Viewpoint

The marriage between stable isotope ecology and plant metabolomics – new perspectives for metabolic flux analysis and the interpretation of ecological archives

Summary

Even though they share many thematical overlaps, plant metabolomics and stable isotope ecology have been rather separate fields mainly due to different mass spectrometry demands. New high-resolution bioanalytical mass spectrometers are now not only offering high-throughput metabolite identification but are also suitable for compound- and intramolecular position-specific isotope analysis in the natural isotope abundance range. In plant metabolomics, label-free metabolic pathway and metabolic flux analysis might become possible when applying this new technology. This is because changes in the commitment of substrates to particular metabolic pathways and the activation or deactivation of others alter enzyme-specific isotope effects. This leads to differences in intramolecular and compound-specific isotope compositions. In plant isotope ecology, position-specific isotope analysis in plant archives informed by metabolic pathway analysis could be used to reconstruct and separate environmental impacts on complex metabolic processes. A technology-driven linkage between the two disciplines could allow us to extract information on environment–metabolism interaction from plant archives such as tree rings but also within ecosystems. This would contribute to a holistic understanding of how plants react to environmental drivers, thus also providing helpful information on the trajectories of the vegetation under the conditions to come.

Introduction

The elements C, H, N, O and S are the building blocks of metabolites. The variations in the natural abundance of the stable isotopes of these elements have been used for more than four decades in plant ecology to assess the response of plants to environmental conditions (e.g. Trust & Fry, 1992; Schulze *et al.*, 1998; Keitel *et al.*, 2003; Helle & Schleser, 2004; Lehmann *et al.*, 2021). Starting somewhat later, at the beginning of this century, plant metabolomics and profiling of a multitude of metabolites became a fast-growing technology for phenotyping and diagnostic analyses of plants (Fiehn *et al.*, 2000; Schauer & Fernie, 2006). Many thematical overlaps exist between plant metabolomics and stable isotope ecology. This is because variations in the isotope composition of plant organic matter as assessed by isotope ecology, as well as metabolite profiles and abundances, which are the focus of metabolomics, depend on enzyme activities and related metabolic pathway preferences (e.g. Tcherkez & Hodges, 2008; Stitt *et al.*, 2009). Until the present, however, they remained rather separate fields with only a few contact points. This is most likely a result of different historical developments in the fields and different mass spectrometry demands. In plant isotope ecology, highly specialised gas sector-field mass spectrometers (isotope ratio mass spectrometer; IRMS) with different peripheries (elemental analysers for overall bulk material and gas and liquid chromatography for compound-specific resolution) have been used for assessing natural abundance δ^2H, $\delta^{13}C$, $\delta^{15}N$, $\delta^{18}O$ and $\delta^{34}S$. IRMS requires the conversion of organic compounds into gas as the multi-collector setup directly measures the different gas isotopologues, which leads to the destruction of the metabolites. The gas is ionised with electron impact (EI) ionisation. For CO_2, as an example, ions with a mass-to-charge ratio of 44, 45 and 46 are detected, corresponding to isotopologues with combinations of ^{12}C, ^{13}C, ^{16}O, ^{17}O and ^{18}O.

By contrast, plant metabolomics are based on bioanalytical mass spectrometers, which combine quadrupole, time of flight, ion trap and mass analysers of other designs with soft ionisation methods that allow the analysis of intact compounds. The recent development of bioanalytical mass spectrometers with perspectives for high-throughput, compound-specific, and intramolecular natural abundance isotope analysis seems to have, however, the potential to tear down existing barriers, bringing the two fields closer together. Here, we outline how a new technological development might, on the one hand, provide new insights into metabolic networks (being thus of strong interest for metabolomics). On the other hand, we show how, in ecological applications, biological archives such as tree rings could unveil unprecedented information on plant–environment interactions by applying compound- and position-specific isotope analysis and by being informed by metabolomics. We use the metaphor of 'marriage' to describe this new and potentially fruitful cooperation between two disciplines.

We first describe how isotopic signals are generated and how the detection of compound-specific and intramolecular isotope signals can be helpful for the interpretation of plant archives. In the next step, we show how metabolic network and flux analyses could be linked to compound-specific and intramolecular isotope information at natural abundance in metabolomic workflows. Informed

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by this new metabolomic-based knowledge, more detailed reconstructions of plant–environment interactions from, for example tree rings might become possible. Further, we explain why methodological issues prevented such detailed insights so far and how new developments in bioanalytical mass spectrometry might be ready to provide analytical solutions. Finally, we focus on the specific example of tree-ring C isotopes to show how a combination of new methodological approaches linked with interdisciplinary interpretation might lead to novel insights. Thereby, we detail which specific analyses and workflows will be necessary for further collaboration between scientists from both disciplines.

Isotope fractionations – how isotope signals are generated

Stable isotope ecology allows an ecologist to trace element cycling in all systems of interest. It thus can disentangle ecological connections within and between organisms in qualitative and quantitative ways (Fry, 2006). A particular focus of stable isotope ecology is on analysing datable biological archives that integrate ecological information and allow the reconstruction of environmental influences on biological processes. In plants, tree-ring stable isotope assessments are a classic example (McCarroll & Loader, 2004). Stable isotope ecology relies on kinetic and equilibrium isotope fractionations (Schmidt, 2003). They occur either in metabolic pathways where one metabolite is the substrate of different, competing enzymatic reactions or as a result of mere physical processes such as the Rayleigh fractionation during the evaporation of water – which vary with the environmental conditions. These fractionations can imprint a specific isotope signal on products of enzymatic reactions and, depending on the turn-over time of the organic matter pools, then provide time-integrated information on plant–environment interactions (e.g. Fotelli *et al.*, 2003). Stored in dateable plant archives such as tree rings (but also in terrestrial and aquatic soils and sediments where plant residues are deposited), the isotopic information allows us to retrospectively assess environmental conditions and plant physiological responses to these conditions over weeks to millennia (e.g. Sachse *et al.*, 2012; Büntgen *et al.*, 2021). The probably most exploited fractionation process in plant and environmental science is photosynthetic carbon isotope discrimination (Farquhar *et al.*, 1982), which is – in a first approximation – depending on the relationship between leaf intercellular (C_i) or chloroplastic (C_c) and ambient CO_2 concentration (C_a).

