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Towards a greener future: The role of sustainable methodologies in metabolomics research

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ABSTRACT

Sustainability is a growing priority in scientific research, and metabolomics is no exception. Traditional metabolomics workflows rely on hazardous solvents, raising concerns regarding their environmental impact. Recent advancements in green analytical chemistry lay the ground for the integration of eco-friendly approaches in metabolomics from matrix collections and pre-treatment, through sample preparation till data analysis. This review explores the current state of sustainable metabolomic workflows, with a particular focus on green sample preparation methods, solvent-free, low-solvent extraction techniques, and energy-efficient instrumental analysis. Computational advancements, including AI-driven models, machine learning-based semi-quantification, and predictive algorithms for solvent selection, further enhance sustainability by reducing resource consumption. The applicability of these approaches in metabolomic studies, particularly in plant and food research is explored. By integrating innovative green methodologies across all stages of metabolomic workflows, researchers can significantly reduce environmental footprints while maintaining analytical rigor.

1. Introduction

As global research increasingly shifts toward sustainable practices, green methodologies have become a milestone of scientific exploration. In analytical chemistry, green practices are studied and applied in the context of targeted analysis, while they are less explored in non-targeted metabolomics – a central core of omics sciences-. Targeted analysis focuses on the detection and quantification of a predefined set of metabolites, while non-targeted analyses focuses on the comprehensive analysis of small molecules within biological systems [1]. In this scenario, metabolomics plays a central role in diverse areas, such as biomarker discovery [2], pharmaceutical and nutraceutical development [3], and ecological monitoring [4]. However, traditional workflows in metabolomics often rely on hazardous chemicals and energy-intensive processes, raising concerns to environmental sustainability and the safety of the operators [5]. To address these concerns, the principles of green chemistry (GC) [6], green analytical chemistry (GAC) [7], white analytical chemistry (WAC) [8] and circular analytical chemistry (CAC) [9] have emerged as guidelines, pressing researchers to prioritize sustainability without compromising analytical rigor. Besides these principles there are also several metrics and index that allow to calculate the greenness score of an entire analytical workflow, like the "National environmental Method index" (NEMI) [10], the "Analytical greenness metric for sample preparation" (AGREEprep) [11], the "Analytical GREEnness" metric (AGREE) [12], the "Green Analytical Procedure Index" (GAPI) [13], and the "Blue Analytical Green Index" (BAGI) [14].

This review explores the status and advancements of sustainable methodologies in metabolomics, with a particular emphasis on the sample preparation step. However, because several steps are included in the metabolomics workflow (as shown in Fig. 1), we aim at critically review how (and if it's possible) to make each step of the metabolomic workflow greener and highlight how innovative solutions might transform the field.

We decided to specifically focus on metabolomics research related to plants and food, highlighting sustainable practices and advancements in

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the analysis of these natural matrices, which is essential for understanding their biochemical composition, nutritional value, and potential bioactivities. In this framework, we considered the application of green sample preparation (GSP) method, sustainable separation and detection techniques and the implementation of computational and AI-driven model as crucial in the development of an eco-conscious workflow.

2. State of the art: green workflows for metabolomic analysis in plants and food systems

The main contributions of green metabolomics-based analytical methods are summarized in Table 1. While the scientific literature is abundant with studies on sustainable target analysis methods, research on green non-target metabolomics remains relatively underexplored. Nonetheless, a few papers have been recently published and are summarized in Table 1. The studies listed were selected based on the implementation of at least one green practice within the metabolomics workflow. The literature search was conducted across various scientific platforms (Semantic Scholar [15], Scopus [16] and Web of Science [17]) including plants, metabolomics, non- target analysis, green solvents, green sample preparation as the main keywords. After compiling the state-of-the-art studies into a library on Zotero, a secondary research was performed using Rabbit Research [18] to establish connections among the selected papers. Results from literature research are summarized in Table 1. As shown in both Table 1 and Fig. 1, various sample pre-treatments and preparations, strategies and software are continuously developed to extract information from metabolomics data.

Different matrix pre-treatment methods are used and can be classified as green. Among these, we found air drying, oven drying and freeze-drying (see Table 1) as the most common ones. Regarding sample preparation within the metabolomic workflow, the inclusion of Design of experiment (DoE) in the preliminary phase represents a significant added value, as it allows the selection of the most effective conditions from a large panel of experiments. This approach enables the achievement of the desired results and leads to a more precise and optimized sample preparation (Fig. 1 and Table 1). Additionally, various strategies are involved in the pre-analytical phase, such as the use of green solvents, microextraction techniques and others that further enhance the sustainability and efficiency of the process (see Fig. 1 and Table 1). Moreover, computational tools like COSMO-RS (Conductor-like Screening Model for Real Solvents) [19] optimize solvent selection, supporting the adoption of Natural Deep Eutectic Solvents (NADES) in metabolomics, which drastically reduces toxic solvent waste. Once the sample is ready, in addition to proper storage conditions (as will be discussed later in Section 3), the workflow proceed to sample analysis. This step can also be greener by implementing the use of NADES as eluents, short chromatographic runs and alternative analytical technique.

Finally, data analysis, driven by the development of computational tools in data mining, the rise of artificial intelligence and automated softwares, has already transformed and continuous to revolutionized the field of metabolomics. Emerging computational strategies and advanced software solutions such as MzMine, MSDial, Progenesis (QI), molecular networking methods, and MetaboAnalyst have significantly improved the efficiency of metabolomic data processing by automating peak detection, alignment, and annotation, thereby reducing the need for manual curation and chemical standards [19–23]. Machine learning-driven models like MS2Quant further contribute to sustainability by enabling standard-free semi-quantification, reducing the reliance on exhaustive calibration curves and minimizing solvent and reagent use. AI-powered platforms such as FERMO [24] and NP Analyst [25] facilitate the integration of metabolomic datasets with biological information, streamlining the identification of bioactive compounds without requiring extensive wet-lab validation.

These advancements support the transition of metabolomics workflow (Fig. 1) toward green and eco-conscious practices and considerations. These advancements collectively enable a more sustainable metabolomics workflow, as they reduce the need for resource-intensive experimental approaches, promote data reusability, and encourage the

