

Establishment and Validation of a Targeted Phytohormone Analysis Method Integrated with Untargeted Metabolomics to Study Metabolic Adaptations in Solanaceae Food Crops

Zur Erlangung des akademischen Grades eines

DOKTORS DER NATURWISSENSCHAFTEN

(Dr. rer. nat.)

von der KIT-Fakultät für Chemie und Biowissenschaften
des Karlsruher Instituts für Technologie (KIT)
genehmigte

DISSERTATION

von

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Tag der mündlichen Prüfung: 17.04.2026

Erklärung zu Fremd- und Eigenleistungen

Die Arbeiten dieser Dissertation wurden zwischen August 2022 und März 2026 am Institut für Sicherheit und Qualität bei Obst und Gemüse (Institut OG) des Max Rubner-Instituts (MRI) durchgeführt. Die Ergebnisse wurden bislang nicht in Fachzeitschriften veröffentlicht, einzelne Aspekte wurden jedoch in Form von Vorträgen und Posterbeiträgen auf Fachtagungen präsentiert. Diese Publikationen sind in Kapitel 9 dieser Arbeit aufgelistet. Sie werden in anderen Abschnitten dieser Arbeit nicht weiter hervorgehoben oder zitiert.

Im Verlauf dieser Arbeit wurden Metabolomanalysen und Phytohormonanalysen in verschiedenen Pflanzenmaterialien durchgeführt. Die Kultivierung der Pflanzen für die Untersuchung fand dabei zum Teil im Rahmen kooperativer Projekte statt. Die in dieser Arbeit untersuchten Tomatenpflanzen wurden jedoch vollständig am Institut OG kultiviert. Dabei wurde die Anzucht der Pflanzen und der *Trichoderma afroharzianum*-Kulturen von technischen Mitarbeitenden des Instituts OG unterstützt. Die Probenaufarbeitung und Messung der Metabolomanalysen wurde teilweise ebenfalls durch Mitarbeitende des Instituts durchgeführt.

Die in dieser Arbeit untersuchten, transgenen Kartoffelpflanzen wurden von der Arbeitsgruppe für Molekulare Phytopathologie des KIT unter der Leitung von Prof. Dr. Natalia Requena erzeugt und kultiviert. Die Ernte der Pflanzen erfolgte gemeinsam durch den Autor dieser Arbeit und Mitarbeitende der Arbeitsgruppe. Alle weiteren Aspekte der Metabolomanalysen wurden im Rahmen dieser Arbeit allein bearbeitet. Parallele molekularbiologische Analysen der Arbeitsgruppe für Molekulare Phytopathologie wurden in dieser Arbeit nicht verwendet und werden daher nur in einem organisatorischen Kontext erwähnt.

Die in dieser Arbeit untersuchten Reispflanzen wurden am Botanischen Institut des KIT unter der Leitung von Prof. Dr. Peter Nick kultiviert. Die Kultivierung der Pflanzen sowie die Phytohormonanalytik der gewonnenen Proben wurden im Rahmen der Masterarbeit von Thérèse Dreßler durchgeführt und dort dokumentiert. Die Masterarbeit wurde wissenschaftlich von Prof. Dr. Peter Nick und Prof. Dr. Mirko Bunzel (Arbeitsgruppe Lebensmittelchemie und Phytochemie, KIT) geleitet. Die praktische Durchführung erfolgte zwischen März und Oktober 2024 am Institut OG des MRI unter der Betreuung des Autors dieser Arbeit. Das experimentelle Design der Versuche wurde vom Autor dieser Arbeit entwickelt, während die Reispflanzen gemeinsam mit der Studierenden geerntet wurden. Die Phytohormonanalytik der gewonnenen Proben wird in dieser Arbeit nur knapp zusammengefasst, da die Ergebnisse Forschungslücken schließen, die für den Verlauf der Arbeit von hoher Relevanz waren. Die Metabolomanalyse der Reispflanzen wurde in praktischen Aspekten zum Teil von der Studierenden ausgeführt. Die in dieser Arbeit dargestellte Auswertung, Interpretation und wissenschaftliche Einordnung der Daten erfolgte ausschließlich durch den Autor dieser Dissertation. Diese Beteiligungen sind in den entsprechenden Abschnitten der Arbeit nochmals explizit hervorgehoben.

Erklärung zur Nutzung von Künstlicher Intelligenz (KI)

Der in dieser Arbeit präsentierte Text wurde vom Autor selbst verfasst. Generative KI wurde nicht zur Erstellung ganzer Textpassagen eingesetzt. Zur sprachlichen Korrektur einzelner Sätze und kurzer Textabschnitte wurden das KI-basierte Tool DeepL Write (DeepL SE, Köln) und der KI-Chatbot Gemini 3 (Google Ireland Limited, Dublin) unterstützend verwendet.

Darüber hinaus kamen bei der Erstellung des R-Codes, der zur Datenauswertung in dieser Arbeit eingesetzt wurde, verschiedene KI-Tools unterstützend zum Einsatz. Dazu gehören Gemini 3, ChatGPT 4.o und 5 (OpenAI Ireland Limited, Dublin) sowie Claude Sonnet 4 (Anthropic Ireland Limited, Dublin). Dabei wurden KI-generierte Codeabschnitte sorgfältig geprüft, angepasst, und eigenständig in übergeordnete Strukturen eingearbeitet. Insbesondere die Codearchitektur der in dieser Arbeit vorgestellten Datenanalyse-Pipeline („*data analysis pipeline*“, s. Kapitel 3.2.5), wurde eigenständig entwickelt. Alle Teile des Codes wurden kommentiert, um die jeweiligen Funktionen darzulegen und den Code anpassbar und vielseitig nutzbar zu machen.

KI-Tools wurden nicht für Zwecke der Datenanalyse und -interpretation, der Ergebnisinterpretation oder wissenschaftlichen Schlussfolgerung eingesetzt.

Deutsche Zusammenfassung der Arbeit

Pflanzen passen sich mithilfe chemischer Signalnetzwerke an Umweltveränderungen an. Diese Netzwerke werden durch Pflanzenhormone (Phytohormone) gesteuert, die Stoffwechselprozesse regulieren und so Reaktionen auf Stress sowie mikrobielle Interaktionen vermitteln. Ein tieferes Verständnis dieser Signalwege und Anpassungsmechanismen ist entscheidend, um die Widerstandsfähigkeit von Nutzpflanzen unter sich verändernden Umweltbedingungen zu erhöhen. Dies ist insbesondere im Hinblick auf den fortschreitenden Klimawandel von hoher Bedeutung.

Ungerichtete Metabolomanalysen sind leistungsfähige Instrumente, um den physiologischen Zustand von Nutzpflanzen zu erfassen und die Qualität ihrer Produkte zu bewerten. Die Sensitivität dieser breit angelegten Methoden reicht jedoch oft nicht aus, um endogen niedrig konzentrierte Phytohormone zu detektieren. In dieser Arbeit wurde daher ein dualer analytischer Ansatz etabliert, der die ungerichtete Metabolomanalyse mit einer hochsensitiven, gezielten Phytohormonanalyse kombiniert. Mit dieser Strategie wurden anschließend die Anpassungsmechanismen von Kulturpflanzen der Familie der Nachtschattengewächse (Solanaceae) sowie ihre Wechselwirkungen mit Bodenmikroorganismen untersucht.

Zunächst zeigte die UHPLC-MS-Metabolomanalyse transgener Kartoffelpflanzen, die Effektoren arbuskulärer Mykorrhizapilze exprimierten, deutliche Verschiebungen im Phenylpropanoid-Stoffwechsel. Dies lieferte wertvolle Einblicke in die Auswirkungen einer Pilz-Pflanzen-Symbiose. Eine mangelnde Phytohormondetektion verhinderte jedoch die vollständige Aufklärung der zugrunde liegenden Mechanismen. Um diese Lücke zu schließen, wurde eine gezielte Analysemethode für 63 Metabolite aus acht Phytohormon-Stoffwechselwegen entwickelt und nach SANTE-Richtlinien umfassend validiert. Dazu wurde eine kürzlich publizierte, breit angelegte UHPLC-MS/MS Methode adaptiert und in entscheidenden Aspekten optimiert. Neben einer Verbesserung der chromatographischen Auflösung wurde die Probenaufarbeitung für gefriergetrocknetes Pflanzenmaterial angepasst und eine Datenanalyse-Pipeline eigens in R entwickelt. Analysen an Kartoffel- und Reispflanzen während der Methodenentwicklung unterstützten diese Anpassungen.

Die finale, validierte Methode wurde auf Tomatenpflanzen angewandt, die Hitzestress ausgesetzt wurden und mit dem pflanzenförderlichen Pilz *Trichoderma afroharzianum* inokuliert wurden. Dabei wurden über 40 Analyten detektiert, von denen 35 eindeutig identifiziert wurden. Es wurde gezeigt, dass die Methode sowohl umfangreiche metabolische Veränderungen unter Hitzestress als auch subtile Modulationen der Pilzinteraktion umfassend und zuverlässig erfasst. Die kombinierte Anwendung mit Metabolomanalysen führte zu einem deutlichen synergistischen Informationsgewinn, da regulatorische Signale mit einem breiteren metabolischen Kontext verknüpft werden konnten. Dieser duale Ansatz stellt somit ein wertvolles Werkzeug für eine ganzheitliche Betrachtung pflanzlicher Anpassungsstrategien dar.

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Table of abbreviations

2D	Two-dimensional
30% ACN	Acetonitrile : water (30:70, v/v)
50% ACN	Acetonitrile : water (50:50, v/v)
abf	Analysis base file (format)
ACN	Acetonitrile
ADP/ATP	Adenosine di-/triphosphate
AM	Arbuscular mycorrhiza(e)
ANOVA	Analysis of variance
CE	Collision energy
CLD	Compact letter display
CoA	Coenzyme A
CSH	Charged surface hybrid
CT	Control (sample)
CV	Coefficient of variation
CXP	Cell exit potential
DMAPP	Dimethylallyl diphosphate
DP	Declustering potential
ESI	Electrospray ionization
ETI	Effector-triggered immunity
EV	Empty vector
FA	Formic acid
FAO	Food and Agriculture Organization of the United Nations
FC	Fold change
FDR	False discovery rate
FDR-p	FDR corrected p-values
FW	Fresh weight
GC	Gas chromatography
GCB	Graphitized carbon black
HCT	Hydroxycinnamoyl-CoA:quinic acid hydroxycinnamoyl transferase
HLB	Hydrophilic-Lipophilic-Balanced
HT (plants)	High temperature (tomato plants)
ICH	International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use
ID	Identifier
IDA	Information-dependent acquisition
IS	Internal standard(s)
LC	Liquid chromatography
LLOQ	Lower limit of quantification

LOD	Limit of detection
LT (plants)	Low temperature (tomato plants)
MeOH	Methanol
MRM	Multiple reaction monitoring
MS	Mass spectrometry / mass spectrometer
MSI	Metabolomics Standards Initiative
NAD(P)	nicotinamide adenine dinucleotide (phosphate)
NMR	Nuclear magnetic resonance
PAL	Phenylalanine ammonia-lyase
PAMP	Pathogen-associated molecular patterns
PAMP	Pathogen-associated molecular patterns
PC	Principal component
PCA	Principal component analysis
PGPR	Plant growth-promoting rhizobacteria
PLP	Pyridoxal phosphate
PPFD	Photosynthetic photon flux density
PTFE	Polytetrafluoroethylene
PTI	Pattern-triggered immunity
Q1	Quadrupole 1
QC	Quality Control
QTOF-MS	Quadrupole time-of-flight MS
QuEChERS	<u>Q</u> uick, <u>e</u> asy, <u>c</u> heap, <u>e</u> ffective, <u>r</u> ugged, and <u>s</u> afe
R²	Coefficient of determination
RMSE	Root mean square error
RT	Retention time
SANTE	European Commission - Directorate-General for Health and Food Safety
SD	Standard deviation
SPE	Solid phase extraction
StS	Study sample
(U)HPLC	(Ultra) high performance liquid chromatography
ULOQ	Upper limit of quantification

Note: Analyte abbreviations are not listed here; they are provided in the Appendix (Table A-17).

1 Introduction

1.1 Increasing Challenges Affecting Food Crops

To meet the demands of a growing global population, modern agriculture has adopted practices that, in the past decades, have heavily prioritized production efficiency over other factors [1-3]. These practices involve selective breeding of high-yielding varieties of crops and widespread use of pesticides and mineral fertilizers. While global crop yields have been growing for decades as a result (see [4]), these practices have also caused drastic environmental impacts, including soil degradation, large-scale land-use change, loss of biodiversity, and widespread contamination of soils and freshwater resources [3, 5-8]. Crucially, the long-term emphasis on maximizing yields in crop breeding has often occurred at the expense of preserving natural adaptation and resilience mechanisms, rendering modern food crops increasingly vulnerable to the intensifying pressures of a changing environment [9-11].

Additionally, the effects of global climate change have already been shown to significantly impact food crop stability. Numerous reports and meta-analyses provide robust evidence that heat waves, droughts, and extreme weather events are becoming more frequent and severe, resulting in significant crop losses and agricultural land devastation [12-14]. Furthermore, it is expected that climate change will accelerate the anthropogenic degradation of natural biomes [6, 15] and lead to an increase in crop infestation by insects [16] and fungi [17, 18]. These detrimental effects can create self-reinforcing feedback loops, where attempts to counter them further increase the overuse of fertilizers and pesticides, as well as the clearing of natural landscapes to create new agricultural land [3, 11].

As the world population is continuously growing and climate change progresses, the Food and Agriculture Organization of the United Nations (FAO) projects that risks to agricultural productivity and food security are likely to increase, unless effective measures are implemented soon [3, 19]. Consequently, food crops are expected to be exposed to an increasing number of simultaneous stress factors [11, 20]. Generally, plants have the ability to sense and adapt to external stressors. In plant science, stresses exerted on plants are usually categorized as biotic or abiotic, both of which have been extensively reviewed (see [21-24] and [25-28]). Figure 1 summarizes major stress factors, but it is important to note that biotic and abiotic processes are not independent and strongly modulate each other [20, 29].

Because maintaining these complex response mechanisms has historically not been a priority in breeding programs, modern cultivars often lack the robustness of their respective wild types. Despite recent efforts to (re)introduce adaptive genes into food crops [9, 10, 30] and to implement

more sustainable agricultural practices [1, 19, 31, 32], such undertakings require time. Furthermore, the molecular and biochemical mechanisms by which plants react to stress have not been fully elucidated for all food crops, especially when considering combinations of biotic and abiotic stresses [11, 20, 33]. Improving our understanding of these processes is essential for developing the sustainable cultivation and breeding practices needed for future food security.