As a consequence, changes in both main processes that affect C_i and C_c, namely the supply of CO_2 into the leaf via stomata (and thus stomatal conductance; g_s) as well as the demand within the plant (and hence RubisCO activity and assimilation; A), leave a particular isotopic imprint on the new assimilates that is, therefore, a proxy for the intrinsic water use efficiency (iWUE = A/g_s). For example, drought will primarily affect g_s (and to a lesser and more indirect extent A), increasing water use efficiency and decreasing photosynthetic fractionation. The change in fractionation scales with the reduction of g_s and affects the $\delta^{13}C$ in tree-ring cellulose via the newly assimilated carbohydrates used to build it, thus allowing the reconstruction of the intensities of past drought events.

Intramolecular isotope distribution and compound-specific isotope differences – they challenge the traditional interpretation of biological records but also yield new insights

In the past, tree-ring carbon isotope signatures were classically interpreted as solely affected by photosynthetic isotope fractionation and its drivers (see Gessler *et al.*, 2014). For more than 30 years, however, there has been strong evidence that postcarboxylation isotope fractionation causes nonstatistical intramolecular carbon isotope distribution in the organic compounds exported from the chloroplast (Rossmann *et al.*, 1991). This has important implications for isotope signals in compounds from metabolic pathways downstream of the Calvin cycle and, thus, for plant isotope archives.

The isotope effects of the aldolase and *trans*-ketolase reactions in the Calvin cycle, together with metabolic branching due to the export of glyceraldehyde 3-phosphate (GAP) from the chloroplast to the cytoplasm, lead to such intramolecular $\delta^{13}C$ differences in GAP and also in the downstream metabolites such as pyruvate and sucrose. If molecules with nonstatistical intramolecular isotope distribution are fragmented in enzymatic reactions, the products can differ in their global (molecule average) isotope composition from each other and the substrate. An important example is the pyruvate dehydrogenase reaction that converts pyruvate (i.e. relatively enriched in ^{13}C at the carboxyl-C originating from chloroplastic aldolase) to acetyl-CoA and releases CO_2. The CO_2 originating from the carboxyl group is thus relatively ^{13}C enriched, whereas the acetyl-CoA and, for example the fatty acids in the downstream metabolism are depleted (Tcherkez *et al.*, 2003). Consequently, intramolecular differences in the substrate are converted into compound-specific isotope differences, even though we need to acknowledge that additional kinetic isotope effects, together with metabolic branching and pathway commitments, modify the magnitude of this effect.

The aldolase reaction, however, also causes temporal differences in the global isotope composition of sucrose: sucrose exported from the leaves and transported to heterotrophic tissues during the day is ^{13}C depleted compared to that exported during the night (Gessler *et al.*, 2008). This is because in the aldolase reaction, ^{13}C is preferred over ^{12}C in the bond-making reaction (Gilbert *et al.*, 2012a). Bond breaking of ^{12}C–^{12}C bonds is faster than that of ^{12}C–^{13}C bonds, and the reverse is true for bond making. Therefore, in equilibrium, the C-3 and C-4 positions in fructose-1,6-bisphosphate (the product) are enriched in ^{13}C, and at the same time, dihydroxyacetone phosphate and GAP (the substrates) get relatively depleted. The equilibrium isotope effects (on positions C-3 and C-4 of fructose-1,6-bisphosphate are 1.0036 ± 0.0002 and 1.0049 ± 0.0001) (Gleixner & Schmidt, 1997). The ^{13}C depleted GAP is exported from the chloroplast and forms the basis for the sucrose exported during the day. The relatively enriched fructose-1,6-bisphosphate is the substrate for transitory starch production, and thus starch is ^{13}C enriched by up to 4‰ compared to the triose-phosphates (Gleixner *et al.*, 1998). Consequently, the sucrose produced during the night via starch breakdown also carries this enrichment (Gessler *et al.*, 2008). The translation of position-

specific isotope differences into temporal global isotope variations of the same compound strongly impacts the interpretation of isotope archives (Tcherkez *et al.*, 2007). Depending on which proportion of an archive, such as a tree ring, is built from day and night sucrose and assuming the proportion might vary among environmental conditions, ontogeny and species, it might become more challenging to reconstruct photosynthetic physiology from tree-ring $\delta^{13}C$. Moreover, additional CO_2 fixation via phosphoenolpyruvate carboxylase (PEPC) in heterotrophic tissues (Cernusak *et al.*, 2009) might lead to the incorporation of relatively ^{13}C enriched compounds (compared to RubisCO fixed assimilates) with different intramolecular ^{13}C isotope distribution (Gilbert *et al.*, 2009) into the stem metabolome. It is unclear if this directly affects $\delta^{13}C$ of tree-ring cellulose; thus, further research is needed.

In conclusion, the $\delta^{13}C$ isotope signals in tree rings, which are amongst the most important ecological archives (McCarroll & Loader, 2004), are often assumed to reflect the environmental impacts on the interplay between CO_2 supply for (i.e. g_s) and CO_2 use by photosynthesis. They are thus used to reconstruct both climate impact on photosynthetic physiology, and past climatic conditions (Gessler *et al.*, 2014). In reality, however, the tree-ring isotope signals are affected by many different metabolic processes that might be differently influenced by environmental drivers. Consequently, the information on environment–metabolism interaction derived from classical whole wood or cellulose isotope analysis can become blurred (e.g. Wieloch *et al.*, 2018). However, these metabolic processes also leave position-specific isotopic imprints on the chemical compounds the tree ring is made of. We postulate here that the possibility to determine position-specific isotope signatures in compounds of ecological archives routinely will increase the quality and quantity of the information that can be retrieved. We also claim that such an approach needs a better understanding of metabolic fluxes and pathways and their impact on compound- and position-specific isotopic signals in the upstream metabolism in leaves and heterotrophic tissues.