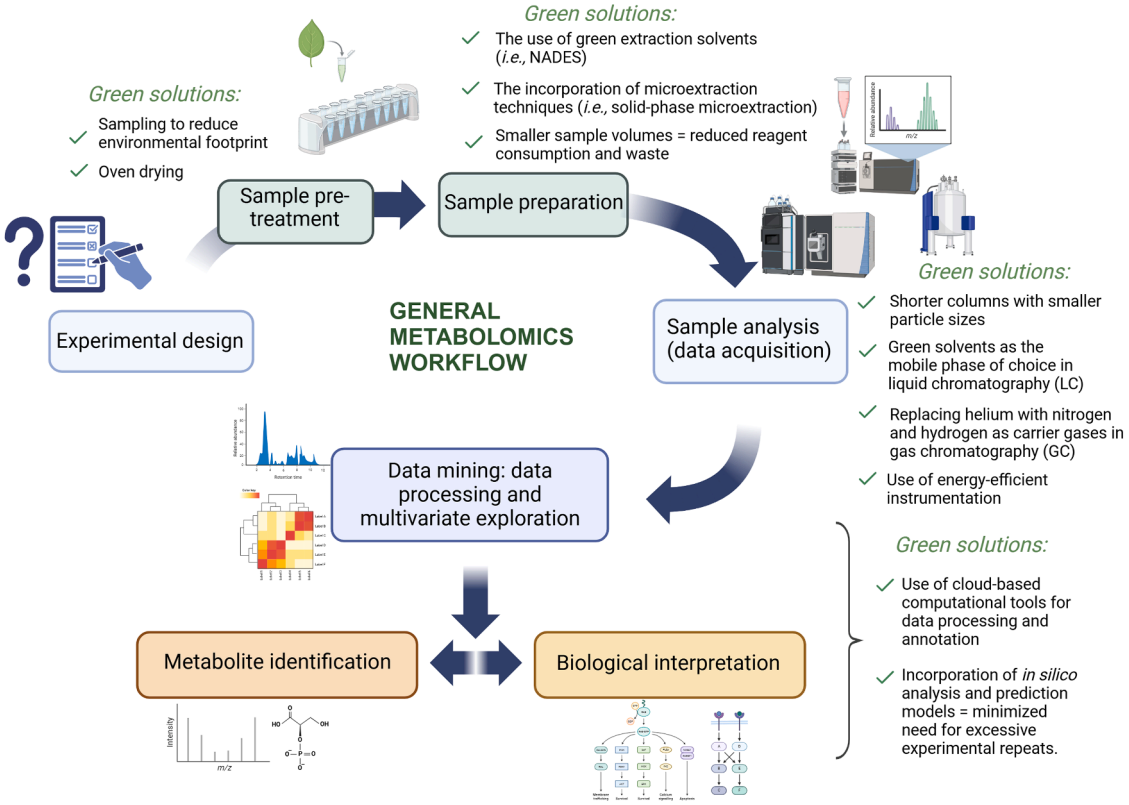

Fig. 1. A multistep metabolomic workflow and how to make it greener. Created in BioRender. Othimire, P. (2025) https://BioRender.com/h25a836.

Table 1
Overview of recent studies on green metabolomics.
Table abbreviations: EtOH (Ethanol); NADES (Natural Deep Eutectic Solvents); LC (Liquid Chromatography); GC (Gas-Chromatography); HRMS (High Resolution Mass Spectrometry); DDA (Data Dependent Analysis); NMR (Nuclear Magnetic Resonance); UAE (Ultrasound-Assisted Extraction), VAE (Vibrocavitation-Assisted extraction), DoE (Design Of Experiment); FBMN (Feature Based Molecular Network), SPME (Solid-Phase Micro Extraction); DVB (Divinylbenzene); CAR (Carboxen); PDMS (Polydimethylsiloxane); HS-SPME (Headspace Solid-phase Micro Extraction); RSLDE (Rapid Solid–Liquid Dynamic Extraction); VOS (Volatile Organic Solvents); PSE (Pressurized Solvent Extractor).

Sample	Pre-treatment	Extraction	Separation	Detection	Data Mining	Reference
Melissa officinalis L.	Oven drying	NADES, maceration	LC C18 - 21 min run	HRMS (DDA)	mzMine-SIRIUS-MS2Quant	[26]
Aralia elata (Miq.) Seem. Var. *mandshurica.*	Oven drying	NADES, UAE and VAE	LC C18 - 17 min	HRMS (DDA)	MSDial	[27]
Opuntia leucotricha Dc.	Oven drying	NADES, UAE	LC C18 - 26 min run	HRMS (DDA)	Xcalibur, knowledge based	[28]
Salvia rosmarinus L.	Air dried	Ethyl lactate, maceration	LC C18 – 47 min run	HRMS (DDA)	Xcalibur, knowledge based	[29]
Sugar cane by-products	Air dried	MeOH/EtOH and water, UAE	GC/LC C18 - 79 min run	HRMS	Knowledge based	[30]
Allium ascalonicum L.	Air dried	EtOH and Water, UAE, Freeze-drying	LC C18 - 35 min run	HRMS (DDA)	MODDE 13.1, Food DB, phenol explorer, SIMCA	[31]
Allium flavum L.	Air dried	EtOH and Water, UAE	LC C18 - 35 min run	HRMS (DDA)	MSDial, food DB and Phenol-explorer, SIMCA	[32]
Thymus vulgaris L.	Air dried	DoE, EtOH and Water, UAE	LC C18 - 35 min run	HRMS (DDA)	MODDE 13.1, Food DB, phenol explorer, SIMCA	[33]
Soy-by-product	Milling	EtOH (70:30), UAE	GC/LC C18- 14 min run	HRMS	Mzmine, FBMN, Protimiza, Prism	[34]
Paullinia cupana Kunth.	Oven drying	ACN and TFA, UAE	LC C18 - 74 min run	HRMS	Chemometrics, smart formula 3D, Line-up,	[35]
Olea europaea L.	Oven drying; deep frozen	MeOH: water (70:30), UAE	LC C18 - 100 min run	HRMS (DDA)	Mzmine, Chemometrics, SIMCA	[36]
Paullinia cupana Kunth.	Oven drying	MeOH: water (70:30), UAE	LC C18 - 19 min	HRMS (DDA)	Chemometrics, Masslinx	[37]
Glycyrrhiza glabra L.	Oven drying; deep frozen	EtOH (70:30), UAE	LC C18- 52 min	HRMS (DDA)	Xcalibur	[38]
Malus domestica Borkh.	Deep frozen	SPME (DVB/CAR/PDMS fibers)	GC - 15 min run	HRMS	ChromaTOF	[39]
Medicago sativa L.	Deep frozen	SPME (DVB/CAR/PDMS fibers)	GC - 7 min run	HRMS	Chemometrics, ADMIS, MET-IDEA, SAS System	[40]
Malus domestica Borkh.	Homogenization	HS-SPME (DVB/CAR/PDMS fibers)	GC - 53 min	HRMS	Chemometrics, MarkerLynx, SIMCA	[41]
Myrtus communis L.	Air dried	Mechanical extraction	LC C18- 40 min	HRMS (DDA)	Knowledge based	[42]
Arabidopsis thaliana (L.) Heynh.	Deep frozen	M1 (3:1): MTBE: MeOH, M2 (3:1): water: MeOH, UAE	LC C18 - 22 min run	HRMS	Progenesis (QI)	[43]
Arabidopsis thaliana (L.) Heynh.	Deep frozen	M1 (3:1): MTBE: MeOH, M2 (3:1): water: MeOH, UAE	GC - 90 min run	HRMS	Knowledge based	[43]
Sorghum bicolor (L.) Moench.	None	MeOH: water (70:30), maceration	LC C18 - 20 min	HRMS (DDA)	Metlin, Massbank	[44]
Corylus avellana l.	Not specified	MeOH: water (70:30), maceration, RSLDE	None	1D and 2D H-NMR	TOPSPIN, SIMCA	[45]
Camellia sinensis (L.) Kuntze.	Freeze-drying	None	None	1D and 2D H—NMR	TOPSPIN, Matlab, SPSS	[46]
Daucus carota L.	Freeze-drying	Buffer and UAE	None	1D and 2D H—NMR	TOPSPIN and SIMCA for statistical	[47]
Cherry tomatoes	Freeze-drying	None	none	HRMAS-NMR	NMR Suite, Matlab	[48]
Capsicum annuum L.	None	None	none	HRMAS-NMR	XWINNMR software, Matlab	[49]
Dry-Sausages	None	None	none	HRMAS-NMR	TOPSPIN, Stratgraphics	[50]
Cheese sample	None	None	None	HRMAS-NMR	TOPSPIN and SIMCA for statistical	[51]
Solanum lycopersicum L.	None	None	None	HRMAS-NMR	Mestre-C Lab, R studio	[52]
Tragopogon lorrifolius L.	Oven drying	MeOH, VOSs, maceration	LC C18 - 20 min run	HRMS (DDA)	Mzmine, FBMN	[53]
Rumex sanguineus L.	Deep frozen and freeze-dried	M1 (3:1): MTBE: MeOH, M2 (3:1): water: MeOH, UAE	LC C18 - 21 min run	HRMS (DDA)	Mzmine, SIRUS, FBMN	[54]
Cinnamomum cassia (L.) J.Presl E Cinnamomum Verum J.Presl.	Oven drying	MeOH:water (7:3), UAE	LC C18 - 38 min	HRMS (DDA)	Progenesis (QI), SIMCA, FBMN	[55]
Yak meat	None	Isopropanol, UAE	LC C18 - 22 min run	HRMS (DDA)	MSDial, SIMCA, FBMN	[56]
Indigofera ammoxylum (Dc.) Polhill.	Oven drying	VOSs, PSE	LC C18 - 12 min run	HRMS (DDA)	Mzmine, FBMN	[57]