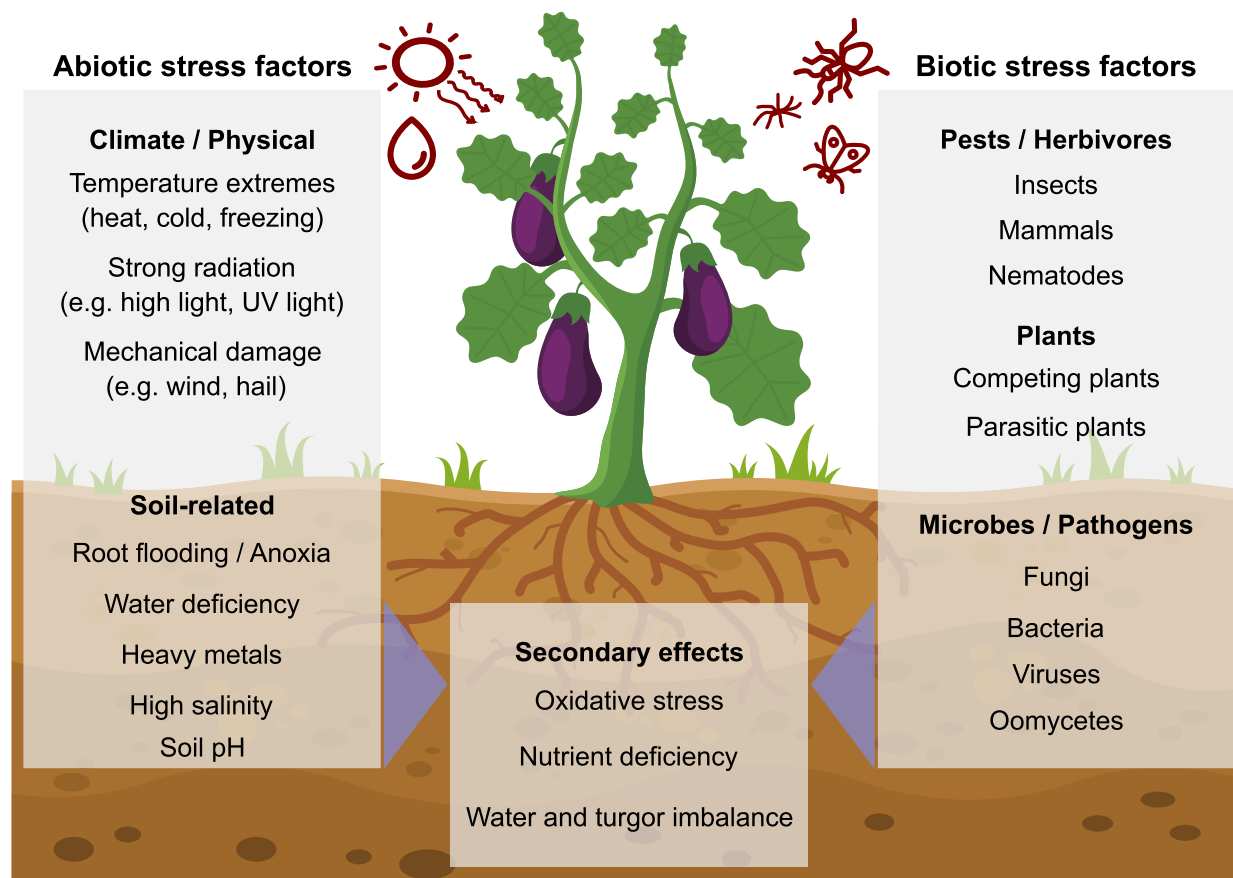


Figure 1: Major abiotic and biotic stressors on plants, as well as secondary effects that can result from either stress type. Sources are listed in the body text.

1.2 Global Relevance of Solanaceae Food Crops

The nightshade family (Solanaceae) is of significant global importance for agriculture, human nutrition, and plant research. This diverse family includes several staple and high-value vegetable crops, such as tomatoes (*Solanum lycopersicum* L.), potatoes (*Solanum tuberosum* L.), eggplants (*Solanum melongena* L.), and peppers (*Capsicum* spp. L.) [34, 35].

Nightshade crops can constitute a source of calorie-dense tubers (e.g. potatoes), but mainly produce various fleshy fruits that differ strongly in their morphologies (e.g. tomatoes, eggplants, peppers, goji berries, and physalis) [35, 36]. These products are sought after for their sensory and/or nutritional qualities, and many are valued specifically for their high content of secondary

metabolites [35, 37]. A metabolic characteristic shared by Solanaceae plants is their production of glycoalkaloids, which function as defensive compounds against herbivores and pathogens, and are frequently toxic to humans at elevated concentrations [38]. However, due to selective breeding practices, the commonly consumed parts of nightshade food crops rarely cause serious human intoxication nowadays [39].

The cultural and culinary importance of these plants is reflected in their global production statistics. Potatoes rank third of all crops in global production tonnage after rice and wheat (when excluding crops used primarily for feed or processing) [40]. Tomatoes have the highest production tonnage among culinary vegetables, while eggplants and peppers also consistently rank among the top ten vegetables produced globally [41].

Besides their economic and nutritional value, the Solanaceae have also served as model systems in plant research since the early nineteenth century [34]. For instance, tomato has been a primary model in plant genetics and genome analysis, tobacco (genus *Nicotiana* L.) has been a primary model for cell culture-based genetics, and potato has been a model plant for research on tuber development. Due to their morphological variety, their wide range of secondary metabolites, and many decades of established research, nightshade plants are exceptionally well-suited for studying plant metabolism and stress responses.

1.3 Analysis of Metabolic Processes in Plants

Metabolic processes in plants are highly complex, and many are still not fully understood [42]. This is partly due to the large diversity of taxa in the plant kingdom, which leads to variations in metabolic pathways and unique processes exclusive to some of these taxa [42, 43]. However, even at the species level, genetic variation and environmental factors can lead to substantial differences in metabolic profiles.

The magnitude of chemically distinct species within plants is immense. Estimates vary considerably, but numbers over 100,000 individual metabolites in the plant kingdom and at least several thousand in individual plants are frequently reported [43, 44]. The term metabolite refers to any small organic molecule, often defined as < 1,500 Da, that is present in a (biological) matrix, while the sum of all those metabolites in a tissue of interest is commonly referred to as the metabolome [45, 46]. In turn, metabolomics refers to a state-of-the-art method used to comprehensively examine the plant metabolome.

Due to analytical and technical challenges, metabolomics techniques were only developed after genomics, transcriptomics and proteomics methods, which also defined the term [45, 47]. Analyzing a plant's metabolome provides a direct reflection of its physiological state. While

genomics, transcriptomics, and proteomics define the potential of a biological system, metabolomics represents the final products of gene expression and therefore the functional outcomes (Figure 2). Furthermore, metabolome analysis also covers exogenous compounds taken up by the plant. Thus, evaluating the plant metabolome provides insight into plant adaptations to external factors but also into the nutritional profile and quality of their produce, which cannot be fully captured by other “omics” methods. Through metabolomics, targeted measures can be developed to improve plant resilience and crop quality [42, 43, 48, 49]. However, the most comprehensive insight into plant metabolism is gained by applying a combination of omics approaches [42, 50, 51].

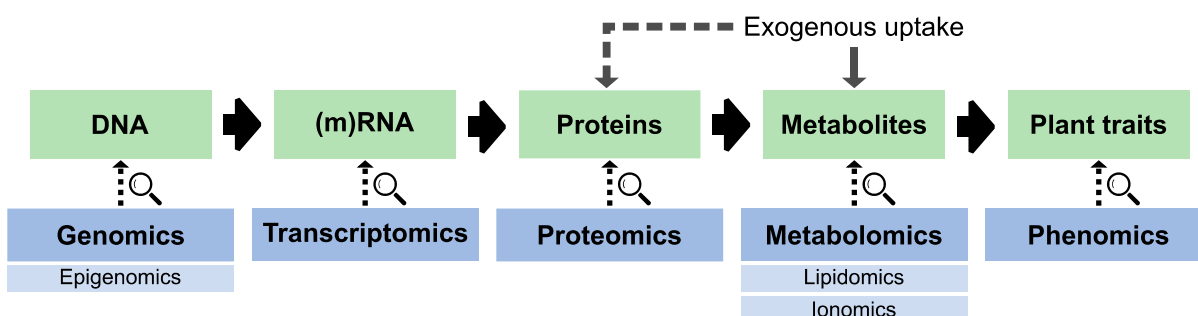


Figure 2: Hierarchical representation of plant trait development levels (green) and the respective comprehensive analysis methods (blue), including closely related but distinct methods (light blue, small print). Sources are listed in the body text.

Metabolome analysis has largely been enabled by modern analytical measurement and detection methods, which allow for the selective and sensitive detection of metabolites [45, 46]. These methods record signals from the numerous metabolites and attempt to annotate or identify them, usually by comparing them with reference libraries comprising authentic reference standards previously measured with the same or comparable methods. Since the first metabolomics applications around the year 2000, two techniques have emerged as the major instrumental metabolomics platforms: nuclear magnetic resonance (NMR)- and mass spectrometry (MS)-based methods [44, 46]. In the latter case, separation by gas chromatography or (ultra) high performance liquid chromatography (GC or (U)HPLC) is most often employed beforehand. NMR-based metabolomics methods offer minimal sample preparation, reproducible quantitative analysis, and non-destructive measurements. However, they lack the wide structural range of MS detection and are more limited in their sensitivity, as concentrations of $> 1 \mu\text{M}$ are often required for analyte detection.

Therefore, MS-based techniques have become the primary choice for metabolomics when capturing low abundance metabolites is intended [44, 46, 49, 52]. In a modern context, MS-based

metabolomics methods are untargeted analysis methods. This means that they aim to capture as many signals as possible to provide a comprehensive profile, often at the expense of absolute quantification and sensitivity for very-low-abundance molecules. In contrast to NMR-based methods, MS methods often require a more complex sample preparation procedure, and usually require internal standards (IS) or quality control strategies to correct for matrix-specific signal interferences and analytical drift.

Naturally, no metabolomics method can capture the entirety of the plant metabolome [53-55]. Each method covers only a subset of metabolites, both in terms of detection and, arguably more importantly, confident identification. Thus, the bias of each method depends greatly on the libraries used for identification. According to the framework set by the Metabolomics Standards Initiative (MSI), the highest level of confidence in metabolite identification is achieved only when a detected signal matches one obtained by measuring an authentic reference standard with the same method and instruments [56]. Other metabolites should be referred to as putatively identified; however, this approach is not universally followed by researchers. These biases, together with the insufficient or differential reporting of biological information for each experiment, make harmonizing metabolome data obtained in various laboratories challenging [53, 57]. To unravel variations in plant metabolism, many more metabolome analysis studies on plants are needed, and these studies must be accompanied by rigorous data reporting.

1.4 Phytohormones as Mediators of Plant Metabolisms

Since plants are sessile organisms, they must be able to adapt to changes in their environment. However, they lack a brain or any other organ that processes information centrally. They also lack a nervous system to relay fast electric signals to and from their various parts. Consequently, they rely primarily on chemical signaling to transmit information. The key metabolites mediating these mechanisms are referred to as plant hormones, or phytohormones.

1.4.1 Concept and Characteristics

Phytohormones act as signal transmitters, regulating the growth and development of plants in balance with environmental changes [58-60]. For instance, they determine whether a plant should prioritize growth or reproduction by either initiating or inhibiting the formation of flowers and fruits. Phytohormones enable plants to react appropriately to abiotic and biotic stress factors, and their research is therefore highly relevant to the understanding of these processes.

The definition of a phytohormone is surprisingly vague. Primarily, they are small organic compounds that cause hormonal action, which involves their recognition by a receptor and the subsequent activation or repression of gene expression [58, 61]. Receptor-mediated signaling

typically involves the induction of a signaling cascade, though some phytohormones can directly bind transcription factors. Other key aspects of phytohormones include their low endogenous concentrations (micromolar to picomolar range, or lower) and their capacity for short- and/or long-distance transport between biosynthesis and receptor binding. This marks a major difference between phytohormones and animal (vertebrate) hormones. In vertebrates, hormones are produced in an endocrine tissue, are transported through the circulatory system, and cause an effect in a different tissue. In contrast, phytohormones can act locally in the tissues where they are synthesized, although transport through the vascular system has been demonstrated for most phytohormone classes [61].

Phytohormones are distinguished by their chemical structures and their associated signaling pathways. However, while these pathways are mostly metabolically distinct, their biological functions are highly interconnected and interregulated [62-65]. When one signaling pathway is activated and induces transcriptional or metabolic effects, feedback loops and competing signals from other signaling pathways often counter or modulate these effects. Capturing and unraveling these manifold interactions between phytohormone signaling pathways, commonly referred to as “crosstalk”, is the key to understanding how plants function and adapt.

A comprehensive review of the complex signaling processes of plants would far exceed the scope of this work. Instead, two major focuses are established. Chapter 1.4.2 provides an overview of the metabolic processes of the most important signaling pathways. Then, Chapter 1.4.3 summarizes the challenges and methods of comprehensive phytohormone metabolite analyses. The complex interactions of signaling pathways are further outlined in Chapter 1.5, in the context of plant interactions with rhizosphere microbes.

1.4.2 Classification and Functions

Phytohormones are divided into several different classes based on their structure and/or function [58, 61, 66, 67]. The classes commonly listed as the most important plant hormones are abscisates, auxins, brassinosteroids, cytokinins, ethylene and its metabolites, gibberellins, jasmonates, salicylates, strigolactones, and peptide hormones. It is important to clarify that these class names function as biological group identifiers rather than strict chemical descriptors. They are intended to group compounds based on their signaling pathways, even when some metabolites are not structurally derived from the hormone that gives the respective class its name.

Furthermore, several other metabolites are considered phytohormones by some researchers, including γ -aminobutyric acid, melatonin, polyamines, and karrikins. While these compounds exhibit mediating functions in plant development, they do not currently meet all the standard

criteria for phytohormones, most notably the requirement for a confirmed receptor-mediated effect. Research advances and arguments for and against the classification of these compounds as phytohormones have been summarized in a recent review [61].

The following chapters outline the metabolism of each major phytohormone class, with the exception of peptide hormones, which are derived directly from protein biosynthesis. Crucially, this work does not detail the receptor interactions or biological effects of each hormone. These aspects are briefly covered for each class, but the main objective is to outline the key metabolites of each phytohormone signaling pathway. Other heavily summarized aspects include the identities of the enzymes catalyzing each metabolic conversion, as well as the tissue specificity and cellular localization of processes. Some exceptions are applied, particularly when these aspects define metabolic branches.

