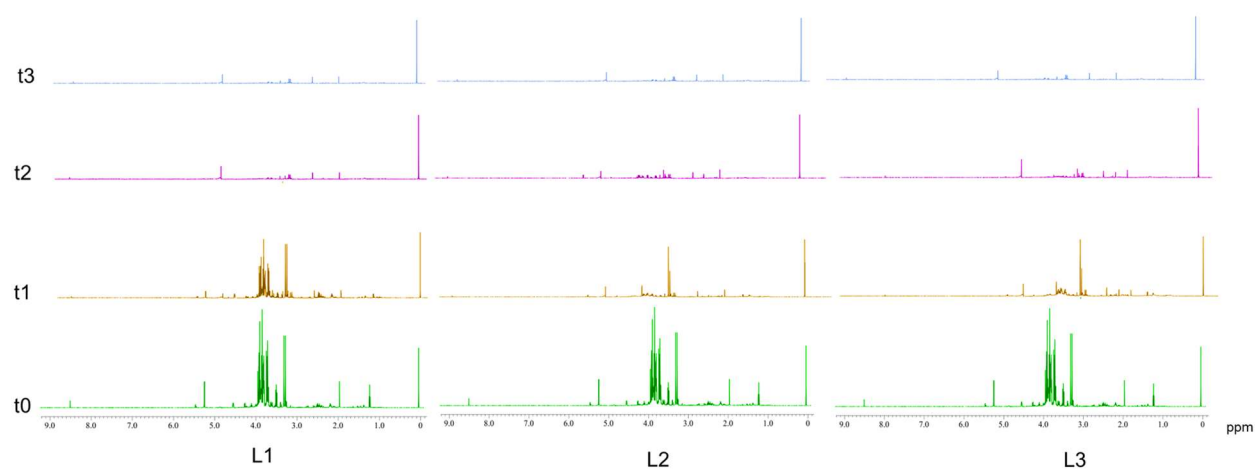


Figure B2.7. Overlapping hydroalcoholic spectra of samples L₁, L₂, L₃ ([TSP]= 0.4 Mm; TSP signal set at 0 ppm water signal suppressed). From bottom to top, the lichen analysis times t₀, t₁, t₂, and t₃.

B1.1.3.3 NMR quantification over time

The metabolites assigned in the reference sample L_0 were, then, quantified at 7 (t_1), 30 (t_2) and 60 (t_3) days for samples L_1 , L_2 , and L_3 . The quantification graphs were obtained on the identified compound classes, which allowed trends in the analysed lichens to be monitored over time, see **Figure B2.8**.