Metabolic networks – fluxes and commitment of substrates to pathways at metabolic branching points and the isotopic fingerprints

Fluxes through plant metabolic networks are generally assessed with steady state or dynamic ^{13}C labelling (Ratcliffe & Shachar-Hill, 2006), but multiple isotope-labelling approaches have also been proposed (Xu *et al.*, 2020). Moreover, machine learning is increasingly applied to interpret ^{13}C-enabled fluxomics (Wu *et al.*, 2022). Because CO_2 fixed via RuBisCo is the primary C source in plants, analysis of transient labelling considering isotopic nonstationarity can be applied (Antoniewicz, 2021). At the same time, many enzymatic reactions in metabolic networks are associated with $^{12}C/^{13}C$ isotope effects, and – depending on the reaction an enzyme catalyses – these effects are position-specific and, for example related to the specific bond made or broken (Hobbie & Werner, 2004). The actually observed position-specific isotope fractionations between substrate and product of an enzymatic reaction depend upon metabolic flux rates and commitments (Schmidt, 2003; Tcherkez *et al.*, 2011). While unbranched irreversible reactions that completely convert a substrate do not fractionate, for two or more reactions that are competitive and consume the same substrate (branched reactions *sensu* Schmidt, 2003), the observed isotope fractionations depend on their relative rates. Tcherkez *et al.* (2011) stated that limiting steps strongly fractionate, whereas rapid, nonlimiting steps fractionate weakly as the larger the commitment into a reaction, the smaller is the observed *in vivo* fractionation. Moreover, when several reactions are successive, the observed isotope fractionation between the substrate and the final product depends on the flux rate balance.

This shows that position- and compound-specific $\delta^{13}C$ values are intrinsically linked to metabolic fluxes and allocation patterns between metabolic pools within metabolic networks. Thus, they contain information similar to that obtained from steady state or dynamic ^{13}C labelling-based metabolic flux assessments without the need to add an artificial isotope label. Moreover, the above-described isotopic fractionations occur not only for C but also for the other elements H, N, O and S in organic molecules, thus allowing us to explore the whole range of the plant's metabolism.

To fully exploit the information on metabolic fluxes imprinted on position- and compound-specific isotope values, more information on isotope effects of central reactions in the primary metabolism is needed. Until now, only few isotope effects are known from *in vitro* studies, but they show good agreement with model-based studies on *in vivo* isotope effects (Tcherkez *et al.*, 2011). With new technologies that allow the determination of high-throughput position-specific isotope distributions in many compounds of the primary and, in the next step, the secondary metabolism, classical ^{13}C label-based fluxomic could be combined with label-free assessments. In paired experiments (with and without labelling), in which plants are either exposed to sets of different environmental conditions or in which different mutants with manipulated metabolic pathways are tested, ^{13}C labelling provides the fluxes and commitments, and the nonlabelled approach allows to determine *in vivo* isotope effects. Once relationships between fluxes and commitments on the one side and isotope effects of key enzymatic reactions on the other are established, position- and compound-specific isotope information can be used for metabolic flux mapping.

Methodological issues that have so far hampered further insights

It is, thus, undisputable that the intramolecular stable isotope distribution provides an information-rich signal that, in principle, allows us to reconstruct commitments of different metabolic pathways and how they are affected by environmental conditions. In particular, compound- and position-specific isotope analysis of metabolic intermediates leading from photosynthetic C assimilation to the compounds laid down in the biological archives could improve the reconstruction of plant responses to environmental drivers. Still, not much work has been done to exploit this information, and we believe this is mainly related to

Table 1 The main differences between isotope ratio mass spectrometers (IRMS) and bioanalytical high-resolution mass spectrometers (HRMS).

	IRMS	Bioanalytical HRMS
Separation of analytes	**GC** or **LC** with aqueous mobile phase; Overall: restricted spectrum of analytes	**LC** as **UHPLC** with organic solvents (**GC** also possible); Overall: large overall spectrum of analytes
Analyte introduced into the MS	**Gas** produced from the original analyte (CO_2 for $\delta^{13}C$, CO for $\delta^{18}O$; N2 for $\delta^{15}N$; H2 for $\delta^{2}H$; SO_2 for $\delta^{34}S$); no structural information retained.	**Unaltered metabolites** with preserved structure
Ionization	**Electron impact ionization (EI)**; hard ionization; but since gases are introduced no fragment information can be exploited	**Electrospray ionization (ESI)**; soft ionization, formation of a pseudo-molecular ion with an intact structure
MS/MS	**No**	**Yes**
First Mass analyser	**Sector field**	**Lower resolution mass analysers** (e.g. linear ion traps or quadrupoles)
Second Mass analyser	**na**	**High-resolution mass analysers** (e.g. time-of-flight, Orbitrap, FT-ICR)
Metabolite identification	**No or only partially** via chromatographic retention time (identification is possible with a split setup, where GC separated compounds are measured in parallel with IRMS on the one hand and a quadrupole or ToF mass analyser on the other)	**Yes** (by mass and via fragments in MS/MS experiments)
Compound-specific isotope analysis	**Yes**	**Yes**
Position-specific isotope analysis	**No** (no structures of the metabolite retained due to conversion of the metabolite into a gas)	**Yes** (via fragmentation in MS/MS experiments)
Simultaneous analysis of the isotope composition of multiple elements	**No** (with some exceptions: bulk tissue C/N/S analysis; C/H/O on purified cellulose with pyrolysis) (Loader *et al*., 2015)	**Yes**