1.4.2.1 Abscisates

(+)-Absciscic acid (ABA) is a key regulator of plant growth and development, as well as plant adaptation to external stress factors [59, 65, 68-70]. While ABA is the only bioactive hormone of its metabolic pathway, all related metabolites are referred to collectively as abscisates. Its biological functions in plant tissues and individual cells depend heavily on its concentration levels, but the specific effects vary by tissue and by the developmental stage of the plant. ABA levels are frequently increased upon stress detection by specialized cells. While ABA is also involved in mediating plant defenses to biotic stressors, its primary function is to maintain plant processes under abiotic stress conditions, such as drought or high temperatures.

Figure 3 shows ABA formation, storage, and inactivation. ABA is synthesized *de novo* from carotenoids located in the plastids of plant cells. Specifically, the C₄₀ carotenoids violaxanthin and neoxanthin are cleaved into xanthoxin, which is then converted in two steps into ABAld and then ABA. ABA levels are regulated by two processes. Bioactive, free ABA is either temporarily inactivated through a reversible glucosylation or oxidatively catabolized into phaseic acid. Phaseic acid is further reduced into DHPA, which can also be glucosylated. In addition to this primary catabolic pathway, alternative routes lead to the formation of the isomer neophaseic acid instead of phaseic acid.

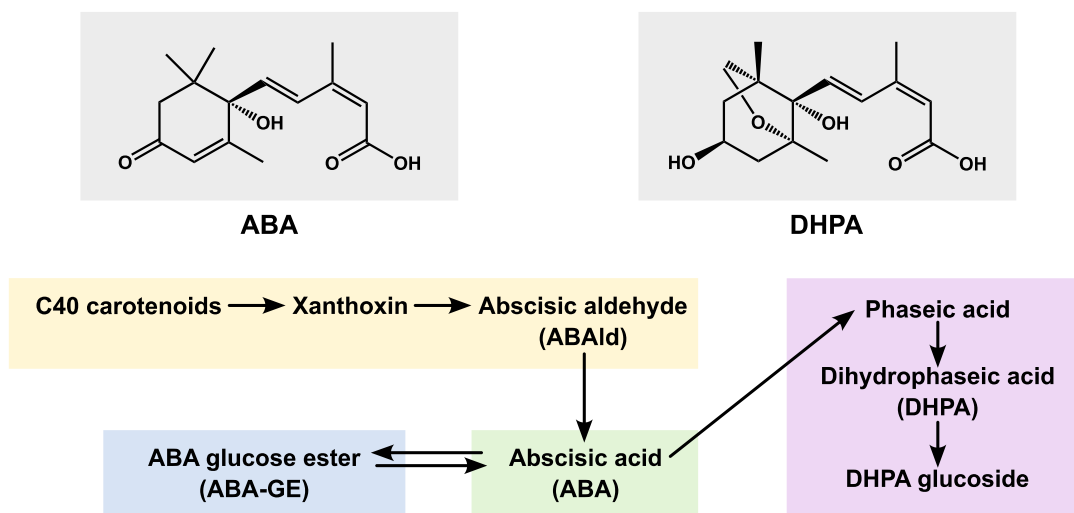


Figure 3: Overview of ABA metabolism. Abbreviated analyte names are provided in parentheses for the metabolites relevant to this work. The metabolites are grouped as bioactive forms (green), biosynthetic precursors (yellow), intermediate forms (blue) and inactivation products (purple). Adapted from [68, 69, 71].

1.4.2.2 Auxins

Auxins are a class of phytohormones spanning multiple bioactive compounds, with indole-3-acetic acid (IAA) being generally regarded as the primary auxin. The functions of IAA and other auxins are numerous and depend heavily on the specific auxin-affected transcription factors expressed by a cell type at a given time [72, 73]. Due to their versatile role in signaling, auxins have been described as “involved in virtually every aspect of plant biology” [73], and basal levels of auxins are present in all plant cells. Hence, extensive coverage of auxin metabolism is required to fully monitor changes in auxin signaling.

The biosynthesis and inactivation of IAA are shown in Figure 4, alongside its main conjugate forms and minor auxins. The central precursor of IAA biosynthesis is the amino acid L-tryptophan, which is transformed into IAA through divergent but interconnected pathways. The immediate precursors of IAA are IAN, IAC, IPY, or indole-3-acetaldehyde, though the biosynthesis route through IPY is considered the primary pathway in many plant species.

Active IAA can be reversibly converted into its glucose ester, into amino acid conjugates, or into its methyl ester. These conjugates primarily serve as storage or transport forms. For example, IAA-ME is less polar than IAA and can diffuse through cell membranes more easily. However, some conjugates of IAA are irreversible, such as IAA-Asp. This constitutes one of the two major IAA inactivation pathways, the other being oxidation at the 2-position of the indole structure,

resulting in OxIAA. Oxidation and conjugation are often combined, with OxIAA subsequently being conjugated or IAA-Asp being oxidized. Alternatively, oxidative carboxylation of IAA can also occur, yielding catabolites such as ICA and indole-3-methanol.

Besides IAA, additional compounds have been described as relevant to auxin signaling. These include several minor bioactive auxins, such as IBA, 4Cl-IAA, IAcrA, and PAA [72, 74]. Furthermore, Indole-3-acetaldoxime and IAN are metabolically connected with the metabolism of various plant defense compounds, such as glucosinolates and camalexin, particularly in Brassicaceae species [72, 75]. Lastly, TPL (indole-3-ethanol) has been hypothesized to serve as a storage pool for indole-3-acetaldehyde [77]. It should be noted that the metabolic pathways presented here focus on well-established routes. However, auxin metabolism is complex, and new pathways or conjugates are regularly discovered; recent advances are reviewed in [78].

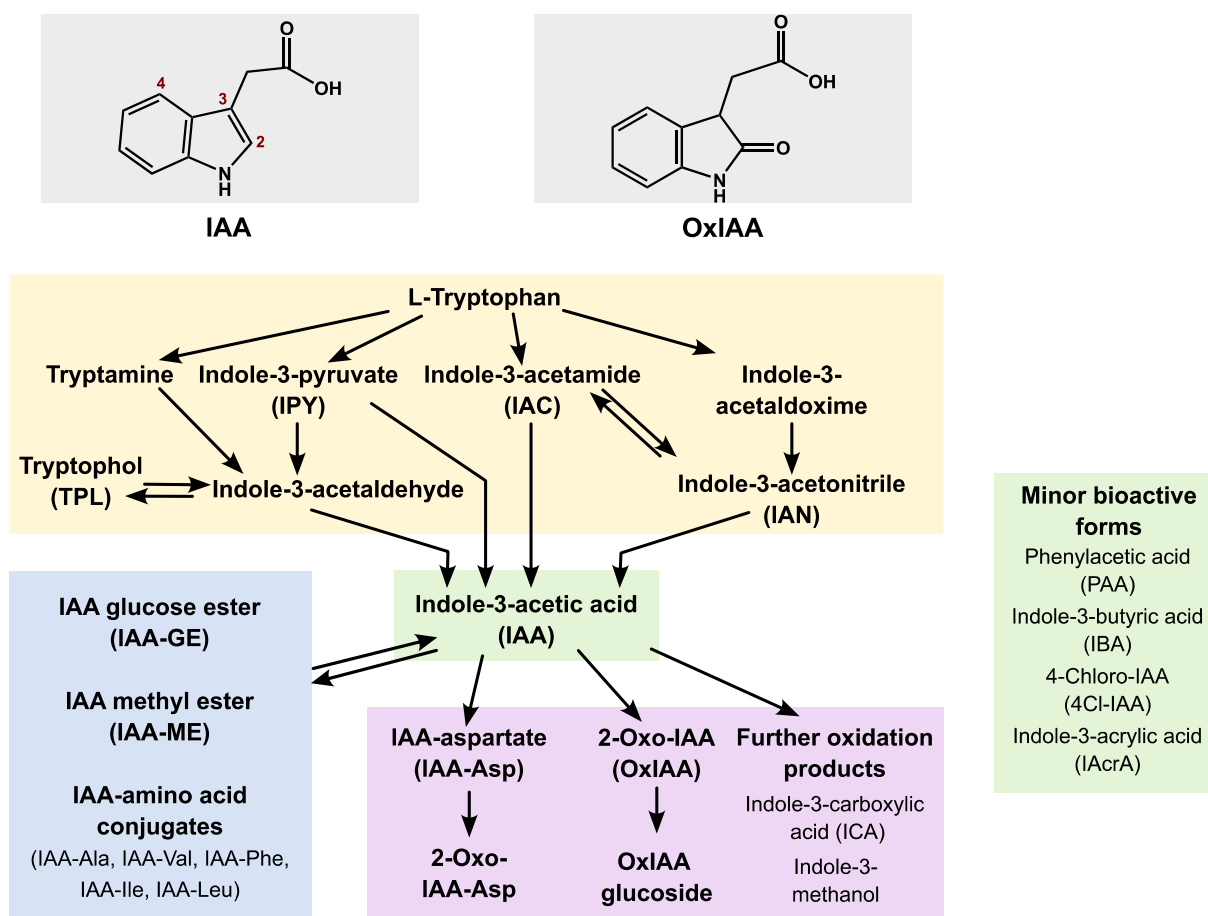


Figure 4: Overview of IAA and auxin metabolism. Abbreviated analyte names are provided in parentheses for the metabolites relevant to this work. The metabolites are grouped as bioactive forms (green), biosynthetic precursors (yellow), intermediate forms (blue) and inactivation products (purple). Adapted from [74-76], with additions from [72, 77].

1.4.2.3 Brassinosteroids

Brassinosteroids are plant steroid hormones that are structurally related to animal steroid hormones [79-83]. However, the functional mechanisms of brassinosteroids differ considerably from their animal counterparts, as they are perceived by membrane-bound receptors instead of intracellular receptors. The primary bioactive brassinosteroid is brassinolide (BL), while other bioactive forms include its epimer, 24-epibrassinolide (epiBL), and its immediate precursor, castasterone (CS).

In plants, brassinosteroids are primarily associated with various functions of plant growth and particularly plant development [79-83]. As such, peak concentrations of brassinosteroids are often found in reproductive plant tissues, such as seeds and pollen. Additionally, brassinosteroids are also involved in plant adaptations to both biotic and abiotic stresses, often modulating the signaling pathways of other plant hormones.

Brassinosteroids comprise an extensive phytohormone class, with over 70 naturally occurring compounds having been reported to date [81, 83], not including their various sterol precursors. Figure 5 summarizes the key biosynthetic precursors and general inactivation mechanisms of BL, starting from cholesterol and campesterol. However, it should be noted that both, the BL biosynthesis and the steroid metabolism preceding cholesterol and campesterol, are a wide, interconnected network of pathways not fully covered here.

From the 3-OH steroid campesterol, a series of oxidation and hydroxylation reactions lead to BL formation, though the order varies between pathways. Other pathways go through either cholesterol or β -sitosterol (not pictured), but all pathways converge at the latest stage in the formation of CS. Interestingly, CS is reported to be the only immediate precursor to BL, and thus presents a crucial metabolic intermediate. BL inactivation is as diverse as its biosynthesis, yielding many potential products, none of which dominate across different plant species. A description of these mechanisms and products would exceed the scope of this work; they are outlined in [83, 84].

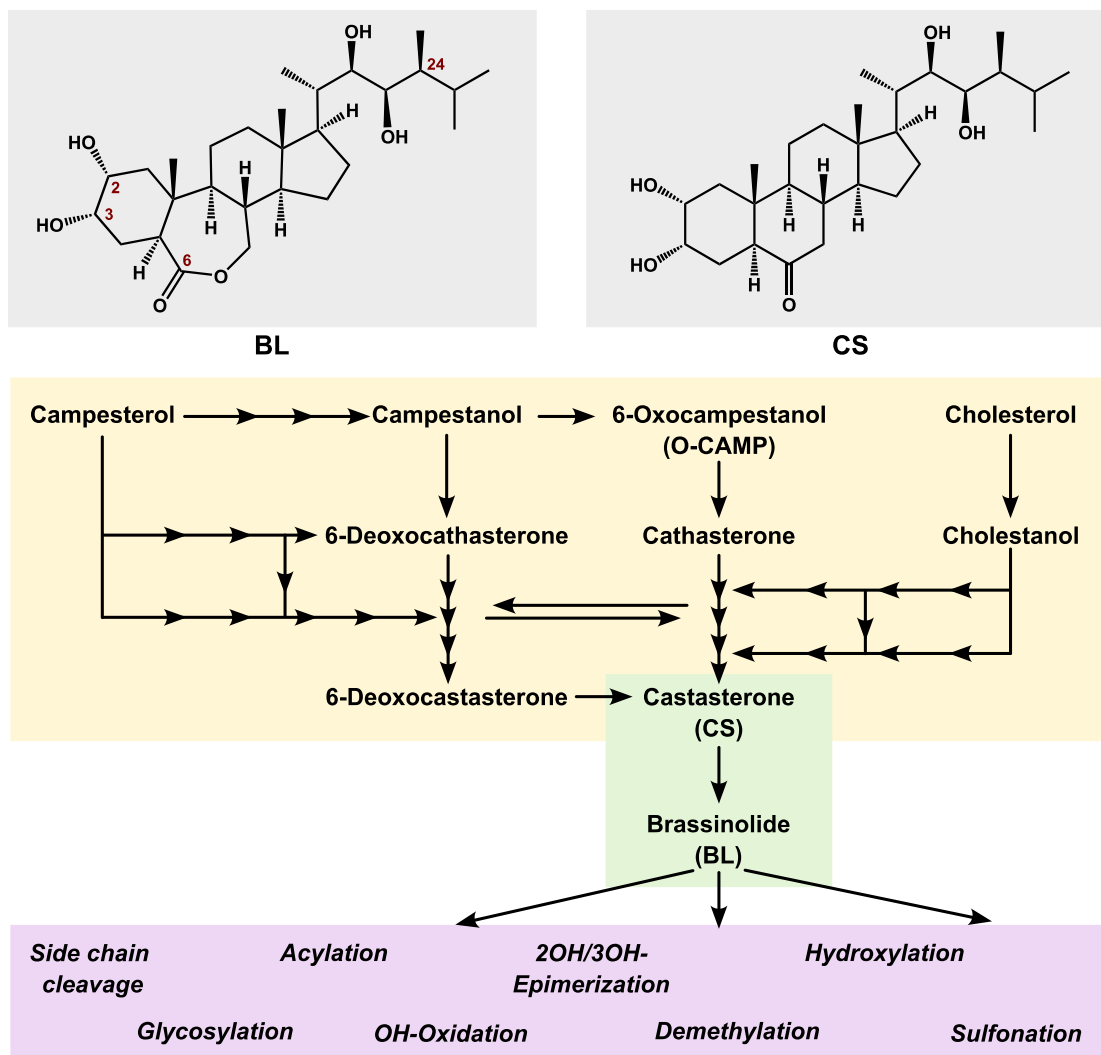


Figure 5: Simplified overview of BL metabolism. Abbreviated analyte names are provided in parentheses for the metabolites relevant to this work. The metabolites are grouped as bioactive forms (green) and biosynthetic precursors (yellow); general mechanisms of BL inactivation are provided in italics (purple). Adapted from [81, 83, 84].