adoption of low-waste, high-efficiency analytical strategies. As green metabolomics evolves (Section 6), future computational developments will likely focus on AI-driven predictive analytics, real-time metabolite profiling, and enhanced integration of machine learning models, further minimizing the ecological footprint of metabolomics research while maintaining analytical rigor and biological relevance. By shifting towards data-centric, automation-driven methodologies, computational tools are playing a pivotal role in transforming metabolomics into a field that aligns seamlessly with green chemistry and circular scientific principles.

3. Sample preparation

3.1. Matrix pretreatment: balancing energy consumption and metabolite integrity

Implementing non-targeted strategies often presents significant challenges, particularly in obtaining homogenous samples, transporting them, and ensuring their proper storage to minimize chemical or physical changes following a green route. The most used matrix pretreatment methods in green metabolomics experiments include air drying, oven drying (typically at 40–60 °C), spray-drying, freeze-drying, and frozen storage (see Table 1). Each of these methods has its own advantages and disadvantages, both in terms of energy consumption and its impact on the composition of metabolites. Air drying is energy-efficient and practical, making it an accessible option, but it may lead to the degradation of compounds due to prolonged exposure to ambient conditions [58]. Oven drying, performed at moderate temperatures of 40–60 °C, strikes a balance between preserving metabolites and operational efficiency. However, higher temperatures can cause partial loss of volatile or heat-sensitive metabolites. In contrast to air-drying which does not require any energy consumption, convection drying requires low to moderate (0.5–1.5 kWh/kg) energy usage depending on temperature and duration [59]. Spray drying is a fast and efficient method for liquid matrices, producing fine, uniform powders, but its reliance on high inlet air temperatures may result in degradation of sensitive bioactive compounds. Furthermore, spray drying has a higher (1.0–1.5 kWh/kg water) energy consumption rate compared to convection drying [60], but it can be considered as green depending on the conditions [61]. Freeze-drying, while preserving the most sensitive and volatile metabolites, is the most energy-intensive (1.5–2.5 kWh/kg) method due to the need for freezing, vacuum, and sublimation processes [62]. Beside energy consumption it is the best in preserving the metabolites composition, as discussed by Roshanak et al. [63] and Wu et al. [64] and the most used to obtain homogenous samples. However, it cannot be generally recommended as a green pre-treatment due to its high energy demand. It can only be considered a green alternative if powered by renewable energy sources. In fact, preservation methods in general can be "greened" if renewable energy is used to reduce their environmental impact. Lastly, frozen storage at temperatures ranging from −20 °C to −80 °C is effective for maintaining metabolite integrity over short to medium durations but it requires continuous energy input (0.75–12 kWh/day) for extended storage, thus it is the best choice for short-term preservation [65,66]. For instance, to sensibilize the use of freezer or frozen storage techniques, laboratories can participate in the Freezer Challenge, an initiative led by non-profit organizations that provides practical guidelines and strategies for improving the efficiency of cold storage practices, including tips on optimizing freezer temperatures, reducing energy consumption, organizing samples, and maintaining equipment for long-term sustainability [67]. Pulsed Electric Field (PEF) treatment, although less commonly used as direct preservation method, offers unique advantages such as short processing time often reducing energy consumption up to 50–80 %. Nonetheless, PEF has shown potential in changing the metabolomic profile of treated products, including the polyphenolic pathway and volatile compounds [68]. On this matter, the choice of pretreatment method should consider the balance between maintaining metabolites integrity and minimizing energy consumption, as each method has its own pros and cons regarding both energy consumption and potential metabolites degradation, which can ultimately alter the metabolomic profile in distinct ways, potentially affecting downstream analysis.

3.2. Innovative green extraction techniques for metabolomics applications

Sample preparation is the most crucial step as it ensures that the metabolites are extracted from the matrix of interest (plant, food, vegetable, and others) making them measurable by different analytical techniques. In 2022, Lopez-Lorente et al. [69] published the ten principles of GSP which outline a comprehensive framework for implementing sustainable practices in sample preparation methods. In summary, these principles aim to minimize the environmental impact by reducing sample size, minimize hazardous reagents and promote the use of renewable solvents while maintaining efficiency and quality of the produced samples.

Traditional solvent-extraction methods account for approximately 80 % of the total processing time, 90 % of the energy consumption, and over 99 % of the solvent used throughout the entire analytical workflow [70]. To align with sustainable standards, contemporary analytical approaches emphasize miniaturization and automation, giving rise to techniques like micro solid-phase extraction (μ-SPE) and solid-phase microextraction (SPME) [39,40,71,72] (Fig. 2). Similarly, modern liquid–liquid extraction (LLE) variants, including micro-LLE [73], dispersive–LLME (DLLME) [74], and hollow fiber liquid–phase microextraction (HF–LPME) [56], address the excessive solvent use and time-intensive processes inherent to traditional LLE [75]. Miniaturization techniques are widely used in analytical chemistry but have limitations that make them less suitable for comprehensive metabolomics analysis. These techniques are typically designed to target specific classes of compounds, such as volatile or semi-volatile compounds (in the case of SPME) or compounds with specific polarity ranges (in the case of DLLME) [76]. Whereas, aligned with principles 2, 3, and 8 of GSP [69], NADES emerge as an ideal green choice. NADES are formed by mixing a hydrogen bond acceptor (HBA) and a hydrogen bond donor (HBD) component at different ratios. The resulting mixture has a melting point lower than its individual components because of the formation of intramolecular hydrogen bonds. These solvents are considered eco-friendly, non-toxic, and biodegradable with a relatively low cost, being easy to produce in the laboratory, which fulfills the GSP principles. NADES were successfully employed to investigate the metabolic profile of *Melissa officinalis L* [26]. The results demonstrated that NADES can be advantageous for obtaining extracts enriched with specific biochemical classes, such as sugars, organic acids, or triterpenes. Furthermore, results highlighted that the NADES composed of thymol and menthol could serve as an alternative to ethanol, as the results from semi-quantitative analysis revealed a comparable metabolome profile. Similarly, in a study published in 2024 by Kaleta et al. the effect of extraction methods and NADES was investigated and results demonstrated that NADES composed of choline chloride and malic acid was the best extractant [27]. In the same study, the roots of *A. elata* were extracted by maceration, ultrasound-assisted extraction (UAE), and vibrocavitation-assisted extraction (VAE), results from statistical analysis highlights that 46 metabolites were most abundant in the extract obtain via VAE with NADES.