methodological issues. Compound-specific stable isotope analysis is more common than position-specific one. It is based either on offline chemical purification of particular compounds, as often performed for α-cellulose (Boettger *et al*., 2007) or on frequently called 'hyphenated' techniques where a GC or HPLC separation is coupled online to an IRMS (Rinne *et al*., 2012; Rinne-Garmston *et al*., 2022). GC-IRMS is restricted to either volatile metabolites or compounds that can be derivatised to become volatile. HPLC separation combined with IRMS requires water-based mobile phases (because of the oxidation step before analysis), limiting the spectrum of analytes and the chromatographic resolution (Perini & Bontempo, 2022). The principle of IRMS additionally requires that the particular analyte is converted to a gas (e.g. CO_2 for determining $\delta^{13}C$ or CO for $\delta^{18}O$) and thus, all structural and position-specific information gets lost (Table 1). Therefore, two different approaches have been mainly carried out in the past to still obtain position-specific isotope information. (1) Purified compounds are differentially degraded (Rossmann *et al*., 1991) or derivatised (Waterhouse *et al*., 2013) to obtain products containing atoms from different positions of the initial molecule, which are then combusted or pyrolysed in an elemental analyser coupled to an IRMS. These procedures are extremely time-consuming, can only be applied to particular purified compounds and not to a whole set of metabolites, and are thus not applicable to high-throughput measurements. (2) With nuclear magnetic resonance spectrometry (NMR), position-specific carbon, nitrogen, and deuterium isotope distributions can be assessed in organic molecules (Gilbert, 2021). However, the need for purification of the analyte (Gilbert *et al*., 2009) and the low sensitivity of NMR, requiring high amounts of purified analytes, preclude high-throughput routine analyses.

New developments in bioanalytical mass spectrometry might provide analytical solutions

In metabolomics (as in proteomics), ultra-high-performance liquid chromatography – high-resolution mass spectrometry (UHPLC-HRMS) is more and more routinely applied to tackle the challenge of the complexity of metabolites in plants (Perez de Souza *et al*., 2021). While UHPLC provides efficient separation, modern HRMS systems with pulse or ion counting detection methods involving preamplifiers, discriminators, counters, time-to-digital converters (TDCs), digital oscilloscopes and digitisers offer high-sensitivity detection. HRMS systems such as Orbitraps, time-of-flight (ToF) or Fourier Transform ion cyclotron resonance (FT-ICR) analysers combined with additional lower resolution mass analysers (e.g. linear ion traps or quadrupoles) allow MS–MS workflows for sensitive detection and unequivocal identification of metabolites (Perez de Souza *et al*., 2019; Schroeder *et al*., 2020). Only recently, Neubauer *et al*. (2023) suggested that such setups are, in principle, applicable for compound-specific isotope analysis and position-specific isotope assessments in the natural abundance range. Comparing results of MS/MS measurements of ethyl toluene with known $\delta^{13}C$ values provided the proof of concept that compound-specific natural abundance isotope analysis is possible with the latest generation of bioanalytical mass spectrometry setups. In principle, liquid chromatography linked via electrospray ionisation to an orbitrap is sufficiently sensitive to measure the

natural isotope composition of single compounds (Griep-Raming, 2017), and it has been shown that such an approach was also suitable for detecting low levels of deuterium tracer in lipids (Neubauer *et al.*, 2018a). These findings were corroborated by Merder *et al.* (2020) for complex analyte mixtures. For these measurements to determine a real isotope pattern, high-performance detection systems with a low detector dead time in relation to the sampling rate are used.

Moreover, Eiler *et al.* (2017) and Weiss *et al.* (2023) showed for alanine and Neubauer *et al.* (2018b) for methionine that position-specific assessment of the carbon isotope composition is analytically feasible. In addition, Gharibi *et al.* (2022b) showed that Orbitrap-based proteomic approaches can provide the amino acid composition and sequence of proteins together with C, H, N and O isotopic ratios. This allows amino acid metabolism to be linked to protein synthesis with the help of isotopes, especially when particular proteins contain amino acids with anomalous isotopic compositions (Gharibi *et al.*, 2022a). Moreover, proteomics-compatible isotope ratio mass spectrometry of polypeptides (Gharibi *et al.*, 2022b) might allow better use of animal-derived archives such as bones, hairs, and feathers to reconstruct environment–metabolism interactions.

With the new HRMS technology the analytical wedding gift for the marriage between plant metabolomics and plant isotope ecology seems within reach. Given the fast technological development and availability in metabolomics facilities, such setups might soon become the basis of routinely combined metabolite and isotope measurements. This might have the potential to boost the application of stable isotope measurements as the switch from dual-inlet IRMS with offline production of the gas to be analysed to continuous flow IRMS (coupled to elemental analysers or GC) did in the past (Brenna *et al.*, 1997).

In general, UHPLC-HRMS provides – compared – to classical IRMS, several advantages (see Table 1 for the comparison). (1) There is no requirement for combustion of the metabolites, converting the analyte to a gas, but the UHPLC eluent containing the analyte is softly ionised by electrospray ionisation resulting in the formation of a pseudo-molecular ion with an intact structure. (2) In MS/MS experiments, mass/charge (*m/z*) selected analytes can be fragmented via *collision-induced dissociation* (*CID*) or *higher-energy collisional dissociation* (*HCD*), and the different fragments are analysed with high-resolution mass detectors. Different fragments contain overlapping atomic sites, and with such redundant information in the mass spectra, position-specific isotope composition can be calculated (e.g. Eiler *et al.*, 2017; Weiss *et al.*, 2023). (3) In a given analyte, isotopes of multiple elements can be detected simultaneously, resulting in a 'multidimensional isotopic fingerprint' (*sensu* Neubauer *et al.*, 2023). The multi-dimensionality is given by the position-specific isotope information for multiple elements. (4) In a UHPLC-HRMS system, many analytes in a plant extract can, in principle, be identified and analysed for concentration as well as for compound and position-specific isotope composition, opening the path to high-throughput analysis.

Future research and development still need to tackle challenges. These include an accurate accounting of isotope fractionation during the MS–MS procedures, the availability of standard materials with known position-specific isotope composition, and the detection of position-specific isotope composition in analytes in complex mixtures.