1.4.2.4 Cytokinins

Cytokinins are another class of phytohormones that are involved in a wide array of growth and development processes in plants [85-88]. One of their main functions is regulating tissue growth and differentiation processes, where they often interconnect with auxin signaling. Cytokinins exhibit effects locally where they are synthesized, but are also often transported throughout the entire plant. All cytokinins are structurally derived from adenine, and the primary bioactive cytokinins are *N*6-isopentenyladenine (iP) and its hydroxylated derivative *trans*-zeatin (tZ). Although they are less active in various bioassays and have been historically considered less

important, the bioactivity of the cytokinins dehydrozeatin (DHZ) and *cis*-zeatin (cZ) has also been demonstrated. However, their biological relevance and the interconversion of cZ and tZ remain subjects of ongoing research and debate. Aromatic cytokinins with separate metabolic pathways, which are not detailed here, include the hydroxylated 6-benzylaminopurines, meta-topolin and ortho-topolin [89, 90], as well as kinetin. The latter was the first cytokinin discovered, but is only rarely found endogenously in plants [91].

In addition to the *N*6-prenylated nucleobases, the respective nucleosides (nucleobase ribosides) and nucleotides (mono-, di-, and triphosphorylated nucleosides) are important to the cytokinin metabolism (Figure 6).

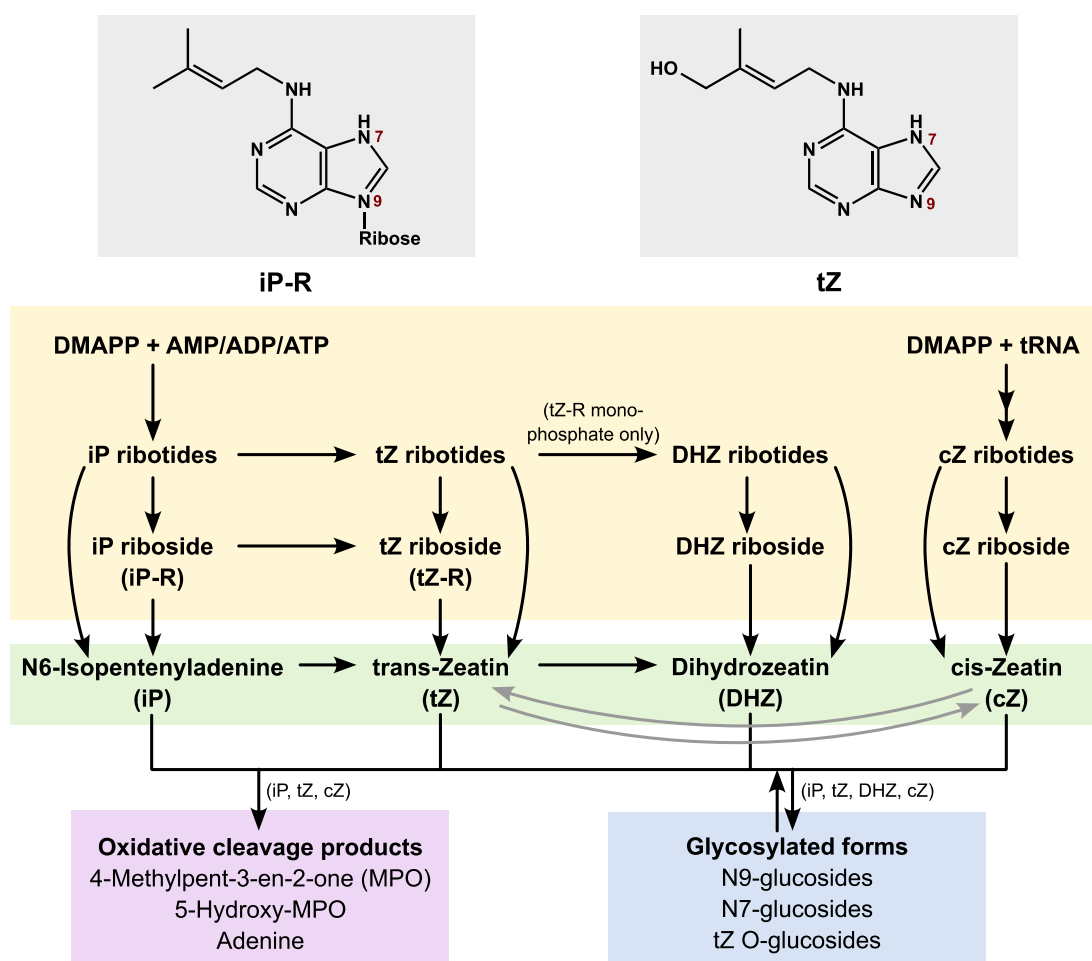


Figure 6: Overview of cytokinin metabolism in plants. Abbreviated analyte names are provided in parentheses for the metabolites relevant to this work. The metabolites are grouped as bioactive forms (green), biosynthetic precursors (yellow), intermediate forms (blue) and inactivation products (purple). Gray arrows mark steps that are known to take place but with unclear conversion rates. Adapted from [85, 88, 91, 92].

The biosynthetic origin differs for cZ nucleotides and iP/tZ/DHZ nucleotides. The former are synthesized through the degradation of *N*6-prenylated tRNA, while the latter are formed through the reaction of dimethylallyl diphosphate (DMAPP) and adenosine di-/triphosphate (ADP/ATP). Free nucleobases (iP, tZ, cZ, and DHZ) can be released directly from nucleotides or in a two-step process involving nucleosides as intermediates. tZ is synthesized through hydroxylation of iP's prenyl sidechain, while DHZ is in turn synthesized through the reduction of tZ's hydroxyprenyl sidechain. These conversions also occur for the respective nucleotides.

Cytokinins can be reversibly inactivated through conjugation with sugars, most often by glucosylating adenine at positions 7 or 9. However, they can also be irreversibly catabolized in an oxidative cleavage reaction, yielding adenine and a side-chain specific carbonyl compound as products. For iP, this reaction produces MPO, while two 5-hydroxy-MPO isomers are produced from tZ and cZ, respectively. DHZ, however, is not a substrate for the enzyme catalyzing this reaction and is not degraded this way.

1.4.2.5 Gibberellins

Gibberellins are an extensive class of tetracyclic diterpenoids formed not only by plants, but also by fungi and bacteria [93-96]. Alongside other unique properties, gibberellins are the only major phytohormone class with a systematic numbering system. Starting from GA1 (gibberellin A1), sequential numbers were assigned to all newly discovered compounds. To date, over 130 gibberellins have been discovered, though not all are synthesized by plants. The main bioactive gibberellins are GA1 and GA4, but GA3 (gibberellic acid) and GA7 also exhibit bioactivity. However, the latter are only synthesized in small quantities in plants while they are produced in higher quantities by fungi. In plants, gibberellins mostly function as positive regulators that degrade nuclear repressor proteins of the DELLA¹ family. Thus, the specific effects of gibberellin signaling depend on the type of repressor protein expressed by the plant cell, but the degradation of DELLA proteins often initiates growth and development processes.

Figure 7 outlines the biosynthesis of gibberellins, beginning from ent-kaurene, a tetracyclic diterpene derived from the cyclization of geranylpyrophosphate. This fully aliphatic molecule is then oxidized into eKA in a multi-step process. Another two-step process introduces an additional aldehyde function to form GA12-Ald, the precursor of all gibberellins in both plants and fungi. GA12-Ald is then oxidized into the dicarboxylic acid GA12, which can subsequently be hydroxylated at the C13 atom to form GA53. Two parallel pathways then continue from GA12 and GA53, which are analogous in their enzymatic steps. The only structural difference is that all

¹ DELLA is not an acronym but an amino acid motif.

GA53-derived metabolites are C13-hydroxyls. After several intermediate steps, GA9 and GA20 are formed, which are the direct precursors to the bioactive GA4 and GA1, respectively. It should be noted that these pathways are not fully decoupled, and interconnecting reactions have been reported. However, the metabolic flux through these interconnecting reactions is generally low, and they have only been demonstrated in a few plant species.

Interestingly, the inactivation of gibberellins is not specific to bioactive forms, but rather targets multiple key gibberellins. While most phytohormone classes exhibit feedback loops that inhibit their biosynthesis when their active hormones are produced at high levels, gibberellins have been described as particularly fine-tuned in this regard. The three main mechanisms are C2-hydroxylation, epoxidation at C16 and C17, and methylation of the free carboxyl group. These mechanisms primarily, but not exclusively, target the bioactive gibberellins GA1 and GA4, their immediate precursors GA9 and GA20, and their common precursor GA12. The primary inactivated forms are the products of C2-hydroxylation (GA29, GA34, GA8, and GA51).

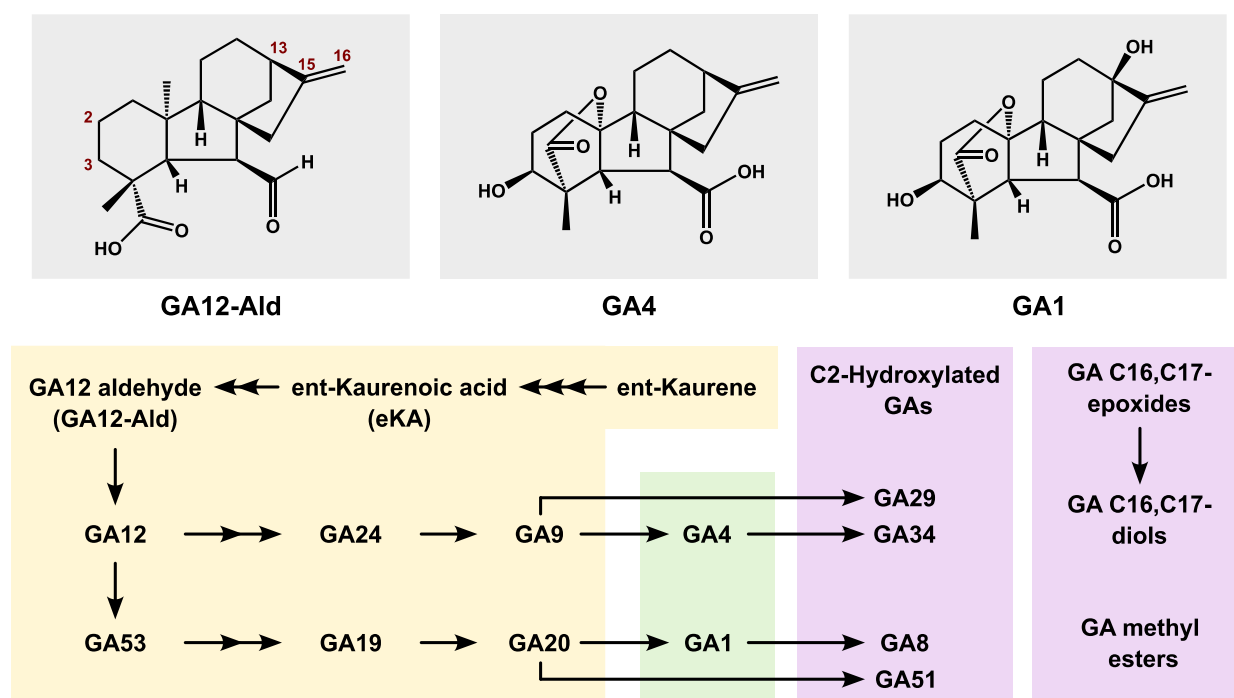


Figure 7: Overview of gibberellin metabolism in plants. Abbreviated analyte names are provided in parentheses for the metabolites relevant to this work; all gibberellins are abbreviated. The metabolites are grouped as bioactive forms (green), biosynthetic precursors (yellow) and inactivation products (purple). Adapted from [97-100].

1.4.2.6 Jasmonates

The signaling pathway of jasmonates is a primary mechanism by which plants adapt to environmental factors, and it is especially relevant to their stress response [101-104]. Jasmonate-dependent responses are often accompanied by a rapid accumulation of phytohormone metabolites. Although the hormone class is named after jasmonic acid (JA), the primary bioactive hormone is its conjugate with isoleucine (JA-Ile). Most jasmonates have multiple stereoisomers, with the bioactive form being specifically (+)-7-iso-JA-Ile. Biological activity has also been reported for other jasmonates. Most notably, the JA precursor 12-oxophytodienoic acid (OPDA) and its amino acid conjugates have garnered significant interest in recent years [105, 106].

Jasmonates are often referred to as oxylipins, because they are derived from oxygenated fatty acids. Figure 8 shows the jasmonate metabolism, starting from α -linolenic acid (18:3, n-3), which is released from membrane lipids and constitutes the primary source of JA. However, it should be noted that alternative pathways have been described that start from other unsaturated fatty acids, such as hexadecatrienoic acid (16:3, n-3), and produce slightly different intermediates. Furthermore, the synthesis of jasmonates is heavily compartmentalized within plant cells, though this is only indicated here to distinguish between specific metabolic and catabolic pathways. JA biosynthesis begins in chloroplasts, where α -linolenic acid is oxidized and cyclized into OPDA in multiple reactions. OPDA is then exported to the peroxisome, where it is converted into JA in a multi-step reaction involving multiple β -oxidations. Free JA is either oxidatively catabolized, conjugated to be inactivated, or conjugated to form JA-Ile. JA-Ile then translocates to the nucleus, where it interacts with specific binding proteins and induces transcriptional changes.

Inactivation products of JA include 12-OH-JA, 12-OH-JA-G, 12-COOH-JA, JSMO, JA glucose ester, 12-sulfo-JA, and several non-isoleucine amino acid conjugates. Several of these conjugates and other metabolites have also been described for JA-Ile. In addition, JA can be reversibly converted into its methyl ester. Several theories have been proposed for the functionality of MeJA, including a role in interplant communication due to its enhanced diffusion properties and increased volatility. However, several studies have concluded that its main purpose is likely to serve as an additional sink for unconjugated JA (see [101]).