In metabolomic experiments, particularly those aimed at analyzing bioactivity, it is a common practice to prioritize a fractionated extraction approach rather than optimizing a single protocol capable of ensuring exhaustive extraction of the entire matrix. This fractionated method typically involves sequential extractions using solvents of increasing polarity, from low to high [77–79]. While this strategy ensures comprehensive coverage of the metabolome of interest, it often relies on the use of highly toxic organic solvents. Such solvents, despite their effectiveness in isolating a wide range of metabolites, pose significant environmental and health risks, contradicting the principles of green and white analytical chemistry [8,9] resulting in an overall non-sustainable workflow. Consequently, there is a pressing need to develop alternative fractionation extraction protocols that balance metabolomic comprehensiveness with ecological and safety considerations, such as the use of hydrophobic and hydrophilic NADES [80]. These offer a safer and more sustainable approach without compromising analytical outcomes. Rebocho et al., (2022) proposed a new fractionation methodology in which they used different NADES systems (hydrophobic and hydrophilic), to perform a fractionated extraction of mate leaves [80]. The first extraction step employs a menthol-based

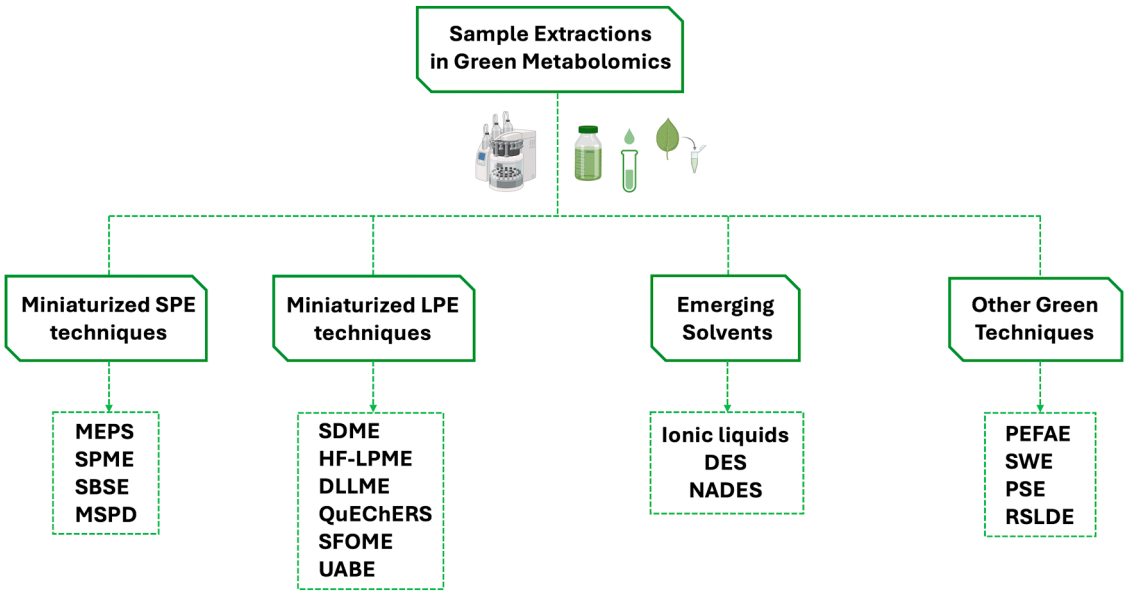

Fig. 2. Classification of the most important sample extraction techniques used in green metabolomics. *Abbreviations*: solid-phase extraction (SPE); liquid-phase extraction (LPE); microextraction by packed sorbent (MEPS); solid-phase microextraction (SPME); stir-bar sorptive extraction (SBSE); Matrix solid phase dispersion (MSPD); single-drop microextraction (SDME); hollow-fiber liquid-phase microextraction HF-LPME); dispersive liquid–liquid microextraction (DLLME); quick, easy, cheap, effective, rugged, and safe (QuEChERS); Solidification of Floating Organic Drop Microextraction (SFOME); Ultrasound-assisted back extraction (UABE); deep eutectic solvents (DES); natural deep eutectic solvents (NADES); pulsed electric-field-assisted extraction (PEFAE); subcritical water extraction (SWE); pressurized solvent extraction (PSE); rapid solid–liquid dynamic extraction (RSLDE).

NADES (menthol:lauric acid, 2:1) to selectively extract chlorophyll. The second step targets phenolic compounds using high-polarity NADES systems, lactic acid:glucose:water (5:1:3) and lactic acid:glycerol:water (3:1:3), which achieved the highest phenolic yields of 12.2 ± 1.0 and 13.5 ± 1.0 % (mg gallic acid equivalent (GAE)/100 mg leaves), respectively. To date, the only study utilizing NADES for fractionated extraction is the one mentioned above. However, scientific literature overflows with publications focusing on the physicochemical characterization of these versatile eutectic solvents, including properties such as polarity, viscosity, density, pH and conductivity [81]. We believe that these studies can serve as a starting point for selecting and optimizing fractionated metabolomics experiments using NADES (see Fig. 3). By building this existing knowledge, researchers can design efficient extraction protocols that align with the specific requirements of metabolomic analyses, contributing to a broader adoption of NADES in this field.

In this context, COSMO-RS (Conductor-like Screening Model for Real Solvents) [82] is a computational tool that plays a key role in the rational selection and design of solvents, including NADES. COSMO-RS allows the prediction of solvent-molecule interactions by calculating thermodynamic properties such as solubility, polarity, and hydrogen-bonding capacity [83]. This is particularly valuable for metabolomics experiments, as it allows to predict the extraction efficiency and selectivity of NADES for specific biochemical classes and metabolites before conducting laboratory experiments. By incorporating COSMO-RS into the experimental workflow, researchers can streamline the optimization of fractionated extractions, reduce experimental trial-and-error, and ensure a more efficient and sustainable approach to metabolomic analysis.