A major challenge arising from the isotope analysis of the metabolites is the high requirement for mass spectrometric resolution to recognise random overlaps of the isotope patterns of different metabolites or their artefacts. High resolution can be achieved in modern mass spectrometry using ultrafast scan modes in combination with advanced counting statistics for the selected mass range. Isotopic patterns can also be mathematically corrected using newly developed deconvolution software algorithms if necessary. One notable development is the ENVIPAT package, which includes functions for calculating isotope patterns, profiles, and centroids (Loos *et al.*, 2015). This tool utilizes fast and memory-efficient transition tree algorithms to derive the exact mass and probability of isotopologues. It offers various settings for handling different molecular complexities and pruning thresholds, making it highly versatile for different HRMS applications (EnviPat documentation: https://www.envipat.eawag.ch/). Another significant contribution comes from the isopattern function within the ENVIPAT package. This function allows for the calculation of isotopologues using transition tree updates, which are capable of reproducing the isotope fine structure of molecules. The isopattern function can be customized with different algorithms and pruning strategies, enhancing its efficiency for complex molecules.

In ESI or APCI (*atmospheric pressure chemical ionisation*) measurements, artefacts can also result to a lesser extent from ionisation, depending on the redox stability of the metabolites, although these ionisation methods are referred to as 'soft' ionisation methods.

A further limitation is to be expected in the fragmentation behaviour of the metabolites in the *CID* experiment. Depending on the structure of the metabolites, in some cases, only a few fragments are obtained, so that a structural assignment of individual C atoms to the fragments is difficult or not possible. In these cases, only a limited statement can be made about the metabolic flux or the biosynthesis of the partial structures of the metabolites. The *HCD* cell may be able to provide a solution here. This *HCD* technique enables a nonequilibrium fragmentation mechanism so that multiple fragmentations with deeper structural information can also be achieved. In contrast to classical ESI-*CID* fragmentation, very fragment-rich spectra are already obtained in the ion source in EI ionisation. The reaction mechanism of the fragmentation, therefore, allows a better structural assignment of the C atoms to be made with this method. The newly developed hybrid ionisation method, which enables a combination of ESI and downstream EI ionisation (ZenoTOF 7600; Sciex, Framingham, MA, USA) (Che *et al.*, 2023), could, thus, provide a remedy. This technology works with a reagent-free and tuneable, high-efficiency *electron-activated dissociation* (*EAD*) fragmentation in a separate *EAD* cell, offering alternative fragmentation comparable to the EI Fragmentation. However, this technology is currently only available with a TOF analyser, which does not achieve the resolution of the Orbitrap systems.

There is still a way to go in finding optimal procedures. One critical point is fragmentation and the energy provided for it. For the identification of compounds, comparability between runs and instruments is needed whereas for isotope measurements a sufficient amount and overlap of fragments is required that allows position-specific calculations.

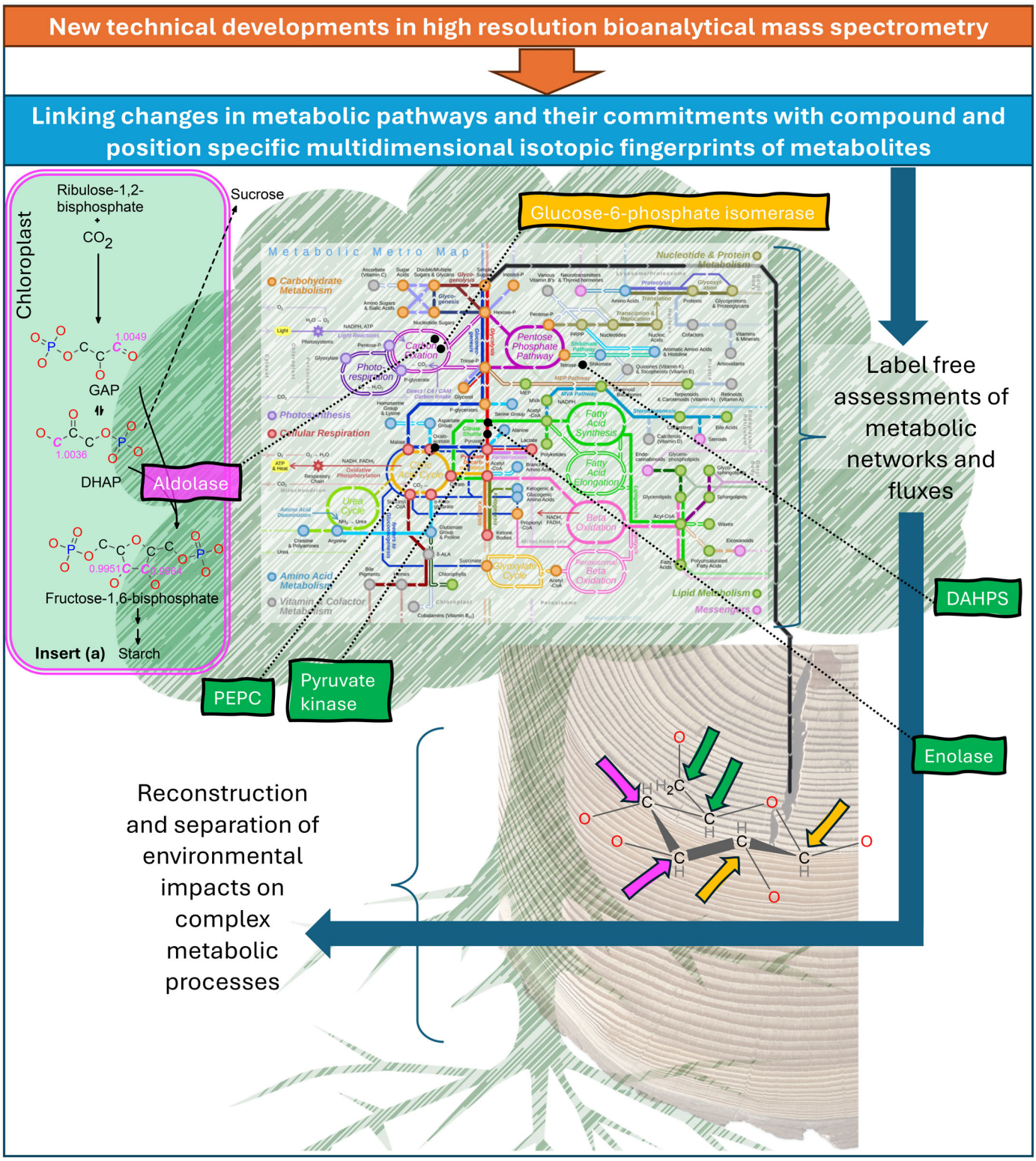
What can such a marriage of two scientific disciplines bring for the future?