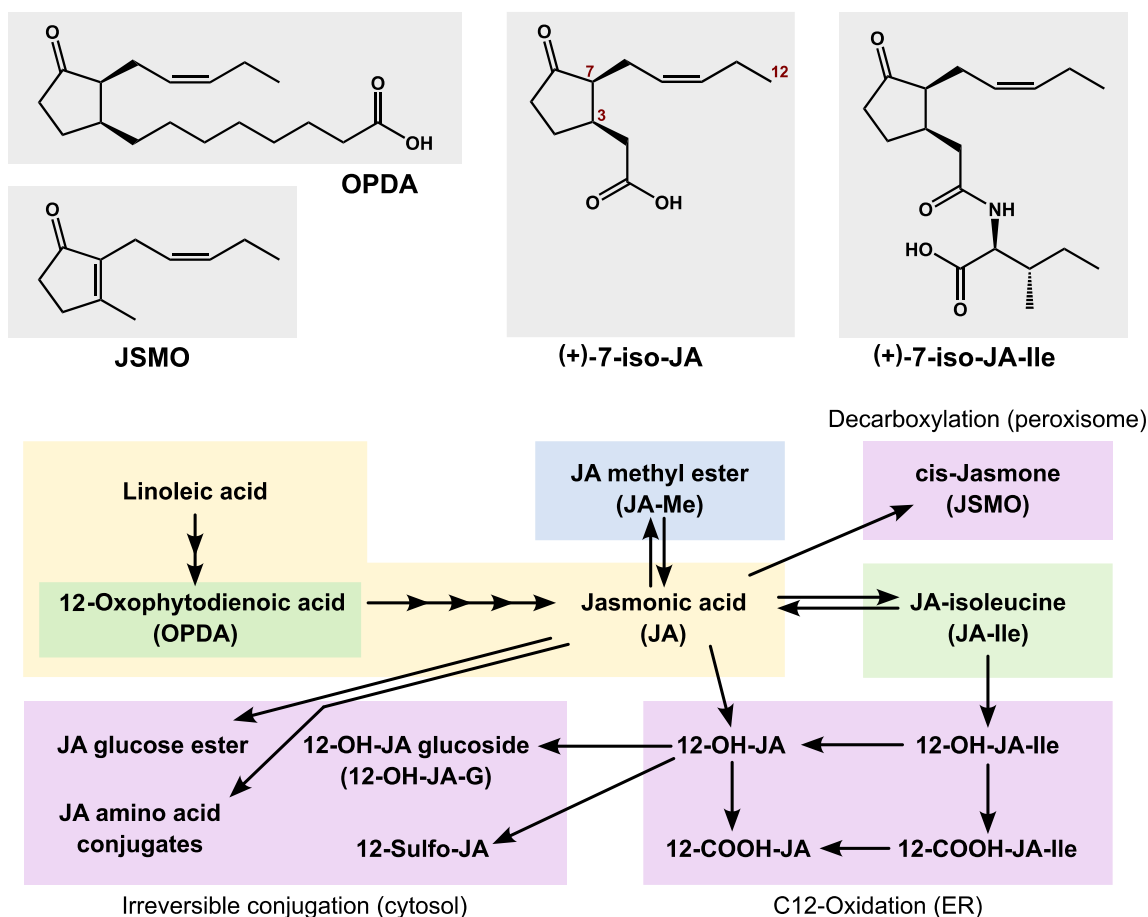


Figure 8: Overview of jasmonate metabolism in plants. Abbreviated analyte names are provided in parentheses for the metabolites relevant to this work. Different inactivation pathways are named, with the respective cell organelle in parentheses. The metabolites are grouped as bioactive forms (green), biosynthetic precursors (yellow), intermediate forms (blue) and inactivation products (purple). Adapted from [101, 102, 104]. ER, endoplasmic reticulum.

1.4.2.7 Salicylates

Alongside jasmonate signaling, salicylic acid (SA)-mediated pathways are the second key mediators of plants' defense systems, but also fulfill other vital roles [23, 107, 108]. As with abscisates and jasmonates, SA and its related metabolites are often collectively referred to as salicylates. Salicylates often accumulate in response to external stressors being sensed by plants. They then take effect both locally and throughout the entire plant. Long-distance signaling predominantly involves the induction of N-hydroxypipecolic acid (NHPA) biosynthesis, which is the primary metabolite transported to distant plant tissues.

Two distinct pathways lead to the formation of SA in plants, as shown in Figure 9. Both pathways start with chorismate, which is a product of the shikimate pathway and a precursor to many

metabolic pathways. The major pathway for SA formation is short, and begins with the formation of isochorismic acid, which is subsequently coupled to glutamate. This intermediate is highly unstable and releases SA in a reaction that can be either enzyme-mediated or spontaneous. The second route of SA biosynthesis follows the phenylalanine ammonia-lyase (PAL) pathway, which also constitutes the beginning of flavonoid and lignin biosynthesis. In the PAL pathway, chorismate is transformed first into phenylalanine, which is then converted into tCA. In contrast to the PAL pathway, tCA is converted into either oCA or BA, which are the immediate SA precursors.

SA is inactivated in various ways. As previously described for other phytohormones, the interconversion of SA and its methyl ester leads to a temporary inactivation as well as increased lipophilicity and thus altered diffusion properties. Permanent inactivation is catalyzed by hydroxylation of the benzene ring, conjugation with aspartic acid, or conjugation with glucose. Both hydroxylation and glucosylation can occur at multiple positions. In many plants, the major flux of SA inactivation is directed towards 2,5-DHBA and SA-G.

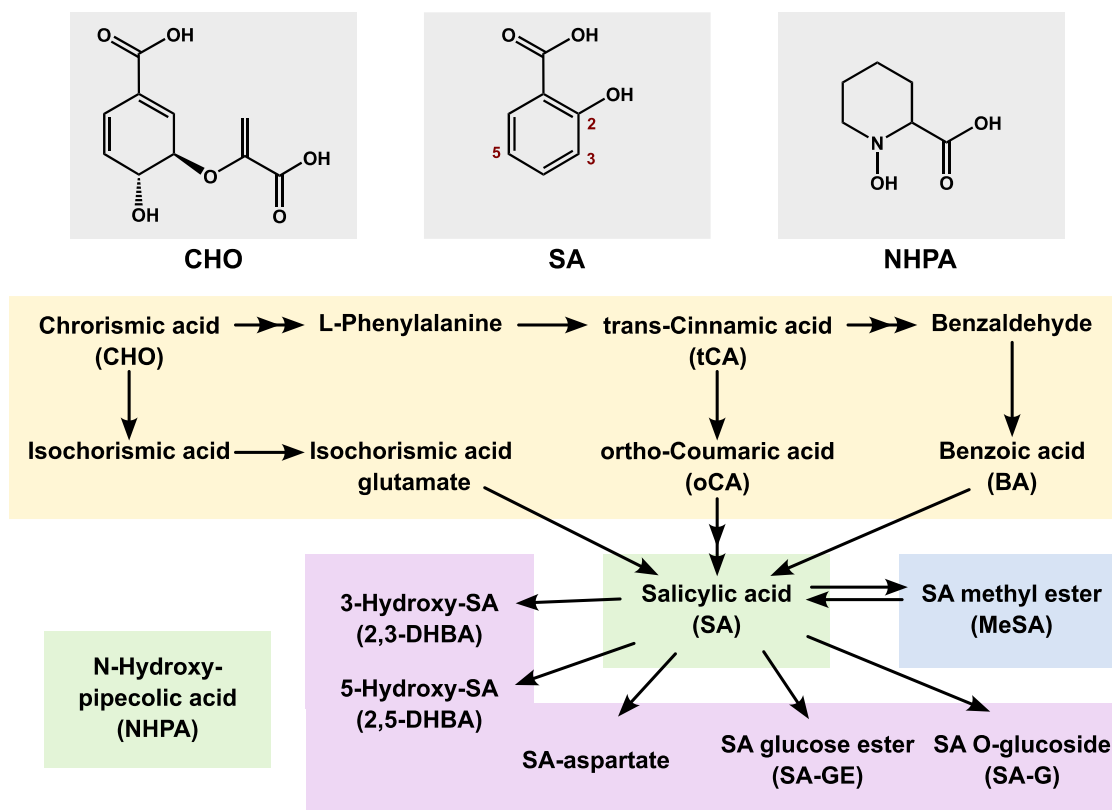


Figure 9: Overview of SA metabolism in plants. Abbreviated analyte names are provided in parentheses for the metabolites relevant to this work. The metabolites are grouped as bioactive forms (green), biosynthetic precursors (yellow), intermediate forms (blue) and inactivation products (purple). Adapted from [107, 109, 110].

1.4.2.8 Strigolactones

Strigolactones are one of the most recently described classes of phytohormones. Initially considered only relevant to plant-microbe interactions in the rhizosphere, they have in the past two decades been shown to exhibit many endogenous signaling functions in plants [111-113]. Strigolactones are primarily synthesized in the roots, from where they are transported to other plant tissues or secreted into the rhizosphere (see next chapter). In plant roots, strigolactones are involved in regulating nutrient uptake and symbiosis with arbuscular mycorrhizal fungi, but they are generally involved in tissue development regulation and plant adaptation to changing environments in many tissue types. Over 30 strigolactones have been identified, which are commonly divided into two major classes: canonical and non-canonical strigolactones (Figure 10). While canonical strigolactones have a conserved core structure consisting of a tricyclic lactone, the structure of non-canonical strigolactones is more diverse. Both types contain a conserved butenolide structure, which is relevant to their biological function.

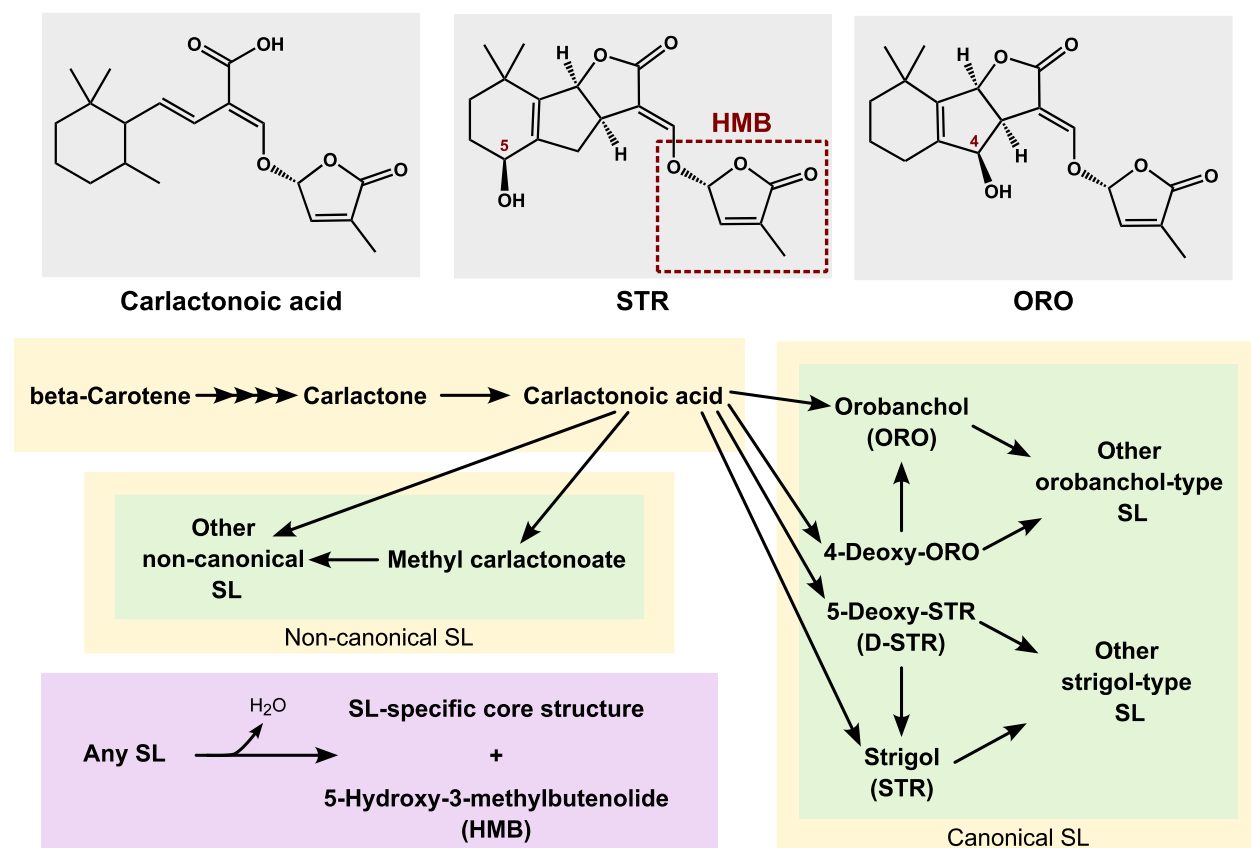


Figure 10: Overview of strigolactone metabolism in plants. Abbreviated analyte names are provided in parentheses for the metabolites relevant to this work. The metabolites are grouped as bioactive forms (green), biosynthetic precursors (yellow) and inactivation products (purple), though biosynthesis and biological activity cannot be separated for many strigolactones. The conserved HMB structure is emphasized in red. Adapted from [113, 114]. SL, Strigolactones.

Figure 10 shows the general formation pathway of canonical and non-canonical strigolactones. All strigolactones are formed from carotenoids. Similar to ABA biosynthesis, the carotenoid precursors undergo various oxidation and cyclization reactions. This process yields carlactone, which is then transformed into carlactonoic acid, the final common precursor of all bioactive strigolactones. Since most subsequently formed strigolactones are bioactive and also precursors to further diversified strigolactones, no differentiation is made in Figure 10. Non-canonical strigolactones can be formed in many ways, often in a plant species-specific manner. Canonical strigolactones, however, are formed through either ORO, 4-deoxy-ORO, STR, or D-STR. Further modified canonical strigolactones are often referred to as orobanchol- or strigol-types.

Strigolactone inactivation is strongly coupled with bioactivity because the strigolactone receptor hydrolyzes the activating molecules. This process always frees the conserved HMB structure from the cleaved strigolactone, along with a strigolactone-specific structure. Thus, despite the highly branched strigolactone formation pathways, strigolactone inactivation generally yields a common catabolite.

1.4.2.9 Ethylene

Ethylene is a unique phytohormone in many ways, but particularly due to its gaseous form. Upon its release from its immediate precursor, 1-aminocyclopropane-1-carboxylic acid (ACC), it is a rapid and potent regulator of many crucial processes in plants, including fruit formation and ripening [115, 116]. It is also involved in plant reactions to stress, where it frequently interacts with the jasmonate signaling pathway.

Biosynthetically, ACC is formed from the Yang cycle, specifically from SAM, as illustrated Figure 11. SAM is a methyl donor in many reactions in plants and usually forms S-adenosylhomocysteine as a byproduct. However, the byproduct of ACC formation from SAM is MTA, which is recycled into SAM in multiple reactions. The enzymatic release of ethylene is highly transcriptionally regulated, leading to accumulation of ACC before ethylene production is initiated by other signals.