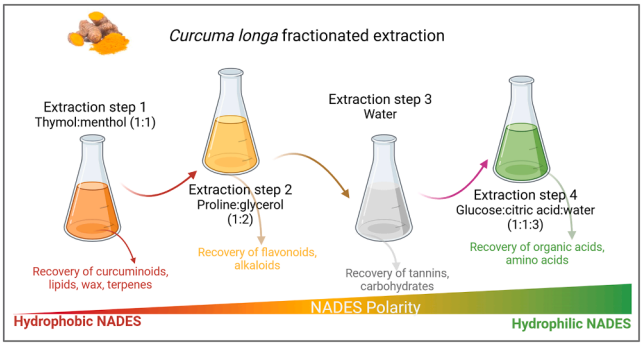

Fig. 3. Fractionated extraction of a wide spectrum of metabolites from *Curcuma longa* using a combination of hydrophobic/ hydrophilic NADES. Adapted from Rebocho et al., (2022). Created in BioRender. Righetti, L. (2025) https://BioRender.com/w70i585.

In addition to NADES-based extractions, other green technologies employing compressed fluids also offer valuable alternatives for fractionated extractions. Among these, supercritical fluid extraction (SFE), pressurized liquid extraction (PLE), and subcritical water extraction (SWE) have gained recognition for their ability to selectively recover metabolites based on polarity, while minimizing solvent use and environmental impact. Although these techniques are not yet considered routine in metabolomic workflows, they provide efficient and scalable strategies that align with green chemistry principles.

Furthermore, it is important to emphasize that sustainability in sample preparation is not solely defined by the extraction technique employed, but also by the nature of the solvent. While neoteric solvents such as NADES have been widely studied for their green potential, other bio-based solvents, such as ethanol derived from renewable biomass, should also be considered as eco-friendly and low-toxicity alternatives to petroleum-derived organic solvents. The integration of these bio-based solvents contributes to a more holistic and sustainable approach to green metabolomics.

In this framework, we also believe that Rapid-solid–liquid dynamic extraction (RSLDE) represents a green alternative for sample treatment for metabolomic experiments. The Naviglio® extractor is an innovative solid-liquid extraction system that utilizes a patented process known as RSLDE designed to optimize the recovery of bioactive compounds from natural matrices [84,85]. This technique relies on the generation of rapid pressure changes within the extraction chamber, which enhances the transfer of bioactive compounds from the solid matrix into the liquid phase. From a green chemistry perspective, the Naviglio® extractor

minimizes the need for high temperatures and toxic organic solvents, reducing both energy consumption and environmental impact. One extraction cycle is usually formed by one static and one dynamic step; these operations are repeated until the solid matrix is exhausted (generally, 20–30 cycles are sufficient and take 2–3 h to complete) [86]. The energy consumption for one full extraction process is estimated to range between 1.0 kWh and 1.5 kWh [86]. Additionally, the gentle extraction conditions help preserve the integrity of thermolabile compounds [88]. A major advantage of the Naviglio® extractor is its ability to achieve exhaustive extraction using only water as solvent, and with significantly shorter extraction times compared to conventional maceration. The amount of solvent required depends on the capacity of the device, which is available in different formats (e.g., 1 L, 40 L, or 125 L), making it suitable for both laboratory-scale and industrial-scale applications. However, it is important to note that the system does not allow for in-process solvent exchange; therefore, the extraction is performed with a single solvent throughout the entire cycle, and fractionation based on solvent polarity is not feasible. The final extract is collected only at the end of the full extraction process. In a study conducted by Ferrara et al. (2014), a comparison was made between a conventional extraction method, UAE and RSLDE to assess the qualitative and quantitative profiles of bioactive compounds in saffron. The findings revealed that RSLDE offered notable advantages, including higher extraction efficiency and superior extract quality [87]. Although the literature on the use of Naviglio ® as an extraction technology is not extensive, its application should be implemented in academic and industrial laboratories. This is particularly important because it is a technology that can also easily enable the direct transfer of metabolomics experiments to industrial applications. Similarly, a previous work by Rocchetti et al. (2019) evaluated the ability of different extraction methods, namely maceration, homogenizer-assisted extraction (HAE), RSLDE, microwave-assisted extraction (MAE) and UAE, to recover phenolic compounds from *Moringa oleifera* leaves [88]. As a general consideration, the authors, by using a non-targeted metabolomic approach to profile phenolic compounds, demonstrated that each extraction method promoted the recovery of specific phenolic subclasses with different efficiencies. Particularly, it was concluded that non-conventional technologies (such as RSLDE) are promising strategies for enhancing the recovery of bioactive and antioxidant polyphenols from *Moringa* leaves. A recent study by Moldovan et al. (2024) evaluated the ability of an ultrasound-assisted sustainable extraction to promote the recovery of phenolics, terpenoids, and sulphur compounds, typically characterizing shallot peel extracts (*Allium ascalonicum* L.) [31]. One of the key points of this work was represented by the utilization of a DoE methodology as a part of the sample preparation step, to better optimize extraction parameters like extraction time, amplitude, and solid-to-liquid ratio. This approach reduces waste, energy consumption, and the use of non-renewable solvents. The authors reported that an extraction time of 10 min, followed by an amplitude of 47 %, and a solid-to-liquid ratio of 1:10 (using hydroethanolic mixture as solvents) were the best parameters to optimize phytochemical recovery and bioactive properties of the extracts. Ethanolic solvents were considered based on their applicability for green and sustainable extraction, since bioethanol can be obtained from renewable sources, presents low toxicity, and ensures high extraction yields while minimizing environmental impact [31]. By using this approach, the authors recovered a total of 8821.5 μg/g of total phenolics and 6172.6 μg/g (both on dry matter basis), representing a very huge amount. As a general consideration, the application of DoE in metabolomics research and phytochemical extraction fosters resource efficiency, reproducibility, and alignment with green chemistry principles. Particularly, by systematically exploring experimental variables, researchers can develop sustainable methodologies that reduce environmental impact while advancing the understanding of bioactive compounds. Therefore, using DoE to fine-tune extraction protocols can lead to scalable, eco-efficient processes suitable for industrial applications, reducing environmental impact at a larger scale.

4. Separation and detection

The development of greener separation and detection methods has become increasingly important in addressing the environmental impact of modern analytical techniques. For example, metabolomic experiments using GC for separation do not produce organic waste, as the technique operates at high temperatures and relies on carrier gases, rather than liquid solvents for sample elution [41,43], but it usually requires samples derivatization [89]. However, there is an associated energy consumption of approximately 1.2 kWh for a 30-min run (one sample), which is primarily attributed to the heating of the column oven and operation of the instrument's components [90]. While, traditional LC—HRMS metabolomics experiments can generate significant amounts of waste and consume considerable energy. For instance, a typical 30-min chromatographic run (one sample) at a flow rate of 0.5 mL/min (average) produces approximately 15 mL of solvent waste, which includes hazardous volatile organic solvents (VOSs). Furthermore, the energy consumption for such a run is estimated at 0.7 kWh for UHPLC system [91] and 1.10 kWh for detector (HRMS) [92], highlighting the high operational energy demands of advanced analytical instrumentation. These problems highlight the need to optimize separation and detection methods to reduce waste generation, minimize energy consumption, and adopt sustainable practices without compromising analytical performance. For instance, in many of the tools and index available online, a low score (often marked in red or below 0.5 points) is primarily attributed to the use of organic solvents and the production of toxic solvent waste, related to separation step, emphasizing the need to improve these aspects.