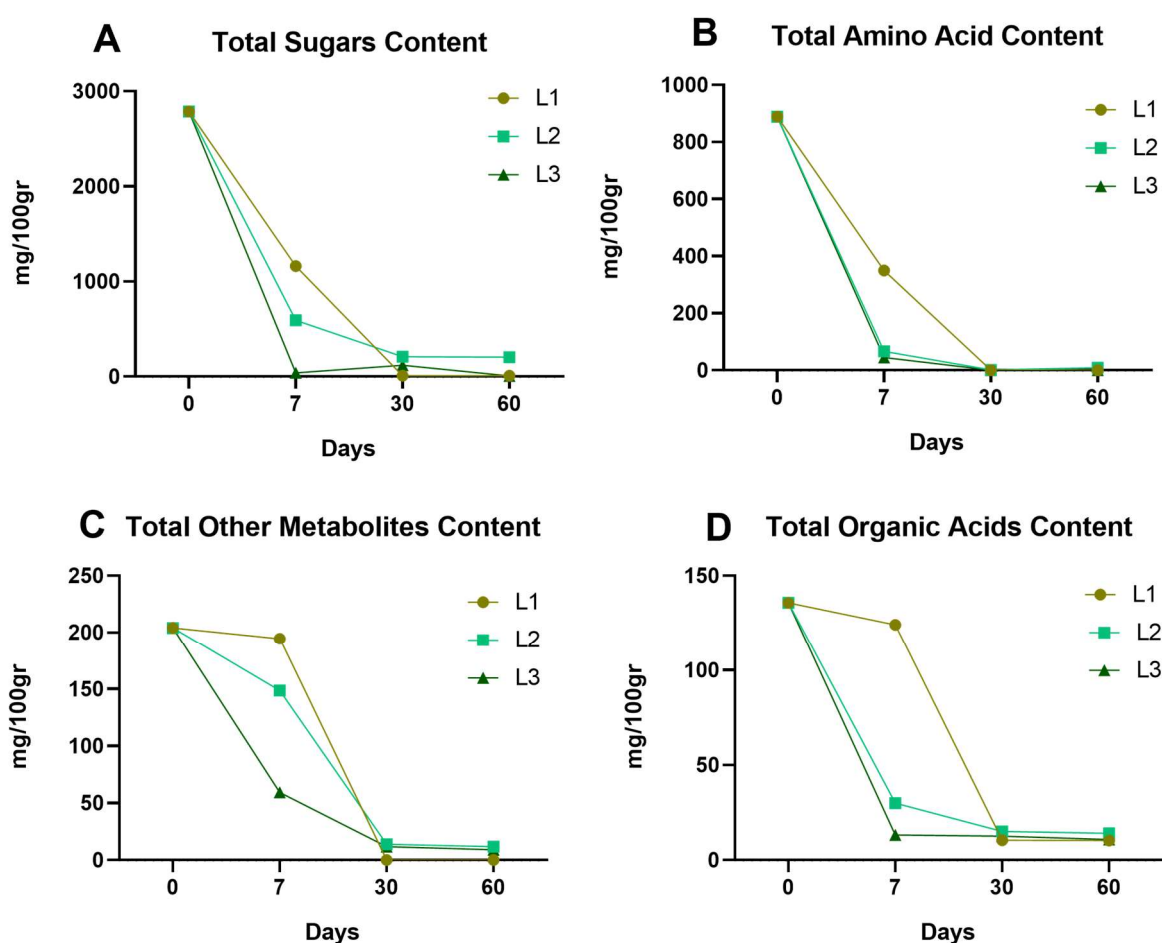


Figure B2.8. Graphs depicting trends in the concentrations of the different metabolite classes according to samples L_1 , L_2 , L_3 over time.

Sugars were the most abundant metabolites measured in lichen kept at room temperature (L_0), while organic acids were the least abundant.

The sugar concentrations of samples L_1 and L_2 were comparable after 7 days to those of L_3 . Furthermore, the amount of sugar in L_2 was more or less constant at times t_2 and t_3 , although it decreased significantly compared to t_0 .

Considering the total amino acid content, already at 30 days, samples L₁ and L₂ had amounts close to zero. At seven days, the concentrations of certain amino acids such as leucine, isoleucine, valine, glutamine, and threonine remained constant compared to t₀.

Among the other metabolites, the levels of phosphorylcholine, choline, and betaine were almost similar at times t₀ and t₁ in sample L₁, then decreased markedly at t₂ and were almost absent at t₃. The concentrations of these compounds in L₂ and L₃ declined from 7 to 30 days, at which time they remained unchanged until 60 days. The amounts of organic acids were detected for all three samples (L₁, L₂, L₃) up to t₃. In particular, while the amounts of L₂ decreased at t₂, in L₁ the citrate levels allowed the total organic acid content to be maintained at around 125 mg/100 g, a value not far from t₀. For L₃, the concentrations of organic acids were constant from 7 to 60 days, while for L₁ and L₂ they remained constant from 30 to 60 days.

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General conclusion

The research outlined in this thesis comprised two main topics under the common theme of NMR-based metabolomics. One focused exclusively on food chemistry, while the other applied an NMR-based metabolomics approach in the field of cultural heritage.

The role of NMR-based metabolomics in food science has expanded over the last decade after being successfully applied to disease, toxicology, and medicine. Recently, NMR-based metabolomics has gained popularity in the food sector and can be applied to food authentication. The low sensitivity of NMR compared to MS is not justified. However, NMR is a method of choice for metabolomic studies due to its reproducibility and quantitative, non-destructive nature. In addition to food authentication studies, i.e. discrimination of the origin of food matrices, NMR-based metabolomics has also recently become established in the evaluation of phytochemicals and their metabolism. Results have shown how factors such as agronomic practices, growing conditions and ecotype of plants can significantly influence metabolic profiles. In particular, the analysis of burdock roots, aerial parts of lemon balm and roots and aerial parts of dandelion allowed the identification of various bioactive compounds, such as amino acids, organic acids, polyphenols and fatty acids, which differed according to tissue type and environmental conditions. Such information is fundamental to furthering our understanding of medicinal plant chemistry, with implications for the nutraceutical and phytopharmaceutical sectors.

Continuing the discussion of officinal plants, a study on wild Corsican lemon balm showed how the combination of NMR and GC-MS can offer an in-depth understanding of the plant's phyto-complex and its biological properties. Extracts of the Corsican lemon balm showed antioxidant, antibacterial and antifungal activity, and the efficacy of the extracts against neurogenic inflammation suggested potential therapeutic applications, particularly for neurodegenerative diseases such as Alzheimer's.

The metabolomics approach has also shown its potential in monitoring food safety and quality by detecting contaminants, spoilage markers and assessing food authenticity. This was particularly useful in the study of food safety, where tomatoes and apples were analyzed to assess risks from pesticides and contaminants, and to investigate the influence of biodegradable packaging materials on food quality during storage. NMR provided crucial

information on changes in primary and secondary metabolites during the storage process, facilitating the understanding of ripening and senescence mechanisms.