Excess ACC is often conjugated, with three primary conjugates having been discovered to date. While jasmonyl-ACC constitutes a link between ethylene and jasmonate signaling pathways, the conjugate formed in the highest concentrations is by far MACC. MACC reconversion into ACC has been demonstrated in plants under specific conditions, but was shown not to be a primary regulatory mechanism, though research on this topic is ongoing (see [116]). A side product of ethylene release from ACC is HCN, which is toxic to plants in higher concentrations. However, plants readily detoxify HCN by converting it into CN-Ala, which is then hydrolyzed by nitrilases.

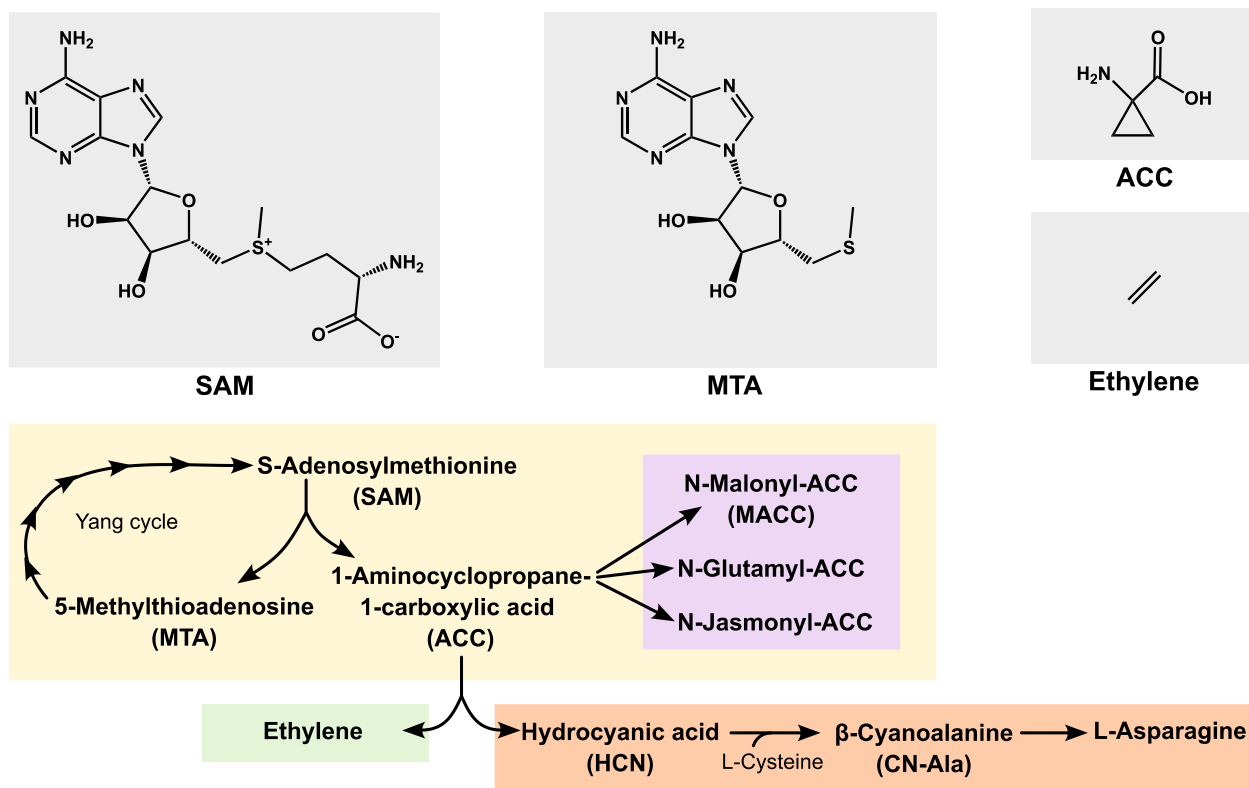


Figure 11: Overview of ethylene metabolism in plants. Abbreviated analyte names are provided in parentheses for the metabolites relevant to this work. The metabolites are grouped as bioactive forms (green), biosynthetic precursors (yellow), inactivation products (purple), and biosynthesis byproducts (orange). Adapted from [116-118], with additions from [119].

1.4.3 Analysis

As emphasized in Chapter 1.4.1, capturing the crosstalk between phytohormones is crucial for impactful plant research. However, simultaneously analyzing the major phytohormone classes presents several challenges. As the metabolic maps of the last chapters demonstrate, chemical diversity between phytohormone classes is high, but biologically distinct metabolites within each class are often closely related in structure. Therefore, analysis methods must cover a wide range of chemical classes, while still resolving analytes within them. Additionally, phytohormone concentrations in plants are very low (usually $\leq$ ng / g fresh weight), and they must be detected and quantified in the presence of thousands of potentially interfering metabolites. Despite these challenges, significant advancements have been made in recent years that enable the simultaneous analysis of a broad range of phytohormone metabolites [66, 67].

Out of the methods published to date, the majority have applied MS-based detection, with LC-MS methods being the most common in both relative and absolute quantification of phytohormone

metabolites [67, 120]. All major phytohormones have been successfully quantified using LC-MS, except for ethylene, which cannot be analyzed using LC-MS due to its volatility. As multiple reviews have covered all the major aspects of LC-MS analysis of phytohormones [66, 67, 121-125], only selected key points will be highlighted here.

A recent meta-analysis examined the state of MS-based phytohormone analysis [120] and assessed the number of analytes covered simultaneously and the amount of tissue used by each method. Both aspects were shown to be primary drivers in the development of new methods, with lower tissue samples allowing for a higher spatial resolution, i.e. the ability to metabolically distinguish smaller tissues. More specifically, the study found that of 122 targeted phytohormone analyses published since 2013 (113 of which were LC-MS based), the vast majority have analyzed fewer than 30 phytohormone metabolites, using an average sample mass of approximately 100 mg of fresh weight (FW). In contrast, state-of-the-art LC-MS phytohormone analysis methods are capable of detecting and absolutely quantifying over 100 phytohormone metabolites at once, all while using low sample masses (20 mg FW) and a simple solid phase extraction (SPE) purification workflow [126]. Other notable methods emphasize an even simpler purification workflow while nonetheless detecting over 50 phytohormone metabolites [127], or prioritize the simultaneous extraction of RNA and phytohormones from the same sample [128]. Through complex derivatization procedures, quantification of 31 phytohormones has reportedly been achieved using sub-milligram FW sample masses [129], allowing resolution of metabolic processes in minimally sized tissues.

Despite these impressive benchmarks and the use of the term "phytohormonomics" by some authors for these techniques [66, 126, 130], these methods have often only been validated for and applied to a limited number of matrices. Further applications are needed to explore the universal suitability of such workflows. Additionally, absolute quantification requires the use of IS to correct for matrix-induced signal changes [66, 120, 122]. However, isotope-labeled IS are often unavailable or too costly for many laboratories. In summary, more research is needed to develop phytohormone analysis methods that can be widely adopted by laboratories.

Considering the quantity of phytohormone analysis studies performed on various plant materials, remarkably few have used freeze-dried plant materials. Freeze-drying (lyophilization) of plant materials has long been a well-established procedure in plant metabolome analysis due to the easier sample handling resulting enhanced reproducibility [42, 56, 131, 132]. Although the chemical composition of a sample is not entirely unaffected by freeze-drying (see [133, 134]), it eliminates chemical and enzymatic reactions that occur when samples are (partially) thawed, e.g. during sample transport or weighing of sample aliquots [131, 132, 134]. Furthermore, lyophilization

provides dry weight as an additional variable and as an option for signal normalization, and it may enhance the extraction efficiency of some metabolites [131, 135]. The preference for freshly frozen plant materials in phytohormone analysis likely stems from the fact that several phytohormones have been reported to chemically degrade under harsh conditions during sample preparation [67, 124].

Nevertheless, early, limited studies have reported moderate to high recovery rates (50–100%) when comparing SA and JA levels in freeze-dried and fresh tissues [136, 137], while sample handling reportedly improved considerably. However, this could be an effect of the extraction, as Trapp et al. tested various solvent combinations for extracting fresh and freeze-dried *A. thaliana* leaves and found differential effects between combinations [138]. Ultimately, their data showed comparable or even higher levels of five phytohormones (JA, JA-Ile, SA, OPDA, ABA) in the freeze-dried material for some solvent mixtures. Similarly positive results were reported by Müller et al., who found marginal differences in the amounts of 11 phytohormones of seven classes that they analyzed in fresh and freeze-dried rosemary leaves [139]. In the validation of a phytohormone analysis method for tomato plants, lyophilized fruits were chosen over freshly frozen fruits, but fresh-frozen leaves over lyophilized ones due to their analytical performance [140]. Other studies report using freeze-dried materials, but do not indicate whether they evaluated it against the use of fresh material [141, 142]. Given the positive findings of some studies, freeze-dried plant materials may offer significant, yet underutilized, advantages for reproducible phytohormone analysis.

In summary, significant progress has been made in phytohormone analysis over the last two decades, and targeted phytohormone analysis has been demonstrated to be a useful tool in plant research, especially when coupled with other omics methods [125]. Further improvements are likely, but a greater emphasis needs to be put on making methods more accessible to various laboratories. Additionally, improvements in sample processing and purification are necessary to achieve high-quality, comprehensive phytohormone analysis.

1.5 Plant Interactions in the Rhizosphere

Plants coexist as part of a multifaceted ecosystem, which holds true even for modern agricultural food crops grown under a high level of pest control [5, 32]. Consequently, plants continuously interact with a variety of microorganisms living in their soil. However, the nature of these interactions can differ greatly for the plants. This chapter outlines general mechanisms of plant-microbe interactions in soil, focusing on phytohormone-related signaling mechanisms.

The rhizosphere, which refers to the narrow band of soil around plant roots (Figure 12), harbors dense and taxonomically diverse microbial communities [144]. These communities include bacteria, fungi, oomycetes, and nematodes, all of which colonize the rhizosphere in much higher numbers and greater diversity than the surrounding soil [144, 145]. Plants actively facilitate and modulate this environment by secreting (exuding) metabolites, including organic acids, sugars, secondary metabolites such as flavonoids or phenolic acids, and even phytohormones such as strigolactones [144, 146, 147]. Rhizosphere microbes are commonly categorized as either plant-beneficial, plant-pathogenic, or commensal [144, 145]. Commensal microbes do not directly affect plants or pathogenic microbes. Beneficial microbes can enhance nutrient acquisition or stress tolerance, whereas pathogenic organisms negatively impact plant fitness. Pathogens are further categorized as necrotrophs, which feed on dead host tissue, or biotrophs, which grow on living tissue [145].

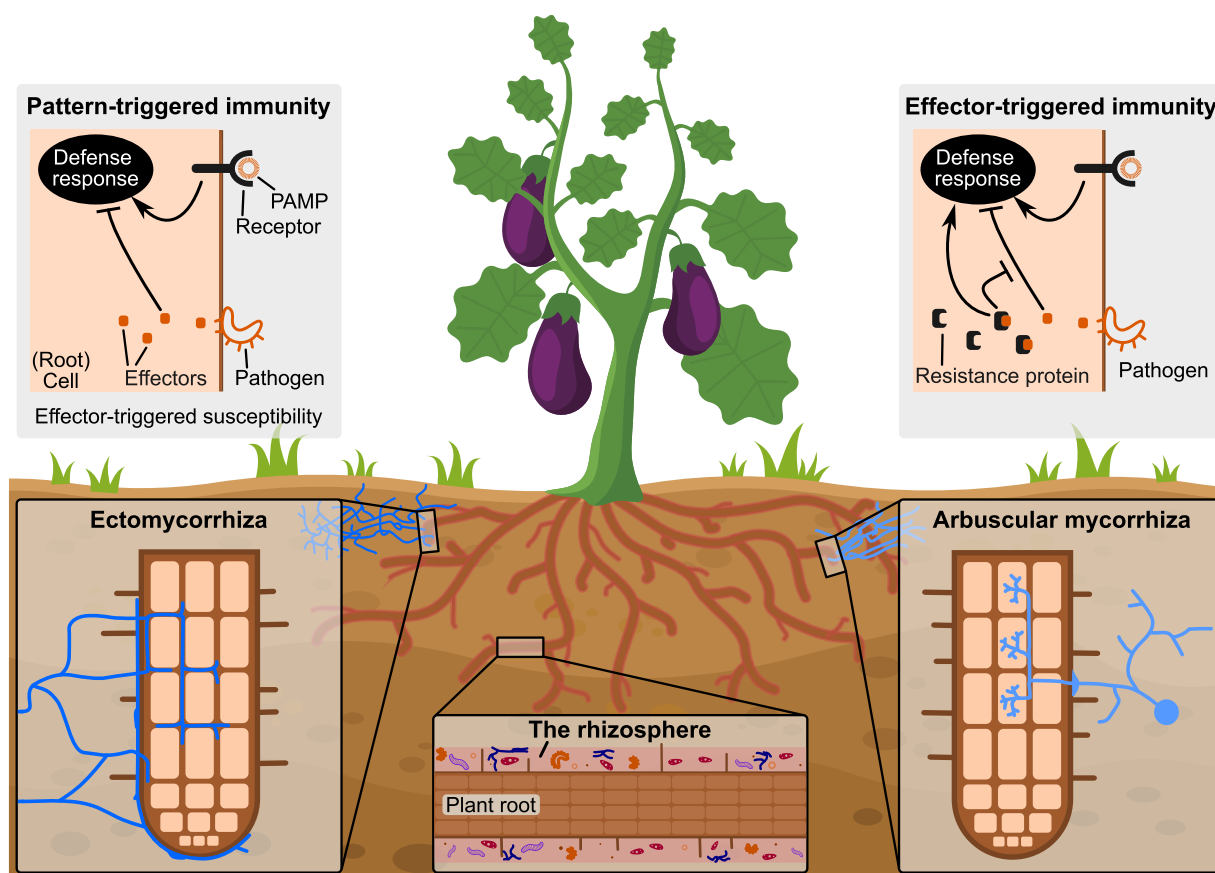


Figure 12: Overview of the plant rhizosphere (bottom center), primary plant defense mechanisms (top left and right), and the two major mycorrhizal types (bottom left and right). Depiction of mycorrhizae adapted from [148]; other sources are cited in the body text. PAMP, Pathogen-associated molecular pattern.

Plants must be able to detect microbes, especially pathogens that threaten their health. This primarily involves two separate mechanisms (Figure 12) [23, 107, 149]. Plant receptors, primarily located on root cell membranes, detect common and often highly conserved structural elements of microbes. These elements are known as pathogen-associated molecular patterns (PAMPs). This recognition leads to the activation of plant defense systems in a process referred to as pattern-triggered immunity (PTI)². Similarly, the detection of effectors by specialized receptors or resistance proteins activates effector-triggered immunity (ETI).