While, until now, plant isotope ecology and plant metabolomics have been two largely separated fields – though with some overlap (e.g. Werner *et al.*, 2011; Gilbert *et al.*, 2012b; Jansen *et al.*, 2014) – the new analytical developments have the potential to bring them closer. Looking at the disparate history, we cannot guarantee it will be a love match from the beginning (but see Shakespeare, 2008; we, however, hope for a better ending). Such a marriage of scientific disciplines will, however, provide short- and long-term improvements for both sides and novel data analysis and bioinformatic tools that take advantage of machine-learning approaches will help (Liebal *et al.*, 2020). As the expression of isotope effects leading to intramolecular (position-) and compound-specific differences depends on the commitment of particular pathways and the activation or deactivation of others, label-free metabolic pathway and metabolic flux analysis might become possible, as described above. Especially position-specific fingerprints of multiple isotopes (i.e. 'multidimensional isotopic fingerprints') of particular compounds will allow to differentiate metabolic information from upstream metabolic pathways. We here provide an example to highlight the potential of both the new technological development as well as the sharing of knowledge between disciplines for obtaining new insights into plant–environment interactions (Fig. 1). It is known that the glucose moieties of cellulose in tree rings, which are the classical dateable biological archive, show position-specific differences in the carbon isotope composition (Wieloch *et al.*, 2018). These authors also observed that not all intramolecular C atoms of the glucose moieties of tree-ring cellulose showed the same relationship to the main environmental drivers of iWUE, thus blurring the overall tree-ring $\delta^{13}C$ signal. Position-specific analysis indicated that especially C-1 and C-2 conserved the original C isotope signal of photosynthetic carbon isotope discrimination. Wieloch *et al.* (2018) postulated that carbon isotope effects in the glucose-6-phosphate isomerase-catalysed reaction from fructose-6-phosphate to glucose-6-phosphate imprinted specific ^{13}C isotope signatures on the C-1 and C-2 positions of the tree-ring glucose moieties. This assumption was based on the fact that this enzyme is the only one that simultaneously modifies the C-1 and C-2 bonds of glucose-6-phosphate and, hence, glucose units in tree-ring cellulose. An isotope effect imprinted by glucose-6-phosphate isomerase would, thus, explain the close similarity in the C-1 and C-2 carbon isotopic signal.

Moreover, the impact of photosynthetic activity on both photosynthetic isotope fractionation and substrate commitment to glucose-6-phosphate isomerase (Wieloch *et al.*, 2024) explains why C-1 and C-2 track drivers of iWUE well. The isotope compositions of C-5 and C-6 were assumed to be influenced by leaf-level enolase, phosphoenolpyruvate carboxylase, pyruvate kinase and 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthase (Fig. 1), the latter being a key enzyme of the shikimate pathway (Wieloch *et al.*, 2022b). In addition, it is well known (as also described above) that chloroplastic aldolase (Fig. 1) affects the C-3 and C-4 positions of hexoses (Rossmann *et al.*, 1991) and that photorespiration and transitory starch accumulation vs export of triose-phosphate out of the chloroplast affects the actual isotope effects (Tcherkez *et al.*, 2004).

It needs to be mentioned that all these findings and postulates are either results of 'reverse modelling' (Tcherkez *et al.*, 2004), where the model is applied to quantify putative isotope effects for enzymatic reactions based on available intramolecular C-isotope distribution of one compound (such as glucose) or rather qualitative estimates of mechanisms based on the most plausible

Fig. 1 Potentials for metabolomics and stable isotope ecology in a combined assessment of compound and position-specific stable isotope signatures. The common basis will be to understand how multiple isotope signatures in various metabolites and of specific atom positions in these metabolites are affected by changes in metabolic pathway commitments. This can be the basis for (a) label-free assessment of metabolic fluxes and (b) the reconstruction of impacts of environmental conditions on the plant metabolism from datable archives (glucose moieties prepared according to Betson *et al*. (2006) of tree-ring cellulose as an example, where the intramolecular position-specific isotope ratios of C, O and H can be determined). Here, we show how different enzymatic reactions in the plant metabolism are assumed to affect the ^{13}C isotope signal at different intramolecular C positions of the glucose moieties in cellulose. Aldolase (magenta box) in the chloroplast is well known to affect the C-3 and C-4 positions of hexose (Rossmann *et al*., 1991) (magenta arrow in the glucose scheme). In insert (a), we depict the aldolase reaction and the C atoms (in magenta) affected in DAHP and GAP (the substrates, which are converted to sugars such as sucrose) as well as in fructose-1,6-bisphosphate (the product, from which transitory starch is produced). The isotope effects are given in magenta next to the affected C atoms. Wieloch *et al*. (2018) suggested that isotope effects in the glucose-6-phosphate reaction (yellow box) imprinted specific isotope signatures on the C-1 and C-2 positions (yellow arrows). The C-5 and C-6 positions (green arrows) were assumed to be concertedly affected by pyruvate kinase, phosphoenolpyruvate carboxylase (PEPC), enolase and 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase (DAHPS) (green boxes) (Wieloch *et al*., 2022b). Note that the list of putative enzyme-specific isotope effects is most likely incomplete. Any change in the commitment of substrates to the particular enzymatic reactions will change their actual isotope effects and thus affect the position-specific isotope signal in the cellulose monomers. Comparable mechanisms apply to position-specific oxygen and hydrogen signals. Hence, the position-specific fingerprint of multiple isotopes in the cellulose of tree rings can be used to reconstruct environment–metabolism relationships in the past. In addition, other compounds in dateable biological archives, such as lignin monomers and storage proteins in tree rings, keratin in animal hairs and feathers or bone collagen, might provide additional information on other metabolic pathways. Such metabolic reconstructions require extensive knowledge of the relationships between metabolic fluxes and commitments on the one side and isotope effects on the other. The Metabolic Metro Map is from https://commons.wikimedia.org/wiki/File:Metabolic_Metro_Map.svg (licensed under the Creative Commons Attribution-Share Alike 4.0 International license).