Another interesting application of metabolomic analysis was the valorization of agricultural by-products, such as cauliflower and artichoke residues, which were re-purposed to produce protein hydrolysates with biostimulant potential. The results showed that these hydrolysates contain a variety of bioactive compounds, which promote germination and early growth of different crops, thus contributing to agricultural sustainability.

Within the framework of cultural heritage, analyses were carried out on black lichens. Lichens, through their combined action as fungus and algae or cyanobacteria, contribute to the aesthetic and chemical degradation of surfaces, with direct implications for the conservation of cultural heritage. In particular, in the study carried out, attention was focused on the archaeological site of the island of Motya (Sicily, Italy), characterized by black lichens and subject to total submersion due to rising seas. Through the simulation of possible future scenarios, it was possible to observe how metabolites (amino acids, polyols, sugars and organic acids) decrease over time following immersion in various saline solutions, conditions similar to those that could occur due to climate change and sea-level rise. Experimental evidence suggested that exposure to saline and aqueous solutions could slow down the biodeterioration process of monuments by reducing metabolites essential for lichen stability.

Ultimately, the application of metabolomics to analyze plants and foodstuffs not only improves the understanding of their chemical profiles, it also opens new perspectives for improving the quality, safety and sustainability of food and plant-based products. The untargeted NMR-based approach could also provide new tools for the protection of cultural heritage in a global context of environmental change, contributing to the conservation and sustainable management of archaeological sites.

List of publications

1. A. Salvo; F. Masciulli; **D. Ambroselli**; E. Romano; C. Ingallin; M. Spano; G. Di Matteo; A. M. Giusti; A. Di Sotto; E. Percaccio; S. Di Giacomo; G. Vinci; S. A. Prencipe; E. Acciaro; A.P. Sobolev; L. Costantini; N. Merendino; R. Giulianelli; E. Campiglia; L. Mannina. *Hydrolysates from cauliflower and artichoke industrial wastes as biostimulants on seed germination and seedling growth: a chemical and biological characterization*. J Sci Food Agric. (2024). DOI: 10.1002/jsfa.13813
2. **D. Ambroselli**; F. Masciulli; E. Romano; R. Guerrini; C. Ingallina; M. Spano; L. Mannina. *NMR Metabolomics of *Arctium lappa* L., *Taraxacum officinale* and *Melissa officinalis*: A Comparison of Spontaneous and Organic Ecotypes*. Foods (2024), 13, 1642.
DOI: 10.3390/foods13111642
3. **D. Ambroselli**, F. Masciulli, E. Romano, G. Catanzaro, Z. M. Besharat, M. C. Massari, E. Ferretti, S. Migliaccio, L. Izzo, A. Ritieni, M. Grosso, C. Formichi, F. Dotta, F. Frigerio, E. Barbiera, A. M. Giusti, C. Ingallina, L. Mannina. *New Advances in Metabolic Syndrome, from Prevention to Treatment: The Role of Diet and Food*. Nutrients (2023), 15, 640.
DOI: 10.3390/nu15030640.
4. M. Spano, G. Di Matteo, C. Ingallina, **D. Ambroselli**, S. Carradori, M. L. Gallorini, A. M. Giusti, A. Salvo, M. Grosso, L. Mannina. *Modulatory Properties of Food and Nutraceutical Components Targeting NLRP3 Inflammasome Activation*. Nutrients (2022), 14, 490.
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5. C. Prezioso, F. Obregon, **D. Ambroselli**, Petrolo, S., P. Checcon, D. M. Rodio, L. Coppola, A. Nardi, C. Vito, Sarmati, L., M. Andreoni, A.T. Palamara, M. Ciotti, V. Pietropaolo. *Merkel Cell Polyomavirus (MCPyV) in the Context of Immunosuppression: Genetic Analysis of Noncoding Control Region (NCCR) Variability among a HIV-1-Positive Population*. Viruses. 2020;12(5):507
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List of posters