4.1. Eutectic solvents to improve greenness separation score

One way to improve the overall greenness score of a liquid chromatography (LC) metabolomics workflow is to shorten the chromatographic time (and column length) to reduce the solvent usage. Interestingly, green solvents have been recently applied as mobile phase additives or as major components of mobile phases, including NADES. In a study published in 2018 by Sutton et al. [93], three different NADES mixtures composed of choline chloride, ethylene-glycol, lactic acid, glucose and water in different molar ratios were used as major components in reverse phase- liquid chromatography (RP-LC-DAD). This study, along with others cited in this section, were performed using LC systems coupled with conventional detectors such as UV–vis and DAD. Despite the successful application of NADES as mobile phase, their routine use in LC faces some challenges. First, NADES's viscosity, which complicates handling and system capability. Limitation that can be mitigated by using instruments capable of handling high pressure and elevated temperature. Another challenge faced was the strong adsorption of NADES to silica-based stationary phases, which reduces chromatographic performance when switching back to traditional mobile phases. Although this issue is less problematic if NADES are used consistently. Furthermore, the lack of "LC-grade" NADES introduces impurities that may interfere with detectors. In addition, NADES removal prior to downstream applications such as metabolomics or in vitro assays can be difficult due to their strong solubilizing properties and viscosity, potentially leading to interference with detection or biological responses. Thus, additional cleanup or dilution steps may be required to avoid matrix effects. Nevertheless, NADES were recently successfully used as mobile phases additives (at 1.0 % v/v) to improve peak separation of a series of alkaloids [94], phenolic acids [95] and isoflavones [96]. Heck et al. [97], stated that the HBA from the NADES can increase column efficiency and peak shape by interacting with silanol on stationary phase, while HBD can decrease the retention time of analytes. Hydrophobic NADES have also been recently used as chromatographic stationary phases with water as eluent or hydrophilic NADES were

employed as stationary phases in normal phase- liquid chromatography (NP-LC), even if notable challenges were noticed [98]. Potential encountered solutions involve co-polymerizing polymerizable HBA and HBD directly onto the matrix like poly (itaconic acid) grafted silica (Sil-PIA) [99] or modification of the silica porous surface in NADES with N-doped carbon dots (NCDs) [100]. While most current applications of NADES in LC have been developed for conventional detection systems, the use of NADES in LC-MS workflows remains an emerging and promising area. Although their high viscosity, non-volatility, and potential ion suppression effects present challenges, these may be addressed through appropriate method development, such as dilution strategies, selection of MS-compatible NADES components, or source optimization. Thus, future research may unlock their broader applicability in MS-based metabolomics.

4.2. Direct injection - leaf spray metabolomics

Direct injection – Leaf Spray (DI-LS) involves the direct ionization of metabolites from biological samples (e.g., plant leaves) without requiring sample preparation. The sample is placed on a conductive surface, sprayed with a solvent, and analyzed directly by a MS-based metabolomics approach [101]. There are several reasons behind the relevance of DI-LS to green metabolomics. Particularly, this approach eliminates labor-intensive extraction and purification steps, drastically reducing solvent use and waste generation. The technique enables basically real-time metabolite profiling, thus significantly cutting down energy and time consumption. The utilization of eco-friendly solvents is another key point; in particular, the solvent spray typically uses small quantities of ethanol or water, further reducing the environmental impact. Finally, the non-destructive nature of this approach, since leaves or plant tissues remain largely intact, making DI-LS suitable for repeated measurements on the same sample, thus avoiding huge sampling resources. The LS-MS technique requires only minor modifications to a nanospray ionization source and is a useful tool to further expand the capabilities of a mass spectrometer. In a previous work by Freund et al. (2018), fresh leaf tissues from *Sceletium tortuosum* (Aizoaceae), a traditional medicinal plant from South Africa, were successfully analyzed using this approach, allowing the detection of numerous mesembrine alkaloids. The authors outlined several features of DI-LS-MS, including its high simplicity, precision, accuracy and quick metabolite detection, and semi-quantitation nature (usually required in level 2-based non-targeted metabolomics experiments) [102]. A similar approach, combined with other instrumental techniques such as HPLC-DAD-MS/MS and NMR, was successfully used by de Lima et al. (2020) for a comprehensive characterization of isoquinoline-derived alkaloids in leaves of *Onychopetalum amazonicum,* an unexplored plant species [103].

4.3. Ion mobility MS

Ion mobility spectrometry (IMS) separates ions based on their shape, size, and charge as they travel through a drift tube under a gas flow. When combined with mass spectrometry, IMS provides an additional dimension of separation, allowing for detailed metabolite characterization [104]. This approach has a great relevance in green metabolomics research; particularly, IMS improves resolution and separation efficiencies, thus reducing reliance on lengthy chromatographic methods that consume large solvent volumes [105]. Basically, one of the main advantages of using IMS is its ability to differentiate isomers and conformers, thus enabling precise identification of metabolites without extensive derivatization or separation [106]. Also, IMS works well with direct injection methods (such as the previously mentioned DI-LS), eliminating pre-analytical procedures that generate chemical waste. By pre-separating metabolites based on their ion mobility, IMS often eliminates the need for traditional chromatographic separations. This dramatically reduces the consumption of organic solvents like acetonitrile or methanol, which are typically required in large quantities for liquid chromatography. Additionally, techniques like ambient desorption electrospray ionization (DESI) and other solvent-minimized ionization methods are easily coupled with IMS, providing a seamless eco-friendly workflow [106]. A literature search dealing with IMS revealed that its main applications are in the field of environmental exposomics, particularly for the analysis of persistent organic pollutants [107], PFAS [108] and indoor dust [109].

4.4. NMR as a green alternative in metabolomic experiments

NMR is one of two methods (in addition to MS) that stands out in metabolomics. One of the advantages of using NMR instead of LC-MS, in the context of green metabolomics, is the elimination of the need for chromatography to achieve the separation of metabolites in the sample. For instance, NMR can be recognized as the greenest detection method which applies only 10 % of deuterated solvents for metabolomics studies [110]. However, one of the primary challenges of NMR remains its high demand for liquid helium, which is necessary to cool the magnets. This issue has been recently addressed through advancements in technology, particularly by Bruker BioSpin, which has developed NMR systems that drastically reduce helium consumption. These systems now include helium recovery technologies that savings up to 95 % of helium, representing a significant step toward enhancing the sustainability of NMR analyses [111]. ^{1}H NMR was used in omics science for the first time to study the human metabolome in 1970s by Radda and Shulman [112] and later used to characterize the metabolic profile of serum [113], blood plasma [114] and urine samples [115] or as reported in Table 1 to develop eco-metabolomics food protocols [46,47]. High-resolution magic angle spinning (HRMAS) NMR spectroscopy was later introduced and used in metabolomics due to its innovative and environmentally friendly approach. HRMAS enables the analysis of semisolid and heterogeneous materials by spinning samples at a "magic" angle of 54.74° relative to the magnetic field. This minimizes dipolar coupling effects and chemical shift anisotropy, resulting in higher-resolution spectra [116]. Samples for HRMAS-NMR are typically analyzed at low temperatures within a short time; only small amounts of samples (10–40 mg) and minimal solvent volume are used, further supporting its alignment with green analytical principles. This technique was successfully used for the eco-metabolomics analysis of *Capsicum annum L* [49]., *Solanum Lycopersicum* L [50] and other food samples [51,117].