Effectors are virulence factors in the form of organic molecules or proteins secreted by microbes intending to colonize a plant. Often, their aim is to inhibit defense responses [24, 149, 150]. Effectors and ETI are the result of evolutionary co-development of plants and microbes, where microbes develop effectors to circumvent defense mechanisms, which consequently drives plants to adapt their recognition of these molecules. PTI and ETI overlap in their downstream signaling effects, but an important factor distinguishing them is the time-intensity balance of their response [24, 145]. Since ETI is generally more specialized, it activates plant defenses more rapidly and intensely to fend off a perceived threat, often by inducing cell death in affected cells.

Phytohormones play a key role in orchestrating these intricate defense systems, but their pathways are not strictly associated with either PTI or ETI. SA-mediated signaling pathways primarily coordinate the defense against biotrophic pathogens, whereas the defense against necrotrophic pathogens and herbivores is mediated by jasmonate and ethylene signaling [29, 59, 107]. Tissue or cell damage particularly activates jasmonate and ethylene signaling. SA-mediated defense often involves long-distance signaling and the induction of systemic acquired resistance. In this process, local PTI and ETI responses trigger signal transmission throughout the entire plant via the signaling molecule NHPA (see Chapter 1.4.2.7), which aims to elicit a preventive immune response in tissues not yet affected [23, 107]. When abiotic stress additionally affects the plant, ABA becomes another key mediator of the defense response. However, all phytohormone classes modulate the two defense signaling pathways in complex ways [29, 59, 64].

Interestingly, commensal and plant-beneficial organisms initially follow the same mechanisms as pathogens. Upon recognition by the plant, they trigger PTI, which the microbes suppress by secreting effectors [149, 151]. However, the specific nature of these effectors leads to different biological outcomes, and cell death is not initiated. Commonly grouped as plant growth-promoting rhizobacteria (PGPR), bacteria can affect nutrient uptake of plants by transforming nitrogen or

² Also referred to more generally as microbe-associated molecular patterns and microbe-triggered immunity by some authors.

enhancing the bioavailability of phosphorus [144, 152, 153]. They can further exert positive effects through their interactions with other rhizobacteria, and some PGPR directly produce growth-inducing phytohormones such as auxins, cytokinins, or gibberellins.

Symbioses between plants and fungi are also common and can have positive effects on plants as well [154]. Many plant-beneficial fungi grow as epiphytes, thriving on and around plant roots, similar to epiphytic PGPR. *Trichoderma* species, in particular, are widely reported to have positive effects on plants and are commonly added to soil in horticulture and agriculture as biocontrol microorganisms [155, 156]. In contrast, other fungi live as endophytes, meaning that they grow within the root cortex. Many species establish a symbiosis with the plant that is referred to as mycorrhiza. There are two types of mycorrhizae (Figure 12): ectomycorrhizae, which colonize the apoplastic space of roots, and arbuscular mycorrhizae, which grow into root cells, where they form branched, tree-shaped structures known as arbuscules [148, 154, 157]. These symbioses are incredibly widespread and occur in over 90% of plant species, including many Solanaceae species [158-160]. The evolution of mycorrhizae can be traced back hundreds of millions of years, shortly after trees first developed. The formation of the symbiosis causes strong metabolic changes in a plant [148, 149], but these changes are overall beneficial, as the establishment of mycorrhizae improves nutrient uptake, has growth-promoting effects, and increases resilience against a wide variety of stress factors [64, 148, 161].

To summarize, the complex communication between plants and their microbial environment, whether pathogenic or symbiotic, fundamentally influences plant metabolism. Characterizing these metabolic fluctuations is of high value for elucidating these processes, and untargeted metabolomics methods can serve as a powerful tool to capture these shifts on a functional level. However, many adaptive responses are coordinated by phytohormones, which often remain undetectable by broad, untargeted approaches due to their low endogenous concentrations and specific chemical properties. An integrated analytical framework combining untargeted metabolomics with targeted phytohormone profiling is thus required to fully unravel the mechanisms of how plants adapt metabolically to external stressors, and to develop strategies for improving crop resilience.

2 Aim and Scope

Modern agriculture has long prioritized production maximization through the selective breeding of high-yielding crops and the intensification of agricultural practices. However, the detrimental effects of these practices, combined with the escalating pressures of climate change, are threatening global food security by increasingly exerting stress on the crops themselves. While plants possess innate mechanisms to adapt to external stressors, the underlying metabolic processes and their influence on the resulting food products remain insufficiently understood, especially when multiple stress factors occur simultaneously.

The primary objective of this thesis is to develop a comprehensive analytical framework to assess plant metabolism, using the Solanaceae family as a model for essential food crops. By integrating untargeted metabolomics with a highly sensitive, targeted phytohormone analysis, this work seeks to simultaneously examine relevant physiological responses and the hormonal signaling pathways that orchestrate them. The combination of these methods may enable a comprehensive view of the plant's physiological state, provide insights into adaptations made under various stressors, and elucidate how plant interactions with rhizosphere microbes factor into these stress responses.

To assess the suitability of an in-house UHPLC-MS/MS metabolomics method in plant research, it is first applied to transgenic potato plants expressing fungal effector proteins. By analyzing potato leaves, stems, and roots, this study aims to characterize the method's analytical performance, while also elucidating metabolic shifts associated with the establishment of beneficial plant-fungus symbioses.

Building upon these findings, a targeted UHPLC-MS/MS method is established to capture crucial metabolites across all major phytohormone signaling pathways, and to enable absolute quantification of key bioactive forms. This involves selecting analytes, optimizing MS detection, UHPLC conditions, and sample purification, and developing a rigorous data analysis workflow in R. To examine the suitability of lyophilized plant material for phytohormone analysis, potato and rice plant materials are analyzed at different stages of method development.

To ensure analytical robustness, the finalized method is fully validated according to SANTE 11312/2021 guidelines, including a comprehensive assessment in tomato leaf and fruit tissues. Finally, the method is applied to the analysis of tomato plants in a complex biological context. This study employs an experimental design combining heat stress and the inoculation of the cultivation soil with the reportedly beneficial fungus *Trichoderma afroharzianum*. A parallel metabolome analysis is performed to compare the individual and combined capabilities of both methods in capturing the effects of multiple, simultaneous influencing factors on the plants.

3 Materials and Methods

This chapter details the materials, experimental designs, and analytical methods used in this work. To ensure clarity, this section is structured to mirror the results presented in Chapter 4. Complete lists of chemicals and instrumentation are provided in Chapter 7, further supporting data is provided in Chapter 8 (Appendix). All supplemental tables and figures in the Chapter 7 and 8 are identified by the prefix "A" (e.g., Table A-1). Contributions from collaborators and individuals other than the author are explicitly acknowledged in the relevant sections.

3.1 Analysis of Potato Plants via LC-MS Metabolomics

To investigate metabolic changes during plant–fungal interactions, a comprehensive metabolomics analysis was performed on potato plants. This research was a part of the “Plants Fit For Future” collaboration project, coordinated by Prof. Dr. Natalia Requena (Molecular Phytopathology Department, KIT). While plant cultivation and molecular biology analyses (such as gene expression studies) were performed at the Molecular Phytopathology Department, this work focuses specifically on the analysis of the potato plant metabolome.

The experimental design is outlined in Chapter 3.1.1, followed by details on cultivation and sampling in Chapter 3.1.2. The analytical procedures are summarized in Chapter 3.1.3, while Chapter 3.1.4 details the subsequent steps of data processing and data analysis.

3.1.1 Background and Experimental Design

The primary goal of the “Plants Fit For Future” umbrella project was to extensively research the growth, health, and produce quality of nightshade plants as well as the quality of foods they produce. A specific focus is on the symbiosis between plants and soil fungi, which have been reported to be beneficial to the plants [161-164]. These interactions are also closely linked with the plants’ uptake of nutrients from the soil, which is another project focus.

In previous works by the Molecular Phytopathology Department at KIT [165, 166], specific fungal effector proteins were putatively identified to be key in the symbiotic interaction between arbuscular mycorrhizal (AM) fungus *Rhizophagus irregularis* and the model plant *Arabidopsis thaliana* (see Chapter 1.5). To further elucidate this communication mechanism and study how these effector proteins alter the metabolism of nightshade food crops, transgenic potato plants were created by the group. These plants were transformed by inserting the effector genes using *Agrobacterium tumefaciens* carrying the genes of interest within a plasmid vector designed for constitutive overexpression. Specifically, plants were transformed to overexpress one of two previously identified fungal effector protein genes (SP5, SP7), while a vector carrying neither was used to create control plants (subsequently referred to as the *empty vector*, or EV, plants).

3.1.2 Plant Cultivation and Sample Acquisition

In total, six potato plant lines were generated, two expressing SP5 (SP5-6 and SP5-8), two expressing SP7 (SP7-7 and SP7-10) and two EV lines (EV-1 and EV-2). The numbering system was continued from previous experiments to ensure data continuity.

For each of these six potato plant lines, six individual seedlings were planted into pots and then cultivated in indoor cultivation chambers at the KIT Molecular Phytopathology Department between July and November of 2022. The plants were harvested after a cultivation period of six weeks. Stems and leaves were sampled separately by combining tissue from various parts of the plant until a volume of approximately 50 mL was acquired. Thus, each potted plant represented one biological replicate, as illustrated in Figure 13. The leaves and stems were shock-frozen in liquid nitrogen immediately after harvesting. Roots were not sampled in this experiment since the cultivation medium (soil) could not be removed without severely damaging the roots, which would have altered the metabolome to an extent that reliable interpretation with respect to the research questions would no longer have been possible.

Instead, a second experiment was conducted at a later stage of the project in order to analyze the roots as well. This was of particular interest since the studied interaction between fungus and plant typically occurs in and around the plant roots. For this cultivation, explants were placed into cultivation boxes filled with Murashige & Skoog medium, adjusted to a pH of 5.5. The composition of the medium is listed in the Appendix (Table A-8). Six explants were placed in each box (8.5 * 6.2 cm base, 9.6 cm height) and six boxes were prepared for each plant line. Cultivation time was 52 days for EV-1, SP5-8, SP7-7, and SP7-10 lines; and 38 days for EV-2 and SP5-6 lines. The latter had to be harvested earlier since their fast growth led to them becoming too large for the culture boxes. Each box represented one biological replicate (Figure 13), and at harvest the roots from all plants were harvested together and pooled. To harvest the roots, the plant was cut off at the stem, and the roots were removed from the medium. They were quickly washed with deionized water to remove residual medium, and then shock-frozen in liquid nitrogen.

For all tissue types, the harvested samples were placed into a stainless-steel sieve and lowered into a Dewar container filled with liquid nitrogen. When no more nitrogen evaporation was observed, the sample was transferred to a 50 mL centrifuge tube. The tube was stored for a few minutes at -20°C to allow the excess liquid nitrogen to evaporate. Then, the lid was put on and the sample was stored at -80°C. All samples were kept in storage at -80°C unless thawing was necessary for subsequent sample preparation steps. For short transports of samples (e.g.

between the KIT Molecular Phytopathology Department and the Max Rubner-Institute) the samples were placed on dry ice in a non-translucent transport box.

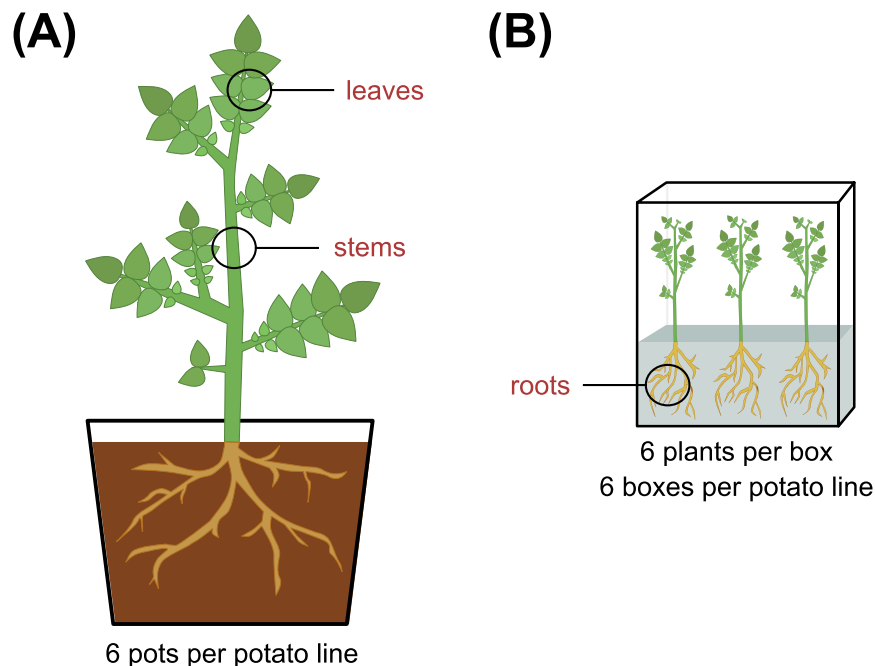


Figure 13: Representation of one biological replicate for each potato cultivation experiment, indicating the respectively sampled plant tissues. (A) Whole potted plant used for the analysis of leaves and stems. (B) Cultivation box containing multiple small plants for a pooled root analysis. In contrast to the illustration, tissue samples from multiple leaves, stems, or roots were pooled for the analysis of each biological replicate.

3.1.3 Sample Preparation and Data Acquisition

Sample analysis was performed in line with an established UHPLC-MS metabolomics method. This method was developed in-house and has been published in application-oriented studies [167]. In summary, samples were freeze-dried and ground to a powder using a ball mill. Weighed, pulverized samples were extracted using methanol and the methanolic extracts subsequently filtered. The filtered extracts were then analyzed by UHPLC coupled to a quadrupole time-of-flight mass spectrometer (QTOF-MS). Importantly, all samples were measured in both positive and negative ESI polarity. The sample preparation and data acquisition for the measurements in each polarity were performed independently of each other, starting from the step of sample weighing. The following subsections describe each step of the metabolome analysis in detail.

Sample Processing

Samples contained in 50 mL centrifuge tubes were placed in a freeze-drying apparatus for 72 h (condenser temperature -80°C , $p < 1$ mbar). The freeze-dried samples were then stored at -80°C until the samples were milled with a ball mill. Leaf and stem samples were processed using a larger ball mill (Retsch MM500, cup volume 125 mL, 8 balls, ball diameter 20 mm, frequency

30 Hz). The milling duration varied between the leaf samples (15 s) and the more fibrous stem samples (20 s). Due to low sample quantities, root samples were milled with a smaller ball mill (Retsch MM400, cup volume 35 mL, 2 balls, ball diameter 12 mm, frequency 30 Hz, duration 15 s). Immediately after milling, the pulverized samples were quickly transferred to a new 50 mL centrifuge tube to minimize adsorption of moisture from the air. The samples were then flushed with argon and stored again at -80°C.