effects in the upstream metabolism (Wieloch *et al.*, 2018). Reverse modelling approaches also provide information on the origin of sucrose, either directly produced via dihydroxyacetone phosphate (and thus during the day) or from starch via maltose (Gessler *et al.*, 2008). This is, hence, a way to disentangle the potentially variable contribution of 'day' vs 'night' sucrose for tree-ring cellulose production.

Reverse modelling is an important and promising approach to linking measured position-specific isotope signatures of a metabolite with the downstream metabolism. Without exact knowledge of

in vivo isotope effects of the major enzymatic reactions and fluxes through different metabolic pathways, however, it remains a blunt sword for reconstructing both, metabolic pathways and the impact of the environment on them, from selected metabolites or constituents of biological archives. Such models are, at present, well suited for testing specific hypotheses on how a specific change in the substrate commitment for an enzyme will affect a downstream metabolite but cannot reflect the real world's complexity. So, we can take the results displayed in Fig. 1 as hypotheses derived from such approaches, which can and need to be tested with novel methodologies as described in the workflow below.

Harnessed with new UHPLC-HRMS methodologies and integrating the knowledge of metabolomics and stable isotope ecology, we will go a step further, being able to quantitatively link the position-specific isotope information of the compounds in biological archives (such as tree rings) with metabolic fluxes and commitments. This will allow us to reconstruct how environmental conditions in the past affected plant metabolism. This not only enables us to verify the assumed effects shown in Fig. 1 but also to discover additional isotope effects and their drivers, especially when isotopes of multiple elements are included. To obtain this aim, we need, amongst others, the following specific analyses and workflows:

(**1**) *Label-free metabolic flux analysis*: as detailed above, paired experiments (with and without labelling) are needed to link leaf-level fluxes and commitments with specific isotope effects.

(**2**) *Environment–metabolic flux interactions*: if, at a later stage, plant–environment interactions based on the environmental impacts on metabolic pathways and fluxes are to be reconstructed from biological archives, the sensitivities of the metabolism to particular environmental drivers need to be well characterised. Soil water availability, temperature, light, water vapour pressure deficit (VPD) and other impacts on pathway commitments, fluxes and related kinetic isotope effects need particular attention.

(**3**) *Linkage between short-term flux assessment and the effects on precursors for building the tree-ring archives*: label-free flux analysis needs to be combined with position-specific isotope analyses of compounds that are the building blocks of archives. For assessing tree rings, in the first step, leaf-exported, phloem-transported sucrose can be targeted. To include metabolic processes in heterotrophic tissues, glucose phosphates in the stem cambial tissues (glucose-1-phosphate, glucose-6-phosphate, UDP-glucose) should be analysed additionally.

(**4**) *Transfer of the position-specific isotopic fingerprints* from chemical precursors (e.g. UDP-glucose) into the biological archive (e.g. glucose moieties in cellulose for tree rings). Biological archives temporally integrate short-term metabolic information, and in tree rings, for example, the annual average isotope signal is often used for climate reconstructions (Treydte *et al.*, 2024). However, already with classical isotope analysis, the comparison between the isotopic signatures of, for example phloem sugars and temporally resolved intra-annual sections of tree rings allowed to quantify the transfer of isotopic information and delays or restrictions thereof into the archive (Gessler *et al.*, 2014; Treydte *et al.*, 2014) with temporal resolutions of days and weeks. Comparable studies need to be performed with position-specific isotope information. The high sensitivity of HRMS might allow to dissect tree rings into even thinner slices as less material is required compared to IRMS, thus increasing the temporal resolution (for intra-annual studies).

(**5**) *Reconstruction of metabolic pathways from biological archives*. Once relationships between metabolic fluxes, isotope effects and the multidimensional isotopic fingerprints in biological archives are established and integration times of archives are defined, better-informed reverse modelling approaches (*sensu* Tcherkez *et al.*, 2004) can be applied to predict metabolic fluxes from the isotope information in the biological archive. In the first experiments, it is advised to carry out experiments under controlled conditions where label-based fluxomics analyses have been performed independently and can serve to validate the predictions.

Overall, with such approaches, the position-specific isotopic fingerprint will allow us to better understand and reconstruct changes in metabolic pathway commitments that go far beyond stomata- and RubisCO-related processes. We focused above on carbon isotopes, but pathway-specific intramolecular isotope fingerprints can also be found for other elements. The comparison of intramolecular hydrogen isotope distribution on sugars from C_3 and C_4 plants can be used to derive information on photorespiration and compartmentation of photosynthesis in the leaves (Ehlers *et al.*, 2015; Zhou *et al.*, 2018) and the intramolecular hydrogen isotope distribution is maintained in tree-ring cellulose (Betson *et al.*, 2006). Moreover, hydrogen at positions 1, 2 and 3 of tree-ring glucose seems particularly responsive to drought conditions, which is probably related to physiological adjustments in the leaf- and stem-level metabolism (Wieloch *et al.*, 2022a, 2024). While not much research has been dedicated to intramolecular nitrogen isotopes (Werner & Schmidt, 2002; Sacks & Brenna, 2005), there is evidence that the position-specific oxygen isotope distribution provides information on the isotopic exchange between sugars and reaction water during heterotrophic cellulose synthesis (Waterhouse *et al.*, 2013). Oxygen at positions 2 and 4 in glucose moieties of cellulose does not exchange with unenriched xylem water during cellulose synthesis in the trunk of trees, thus conserving the evaporative enrichment signal that is passed from leaf water to leaf glucose and which is strongly related to the water vapour pressure of the air. Oxygen atoms at positions 5 and 6, by contrast, undergo *c.* 80% exchange with xylem water, thus providing isotopic information of the tree's water source and precipitation.