Although NMR metabolomics is recognized as a greener approach in metabolomics as no solvents are required as for the LC separation, when we compare AGREE score with that of LC—HRMS-based metabolomics using green solvents (e.g. NADES), the two scores are very similar, as depicted in Fig. 4. NMR surpasses the LC—HRMS workflow in terms of waste generation (principle 7), requiring only a few milliliters of deuterated solvent per sample, while an LC-MS run typically consumes around 15 mL (considering a flow rate of 0.35 ml/min) of non-biobased solvent. However, NMR scores lower for principle 9, as it demands significantly more energy, making it a highly energy-intensive technique. The LC—HRMS-based workflow can be further improved if ACN or methanol in the mobile phase are replaced with biobased and non-toxic solvents (principles 10 and 11). This is possible, as demonstrated by Sutton et al. [93] as they applied NADES as mobile phase additives in their metabolomics analysis. The score that, at least for now, cannot be improved is number 3, as, to the best of our knowledge, there are no portable MS or portable NMR systems capable of achieving the analytical performance required for in situ non-targeted metabolomic analysis.

5. Data mining: math is greener than chemistry

Data mining is a critical step in metabolomic workflows, enabling researchers to extract meaningful information from complex datasets generated by analytical platforms like LC/GC—HRMS. Advanced software tools such as MZmine [21], MSDial [118], MetaboAnalyst, GNPS

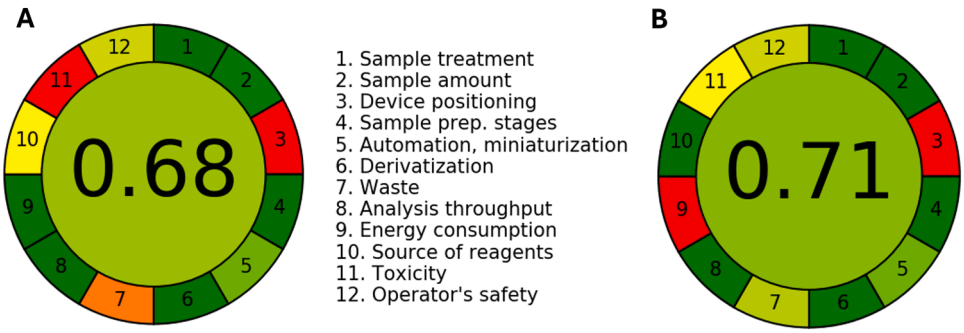

Fig. 4. Greenness evaluation of the A) NADES LC—HRMS metabolomics [26] and B) NMR-based metabolomics workflow [46]. The scores were calculated using the AGREE metric [12].

(Global Natural Products Social Molecular Networking) [119], and feature-based molecular networking (FBMN) have revolutionized the processing, analysis, and interpretation of metabolomic data. These tools streamline tasks like peak detection, alignment, annotation, and pathway analysis, bridging the gap between raw spectral data and biological understanding. For instance, MZmine [21] facilitates comprehensive preprocessing, including feature detection and alignment, while MetaboAnalyst [22] supports statistical and pathway-based analysis of metabolite profiles. GNPS and FBMN enhance metabolite annotation by using collaborative spectral libraries and visualizing compound relationships based on shared structural features, as reported in Table 1 [53,57,64], allowing for more eco-conscious metabolomics. They reduce the need for exhaustive standard libraries and lengthy validation experiments, saving chemicals, solvents, and energy [120]. Compared to traditional knowledge-based methods, which often rely on extensive prior information, manual curation, and large numbers of chemical standards, data-driven approaches like these are inherently greener [121]. Additionally, multivariate analysis and chemometrics [122], integral components of modern metabolomics, allow for the discovery of patterns and relationships in high-dimensional datasets (Table 1). Techniques such as principal component analysis (PCA), partial least squares-discriminant analysis (PLS-DA), and hierarchical clustering help to identify key metabolites, differentiate biological groups, and establish meaningful connections between metabolic changes and biological conditions. The green impact of chemometrics usually comes from the minimization of complex -physical manipulation (not green) of samples and instruments while maximizing the use of existing data [123]. Together, these tools and approaches create robust and environmentally conscious metabolomics for discovery novel metabolites, understanding metabolic pathways, and driving biological discovery with precision and efficiency. Thus, computational metabolomics plays a crucial role in reducing the environmental footprint of chemical research by replacing labor-intensive experimental procedures with data-driven models. By integrating mathematical modeling, AI, and machine learning, computational metabolomics exemplifies how "math is greener than chemistry" by minimizing hazardous waste, reducing experimental costs, and accelerating sustainable chemical discovery. For example, machine learning-based classifiers, and other AI-powered methods [124,125], integrate metabolomics data with pathway analysis to gain novel and actionable insights on biological pathways, but also to infer the biochemical impact of new compounds, allowing scientists to focus only on the most promising candidates for synthesis. Furthermore, in environmental toxicology, computational metabolomics is employed to assess the impact of industrial chemicals on ecosystems without the need for *extensive in vivo* testing. Various studies have highlighted how metabolomics data can be combined with computational methods to predict toxicological effects, enabling regulatory agencies to make data-driven decisions while reducing animal testing [126,127]. Another example is the application of computational models to optimize biotechnological production of green chemicals [128]. By analyzing metabolic fluxes in engineered microorganisms, computational metabolomics facilitates the efficient design of microbial strains that produce biodegradable plastics, biofuels, or pharmaceutical precursors with minimal environmental impact.

5.1. *Standard free semi-quantification*

Advances in data mining for metabolomic experiments have transformed the detection of the chemical metabolic space. One of the major problems related to HRMS analysis, particularly those conducted using electron spray ionization (ESI) as source, is that we cannot rely on intensities or peak area for quantification. These experimental parameters are strongly related to molecules ionization efficiency and consequently the response factor (RF), represented by the slope of the calibration curve, can vary significantly between compounds. Thus, accurate determination of a chemical's concentration requires calibration with analytical standards, and this is costly and time, energy and waste consuming. In this framework, MS2Quant was developed by Sepman et al. [129]. MS2Quant is a machine learning model that enables the concentration prediction of annotated and or unidentified chemicals. MS2Quant can predict ionization efficiency for $[M + H]^+$, $[M]^+$and $[M\text{-}H]^-$, $[M]^-$ ions. To translate the predicted ionization efficiency (IE) value into an instrument- and measurement- specific RF, the model is calibrated by analyzing calibrants (at least 5) alongside the suspects (samples) within the same experimental run. Spaggiari et al. (2025) successfully used this tool to semi-quantify 385 metabolites detected and annotated in *Melissa officinalis* leaves, using only 11 analytical standards [26], improving the greenness of the entire metabolomic workflow.