1. **D. Ambroselli**; F. Masciulli¹; C. Ingallina; V. Vergine; L. Mannina, M. E. Crestoni, G. Vinci. "Multi-methodological analysis along the 'Pomodoro Riccio' supply chain: sustainability and safety", XXI CONGRESSO NAZIONALE SISA (Rome, 3-4/10/2024)
2. **D. Ambroselli**; V. Vergine; C. Ingallina; G. Adiletta; P. Russo; M. E. Crestoni; L. Mannina. "The influence of biodegradable packaging material on the quality and shelf life of fresh apples", 51st National Congress on Magnetic Resonance (Florence, 4-6/09/2024)
3. **D. Ambroselli**; F. Masciulli; E. Romano; C. Ingallina; L. Mannina, "Biodiversity within *Melissa officinalis* by applying NMR based metabolomics", 50th National Congress on Magnetic Resonance (Rome, 6-8/09/2023)
4. **D. Ambroselli**; C. Ingallina; F. Masciulli; E. Romano; G. Di Matteo; M. Spano; A. Salvo; G. Vinci, A. Di Sotto; C. Di Meo; P. Matricardi; L. Mannina. "Valorization of agro-food waste into artichoke and cauliflower by-products-based food supplements", Chimali 2023 (Marsala, 29-31/05/2023)
5. **D. Ambroselli**; F. Masciulli; E. Romano; A. Sobolev; L. Mannina. "Cauliflower by-products valorization by applying NMR based metabolomics", Italian-French International Conference on Magnetic Resonance (Milan, 27-30/09/2022)
6. **D. Ambroselli**; M. Spano; G. Di Matteo; F. Masciulli; E. Romano; C. Ingallina; L. Mannina. "NMR metabolomic profiling of fresh Goji berries from two varieties grown in Lazio", VII workshop - Applicazioni della Risonanza Magnetica nella Scienza degli Alimenti (Rome, 23-24/06/2022)

List of oral communications

1. **D. Ambroselli** "From safety to sustainability along the tomato supply chain", AMYC-BIOMED, Autumn Meeting for Young Chemists in Biomedical Sciences - 5th Edition (Rome, 23 – 25/09/2024)
2. **D. Ambroselli** "Plant-Based Food: From Nutritional Value to Health Benefits", SYNC 2024, Symposium for YouNg Chemists (Rome, 24 – 28/06/2024)
3. **D. Ambroselli** "Profilo fitochimico di *Arctium lappa* L., *Taraxacum officinale* e *Melissa officinalis*: un confronto tra ecotipi spontanei e biologici", VIII Workshop - Applicazioni della Risonanza Magnetica nella Scienza degli Alimenti (Rome, 20 – 21/06/2024)
4. **D. Ambroselli** "Cauliflower by-product valorization through a multi methodological approach", Autumn school in food chemistry (Pavia, 17-18/10/2022)
5. **D. Ambroselli** "Caratterizzazione metabolomica NMR di tre ecotipi di *Melissa officinalis*", Workshop FITOBIO caratterizzazione multi-metodologica di piante officinali (Rome, 9/09/2022)
6. **D. Ambroselli** "Produzione di biostimolanti a partire da scarti di carciofo e cavolfiore", Workshop Ri-CicloHorto - Valorizzazione degli scarti alimentari (Rome, 25/05/2022)

Acknowledgements

This thesis is dedicated to the quest for knowledge and to those who have supported and inspired me along this path.

I would like to thank my tutor Luisa Mannina for welcoming me into the Food Chemistry Laboratory. It was important for me to get closer to analytical study, in particular nuclear magnetic resonance spectroscopy.

I would like to express my heartfelt gratitude to my co-tutor, Cinzia Ingallina, for her invaluable support and unwavering belief in me. Day after day, she has shared in my small successes. Her guidance, suggestions, and encouragement have been essential in helping me overcome the challenges of my research. Her attention to detail and dedication to quality have inspired me to continuously improve. Beyond her exceptional teaching abilities, what truly sets her apart is her remarkable humanity. I will always remember the impact she has had on my journey over these years.

I am grateful to Professors Alain Muselli and Aura Tintaru, who welcomed me into their research groups during my time in France.

Everyone who has been part of the Food Chemistry Laboratory over the years has contributed immensely to my personal and professional development. In particular, I am grateful to Mattia for teaching me about NMR, from spectrometer operation to spectra processing. His explanations, patience (!) and helpfulness were important for my start in the workshop, and without him many of the concepts I now consider acquired would have been much more difficult to grasp.

My heartfelt thanks go to Federica, who, despite being 'only' a thesis student when I entered the lab, played a fundamental role in teaching me how to move confidently and competently among the instruments and techniques. Her helpfulness, patience and clarity in sharing her knowledge enabled me to overcome the first difficulties and face the first steps of research with greater serenity. I thank you for supporting me in moments of uncertainty and for making the working environment more welcoming and stimulating. Her presence was truly invaluable.

I would like to express my sincere and profound thanks to my colleague and dear friend, Fabrizio whose support, collaboration and friendship have been significant during this PhD course. His willingness to share ideas, solve the most complex problems together and deal with everyday

difficulties has been crucial not only for my work, but also for my well-being during these years. I thank him for his constant motivation, long discussions, and laughter that made the days in the lab lighter. I am thankful to have had the opportunity to work alongside you and to have been able to count on your friendship, which has become one of the most precious things of these PhD years.