In summary, the 'multidimensional isotopic fingerprint' consisting of the position-specific isotope composition of multiple elements in metabolites can provide a linkage between metabolism and environment that is traceable with information from biological archives. While we focused on tree rings here, other dateable biological archives (such as plant residues in sediment cores, animal teeth, hairs, feathers, or fish otoliths) could also be assessed.

For the single fields of metabolomics and stable isotope ecology, the technical advancements allowing to assess multidimensional isotopic fingerprints will already lead to new applications and innovations (Fig. 2).

The perspective of label-free metabolic flux analysis alone provides new perspectives for plant phenotyping as it only involves sampling and subsequent analyses and does not require ^{13}C

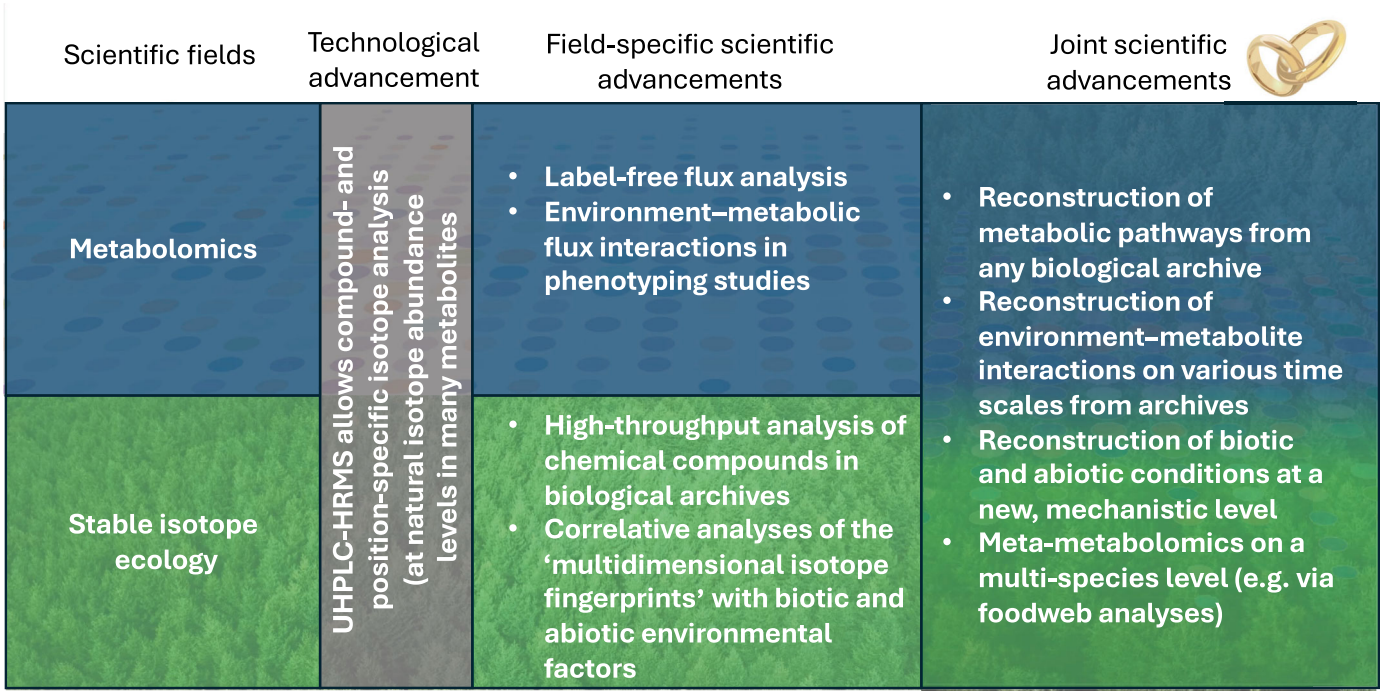


Fig. 2 Scheme showing how the technological advancement brought by new generations of ultra-high-performance liquid chromatography-high-resolution mass spectrometers (UHPLC-HRMS) systems might impact the specific fields of metabolomics and stable isotope ecology. However, if the fields strongly collaborate (and that is what our metaphorical term 'marriage' is meant to represent), many additional and further advancements can become possible.

labelling. For stable isotope ecology, chemical constituents of any biological archive can be assessed for position-specific multi-isotope signals. This will vigorously advance our ability to better reconstruct past environmental impacts on organisms on a statistical basis (as already shown for ^{13}C alone; Wieloch *et al.*, 2018).

The full potential (Fig. 2) will be, however, developed by a metaphorical marriage of metabolomics and stable isotope ecology. Integrated information on the metabolism and its fingerprint in biological archives could be used to better understand mechanistically how plants reacted to past environmental drivers, thus also providing helpful information on the trajectories of the vegetation under the conditions to come. Moreover, new applications might emerge not only for the temporal axis within a single organism. Stable isotope ecology also disentangles ecological connections between organisms, and isotopic foodweb analyses (Layman *et al.*, 2012; Elliott *et al.*, 2021) are a popular example. Applying the new technology and the assessment of 'multidimensional isotopic fingerprints' might have the potential to scale up metabolomics and metabolic flux analysis to a community or ecosystem level in the sense of 'meta-metabolomics' (Hajjar *et al.*, 2023; Fig. 2). This will help to understand better interactions between organisms and to assess how changing environmental conditions affect this interplay. In conclusion, we can state that a strong integration of the two fields provides jointly much more than a single discipline might achieve in the face of new technological developments. Real eco-metabolomics (Nagler *et al.*, 2018) and meta-metabolomics that allow quantifying processes outside the laboratory are now within reach.

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All authors contributed to discussing and creating the new framework of the Viewpoint and were included in writing the manuscript.

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