5.2. *Connecting metabolomics data with biological ones*

In metabolomics experiments, the goal is often to identify molecules or metabolic patterns with potential biological activities, such as antioxidant, anti-inflammatory, antimicrobial effects or any biologically relevant activity. However, a successful metabolomics experiment can yield over 500 tentatively annotated metabolites, making it challenging to attribute specific phenotypic activities to individual compounds [130]. One approach to address this issue is to perform fractionated extractions followed by metabolomics analysis. As we discussed in Section 3, VOSs are frequently used for such extractions making it less sustainable, while NADES are less commonly employed, despite representing a promising green alternative. From our perspective, a way to make this step greener and more sustainable, aligning with the principles of GC, GAC, WAC, and CAC [9], is to integrate the workflow with online tools that prioritize HRMS data with qualitative and quantitative biological observation. Among these, we suggest FERMO [24] and NP Analyst [25] as interesting platforms. The applicability of FERMO was demonstrated in a case study on antibiotic activity of bacterial extracts, where the bioactive molecule siomycin was successfully prioritize out of >143 molecular features [24]. As of today, FERMO is not yet widely

adopted, but it has recently been updated to allow users to work directly online without needing to know Python language. This new functionality makes it more accessible to researchers, while still retaining the option to perform offline analyses using Python script for advanced customization [131]. FERMO is positioning itself as a versatile tool for metabolomics, enabling both novice and experienced users to process HRMS data efficiently and connect them to phenotypical data, improving the overall greenness score of the metabolomic workflows. Similarly, NP analyst was developed as a user-friendly online platform that allowed the correlation of HRMS data with biological ones. NP-analyst facilitates the identification of bioactive compounds, their potential mechanism of action and pathway mapping [25]. NP analyst was successfully used to highlight the toxicity (bioactivity) of studied cyanobacterial extracts without the need to isolate complex extracts [132].

5.3. AI vs manual processing

The field of metabolomics is undergoing a transformative shift with the integration of AI-driven data analysis, significantly enhancing efficiency, reproducibility, and sustainability. Traditional manual data processing—which involves peak picking, feature alignment, metabolite annotation, and statistical modeling—has long been the backbone of metabolomics studies. However, manual workflows are time-consuming, prone to human error, and require repeated computational and experimental validation [133–139]. This results in higher energy consumption and increased data redundancy, making them less aligned with green metabolomics principles. In contrast, AI-powered data analysis automates and optimizes these processes, reducing computational waste and improving data reusability. One of the biggest limitations of manual metabolomics data processing is feature alignment and peak deconvolution in complex datasets. Traditional peak-picking algorithms rely on user-defined parameters and iterative corrections, often requiring extensive manual verification [140–142]. AI-driven alternatives like DeepMetabo, PeakBot, MetaClean, AutoMS and many more employ deep learning to automatically detect, align, and integrate metabolite features without manual intervention, improving both accuracy and efficiency [143–147]. These AI-based tools continuously learn from datasets, reducing false positives and eliminating the need for labor-intensive peak correction steps. Metabolite annotation and classification, another critical step in metabolomics data analysis, has traditionally relied on manual curation of spectral libraries. Experts match MS or NMR spectra against reference databases such as Human Metabolome Database (HMDB), METLIN and other spectral libraries, a process that is both time-consuming and requires multiple rounds of validation. AI-driven tools like computational strategies (e.g., molecular networking methods) housed in the GNPS ecosystem, and other emerging tools such as MS2LDA, ClassyFire, MassSpecGym, matchms, and msFeaST apply self-learning algorithms to (semi)automatically annotated and classify metabolites, significantly reducing the dependency on chemical reference standards while minimizing human error and computational costs [148–152]. These tools also enhance metabolite identification in non-targeted metabolomics by predicting unknown compound structures, eliminating the need for repeated experimental validations. Another area where AI surpasses manual processing is data integration and statistical analysis. Traditional workflows involve using tools like PCA and PLS-DA for pattern recognition, requiring multiple iterations of statistical validation and manual tuning. AI-based models such as XGBoost, LightGBM, artificial neural networks, and random forest classifiers dynamically adapt to large metabolomics datasets, automating feature selection, reducing dimensionality, and minimizing computational load [153–156]. These machine learning models provide better predictive accuracy with less reliance on manual parameter tuning, reducing computational energy use and data redundancy. AI is also revolutionizing big data processing and network-based metabolomics. In multi-omics research, where metabolomic data must be integrated with genomics, transcriptomics or proteomics, manual processing is extremely resource-intensive. AI-powered platforms like NP Analyst and FERMO automate cross-omics integration, enabling faster and more efficient metabolic pathway reconstructions without extensive manual curation. This significantly reduces computational waste and enhances data reusability, a key principle in green metabolomics.

While AI-driven tools are advancing metabolomics data analysis, manual curation remains crucial for biological interpretation and validation. AI models require high-quality training datasets, and without proper human oversight, there is potential for errors in data-driven predictions. However, as AI models become more sophisticated, their role in automated data preprocessing, metabolite identification, and statistical validation will continue to grow, further minimizing computational waste, enhancing sustainability, and contributing to the eco-conscious future of metabolomics.

6. Envisioned green metabolomics workflow

Designing a green workflow for metabolomics requires integrating principles of sustainability, resource efficiency, innovation and reusability at each stage of the analysis (see Fig. 1). Overall, the ideal green metabolomics workflow should be based on a sustainable sampling process (to reduce the environmental and ethical footprint), using eco-friendly but efficient sample preservation techniques (drying processes, that minimize energy consumption and chemical waste), and employing solvent-free or low-solvent techniques as described in the previous sections (such as solid-phase, supercritical fluid, and pressurized extraction methods). As a general consideration, one should go for bio-based or recycled solvents, while reducing extraction times and process temperatures to save energy. Additionally, computational solvent selection tools such as COSMO-RS optimize the use of green solvents like NADES, promoting sustainability in sample preparation. As far as the instrumental analysis is concerned, researchers should prioritize high-efficiency instruments minimizing resource consumption, such as high-throughput UHPLC—HRMS methods with microfluidics, and/or the utilization of DI-MS or NMR (to avoid chromatography). Furthermore, as echoed in the previous sections, a sustainable green metabolomics workflow implies computational strategies and AI-driven methods for efficient data processing and analysis. Machine learning and artificial intelligence optimize workflows by automating peak detection, feature alignment, and metabolite annotation, reducing the reliance on resource-intensive laboratory validation. By integrating these sustainable innovations, metabolomics can continue to evolve as a powerful tool for scientific discovery while reducing its ecological impact.

Although several metrics are available, none of them incorporate the data analysis step into the overall score. While this may not be a major issue for targeted analysis of a few analytes, it becomes crucial for metabolomics. As a result, the assessment of green performance is not fully comprehensive. In fact, despite highlighting the benefits of AI and automation for data analysis, we must also consider the significant energy consumption required by these computational processes. It would therefore be valuable to develop a metric that allows for a more holistic evaluation of performance.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used DeepL and ChatGPT in order to improve the readability and language of the manuscript. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the published article.

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draft, Visualization, Conceptualization. **Kgalaletso Othibeng:** Writing – review & editing, Writing – original draft, Visualization. **Fidele Tugizimana:** Writing – review & editing, Writing – original draft. **Gabriele Rocchetti:** Writing – review & editing, Writing – original draft, Visualization. **Laura Righetti:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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