Since their mass was too low for analysis (< 15 mg), two pairs of root samples were pooled into one single sample. Replicates 2 and 3 of plant line SP5-6, as well as replicates 1 and 4 of plant line SP5-8, were pooled. These pooled replicates were treated the same as all other replicates. Consequently, only five replicates were available for the analysis of the two plant lines instead of six.

Sample Weighing

When aliquots of samples were weighed, the samples were left to warm to room temperature. For each study sample (StS), aliquots of freeze-dried powder were weighed into 2-mL microcentrifuge tubes. For leaf and stem StS two aliquots were weighed, one each for analysis in positive and negative polarity. For root StS, only one aliquot was weighed, since positive and negative measurements were made using the same extract. This was due to the low amount of sample mass available for root samples. An analytical balance with a precision of 0.01 mg was used. 50 ± 0.5 mg were weighed for leaf and stem samples and 15 ± 0.2 mg for root samples. All sample masses were documented. All sample containers and weighed aliquots were always overcoated with argon after being opened.

A pooled sample was also prepared during the weighing process, which later served as the quality control (QC) during data acquisition. For this purpose, material from multiple samples was combined into a 30-mL brown glass bottle that was kept on ice during weighing. Special attention was paid to equally balancing the materials added from each plant line. For leaf and stem analysis, material from both tissues was added to a joint QC pool. The QC pool was thoroughly mixed, flushed with argon and stored at -80°C. The QC samples for analysis were weighed in the same manner as described above for the StS.

Sample Extraction

On each measurement day, the samples scheduled for analysis were taken out of the freezer and allowed to warm to room temperature. Numbers of StS and QC samples varied between study days, an overview is given in Table A-9.

The optimal sample mass to extraction volume ratio was determined in preliminary experiments and found to be 1:30 mg/ μ L for stem and leaf samples. This ratio was chosen in order to keep the ratio of analytes near the detector saturation range sufficiently low ($< 1\%$). For root samples, a ratio of 1:20 mg/ μ L was chosen, representing the maximum concentration feasible, given the limited sample masses available. Hence, either 1,440 μ L (leaf/stem samples) or 300 μ L (root samples) of ice-cold methanol was added to each sample, followed by either 30 μ L (leaf/stem samples) or 6 μ L (root samples) of each IS mix. The composition of the IS mixes is given in Table A-10 and Table A-11. In addition to StS and QC samples, five controls (CTs) were prepared on the first day of measurements for each polarity by adding only the ice-cold methanol to 2 mL microcentrifuge tubes. These controls will later be used for the subtraction of so-called blank features (see Chapter 3.1.4.1).

After solvent addition, all samples (CTs, StS and QC samples) were vortexed for 5 s and then placed in a thermomixer (15 min, 35°C, 1,400 rpm). Afterwards, the samples were immediately placed on ice to cool them down, and then centrifuged (5 min, 16,100 g, 4°C), before being placed on ice again. Next, the supernatant was transferred into a 2 mL syringe and filtered through a polytetrafluoroethylene (PTFE) syringe filter (pore size 0.2 μ m, diameter 4 mm). The filtrate was collected in a fresh 2-mL microcentrifuge tube which was kept on ice during filtration.

Depending on the sample type, different aliquots of the filtered extract were then transferred to clear glass vials with integrated inserts. For each study sample, 100 μ L was pipetted into a vial, as was the case for each CT. For the QC samples, 80 μ L aliquots were pipetted into 5-7 different vials for the leaf/stem analysis, or 1-5 different vials for the root analysis. The vials were immediately transferred to the UHPLC's autosampler, where they were kept at 4°C throughout the data acquisition. The leftover extracts were stored at -80°C.

Data Acquisition

Samples were measured on the same day as extraction to ensure metabolite stability and consistent analytical conditions. Detailed specifications for the instrumentation and analytical parameters are provided in the Appendix. Table A-5 lists the instrumental setup, Table A-12 lists the settings, components, and solutions used for the UHPLC, Table A-13 the UHPLC-gradient, and Table A-14 the MS parameters. The mass spectrometer was operated in Information-dependent acquisition (IDA) mode. In an IDA experiment, the instrument automatically selects quasi-molecular ions for fragmentation (MS/MS) based on user-defined criteria, such as a minimum intensity threshold. These fragmentation events are performed in parallel with the

primary MS survey scan. In this study, MS/MS spectra were acquired exclusively through this automated process. Table A-15 provides the IDA-specific settings.

While the exact sequence of sample measurements (sample batch) is not listed in this work, as it is not of major relevance, the overall batch structure is important for the processing of the acquired data and will therefore be summarized here. In brief, the first samples measured were the CTs, followed by a series of 16 QC samples. These QC samples were not evaluated or processed later and served to equilibrate the system. Then, throughout the rest of the series, blocks of eight samples consisting of three QC and then five StS were measured. Overall, the QCs serve to evaluate and, if necessary, to correct signal drifts occurring over the acquisition process. After each block of eight samples, a mass calibration is performed automatically by the instrument in order to maintain stable mass detection. Since it is known that the first measurement after such a calibration can contain erroneous signals and artifacts, the first QC sample in each block was excluded from further processing. Consequently, the final structure of the acquired data alternated between two QC samples and five StS.

3.1.4 Data Processing and Analysis

3.1.4.1 Initial Data Processing

Raw data were acquired in the Sciex proprietary format (.wiff) and converted to the Analysis Base File format (.abf) using the freely available ABF Converter (Reifycs Inc.). These files were processed using the open-access metabolomics software MS-Dial (version 4.9.2), developed through a collaboration between University of California (UC Davis, Fiehn Lab) and the Institute of Physical and Chemical Research (RIKEN) in Japan [168].

MS-Dial was used for peak detection, integration, annotation, and alignment across samples. Peak annotation is the process of assigning chemical identities to metabolite signals by comparing their quasi-molecular ion mass-to-charge ratio (m/z), their experimental mass spectra and their retention time (RT) with entries in established libraries. These libraries can be specified or provided by the user. MS-Dial accounts for common adduct formations in the annotation of quasi-molecular ions. Peak alignment is the process of matching identical metabolite signals across different samples by correcting for minor variations in retention time and mass accuracy. Additionally, the software also performed a blank subtraction by removing metabolite signals that appeared with similar intensities in the extraction blanks and the samples.

It should be noted that the processing of stem and leaf samples was initially conducted alongside data from a separate potato project. While the potato data are not included in this thesis, this combined processing influenced the initial alignment IDs and metabolite signal numbers for the

stem and leaf datasets. Conversely, data from potato roots were processed as an independent batch. As a consequence, root alignment IDs differ from those of the stems and leaves. Thus, the two datasets were therefore merged only after the analyte identification. The data from analyses in positive and negative polarity were also processed separately.

The output generated by the software is a list of all features matching the specified criteria. Features are metabolite signals with a unique combination of m/z and RTs. It should be noted that one metabolite can produce multiple features, e.g. due to adduct formation or source fragmentation. Four feature lists were obtained in this experiment, two each (positive and negative polarity) for the leaf/stem and the root datasets. Further steps of data analysis were performed on these exported feature lists outside of the software. However, MS-Dial also allows for interaction with the data in its user interface, which was used to assess correct alignment and annotation in later steps.

3.1.4.2 Evaluation of Sample Outliers

For each sample, several indexes were calculated to evaluate whether some samples should be removed as outliers, e.g. due to errors during injection. Amongst these indicator parameters were the ratio of not-detected features, the average signal intensity, and the sum of all signal intensities. Using conditional formatting in Excel, these parameters were then color coded to highlight patterns in the data. Signal intensities of the IS were additionally evaluated.

During sample examination, it was noted that the signal intensities in QC samples showed a high variance. This made the evaluation of potential sample outliers challenging, and ultimately none were identified in this step. However, a clear outlier was later identified during the exploratory data analysis (Chapter 3.1.4.5), where PCA plots splitting samples into tissue groups showed that two samples (with consecutive numbers in the sample preparation) had very likely been swapped by accident. Only one of these samples (Line SP5-8, Replicate 5) was part of the dataset presented in this work and was therefore removed prior to further analysis steps.

3.1.4.3 Elimination of Features

When generating data with the metabolomics method used here, feature numbers are often high (>10,000). However, a substantial proportion of these features is usually not detected reliably, i.e. they have only been detected in few samples or show a high variance in QC samples. Since the identification and interpretation of such features is often not possible, filtering steps are usually employed to focus on reliably detected analytes. Here, two filtering steps are used, one based on QC data and one based on the detection within subgroups.

In the first filtering step, all features were removed that were not detected in at least 25% of QC samples or showed a high variance of signal intensities in the QC samples. Specifically, the coefficient of variation (CV; also: relative standard deviation) in QC samples had to be $\leq 30\%$ for the respective feature to be retained.

For the second filtering step, one feature list was created per plant line and polarity. Thus, six feature lists resulted (two polarities x three tissues) and were then filtered independently as follows. Features were removed if they had more than 25% missing values, unless they were reproducibly detected in at least one plant line. In other words, features were still kept if they were detected in $\geq 80\%$ of samples of at least one plant line. This approach aimed to also keep features detected only in one or a few plant lines and might therefore represent metabolic alterations unique to those plant lines.

3.1.4.4 Data Imputation

Following the data filtration, missing values in the data were imputed. This was also done independently for each of the six feature tables described in the previous chapter. Missing values can cause issues in statistical analyses since they introduce bias or lead to erroneous statistical results, the likelihood of which increases with the ratio of missing values (see [169]). The missing values are therefore substituted with estimated values, which is known as imputation.

In the case of this metabolomics method, imputation is performed by replacing missing values with random numbers between the global intensity minimum and the feature-specific first percentile of intensities across the examined samples. This ensures that features with varying baseline signal intensities are treated consistently.

3.1.4.5 Exploratory Data Analysis

A principal component analysis (PCA) was performed for each of the six feature tables to gain an overview of the datasets. PCAs were performed using the statistics program JMP (version 18.2), which was set to perform a Z-transformation prior to the PCA in order to mitigate effects of signal intensity magnitude.

No joint PCA was performed on multiple tissues together, outside of very early tests not presented in this work, since the sample analyses were conducted separately for roots and leaves/stems. This approach prevents feature merging between the datasets because identifiers such as feature IDs differ. Datasets obtained through measurements at different ESI polarities were not merged either, due to the likelihood of different ion species of the same metabolite overlapping between the datasets, which could lead to distorting effects. The two-dimensional (2D) PCA score plots

were evaluated, and the graphs exported. Due to the large number of variables, loading plots were not evaluated.

3.1.4.6 Univariate Feature Statistics

Univariate statistics were run on each feature using the Response Screening Module in JMP. This module performs an analysis of variance (ANOVA) on a specified factor for each feature.

The analyzed factor was the plant line, and no inputs were given to link the plant lines transformed in the same way (e.g. EV-1 and EV-2), leading to all six plant lines being treated equally in the statistical analysis. A correction of the false discovery rate (FDR) was performed by the software and the resulting FDR-corrected p-values (FDR-p) were also $\log_{10}$ -transformed for easier interpretability. A Tukey Post-Hoc test was subsequently performed on all features in order to assess statistical differences between pairs. The p-values and Compact Letter Display (CLD) labels ($p < 0.05$) were exported.

All statistical results were transformed so that they could be saved in one row of an Excel-table together with all other information regarding the feature. This was done to facilitate the segmentation of data, e.g. into significant features or identifiable features only, while easily carrying along all generated information. Custom JMP scripts, written in the JMP-specific coding language JSL, were used during several steps of the statistical analysis but especially during reformatting. These were self-created in order to execute various steps of the process reproducibly and limit human errors in copying large tables of data.

In addition to the statistical results, fold changes (FCs) were calculated for each feature. This was done by dividing the average signal intensity in a non-control plant line (SP5-6, SP5-8, SP7-7, or SP7-10) by the average signal intensity for both EV-lines. The FC were then $\log_2$ -transformed in order to limit differences in magnitude and enable symmetric and intuitive visualization. Conditional formatting was applied in Excel to highlight value distributions.

3.1.4.7 Visualization of Metabolite Patterns

To highlight some trends seen in the PCA plots as well as the conditionally formatted feature data collected in Excel tables, heatmaps were created. These heatmaps were put together by selecting the features with the 100 highest FDR-p-values, i.e. the features of highest significance in the ANOVA comparing the samples of the different plant lines. Color scales were equalized between the different graphs and limited between +4 and -4, resulting in very few data points lying out of range.

3.1.4.8 Compound Identification

The MS-Dial software performs automated annotation by comparing acquired data against either an in-house library or integrated external databases. The former provides higher confidence (MSI-Level 1, see Table 1) by incorporating matching against precursor ion mass, RTs and collision-induced fragmentation spectra (MS/MS spectra). These are all obtained through the analysis of authentic reference standards using the exact same instrument and analytical method. In contrast, using external databases can be advantageous due to their larger scope, but matching is only performed against precursor ion mass and MS/MS spectra, and the reference data is sourced from various instrumental setups and methods. Regardless of the database used, high-confidence metabolite identification remains labor-intensive and represents a significant analytical bottleneck. Based on the inconsistencies observed between the two SP5-expressing potato lines (see Chapter 4.1.1), it was decided to focus on the SP7-expressing plant lines for the metabolite identification.

Identification was performed at two different stages of the project using different approaches, which are summarized in the following chapters. First, an entirely untargeted identification approach was applied to leaf and stem samples to identify which metabolic pathways were most affected by SP7 expression. This was done before the potato root experiment was devised. Second, all annotation matches with the in-house database were evaluated for all three tissue type samples (leaf, stem and root) regardless of FC or significance. This approach can be considered semi-targeted.

3.1.4.8.1 Identification of Robustly Altered Features in Stem and Leaf Samples

To account for the low throughput and manual curation required for high-confidence identification, a stringent pre-selection of metabolites was necessary. Given the high number of significant metabolic changes between EV and SP7 lines, strict filtration criteria were applied to prioritize features for identification. A feature was only kept if all four pairwise Tukey tests between SP7 lines (SP7-7, SP7-10) and control lines (EV-1, EV-2) were highly significant ($p < 0.01$), otherwise it was removed. This approach ensured that only the most robust alterations associated with SP7 expression were selected.

Initial data processing via MS-Dial was limited by technical constraints, resulting in the processing of leaf and stem samples with the integrated external databases and a reduced in-house database format. This meant that MS/MS spectrum matching was only performed on the external database. Matches with the in-house database relied on precursor ion m/z and retention time only, and MS/MS spectra had to be checked manually. For matches with the external databases, Total

Score values of ≥ 80 were accepted and the feature kept for further identification. For matches with the in-house database Total Score values of ≥ 90 were accepted, since no MS/MS comparison was factored into their calculation. Additionally, in-house database matches were only kept if the acquired RT and precursor mass matched the database entry sufficiently well ($\Delta RT < 0.1$ min, $\Delta m/z < 0.0075$ Da).

A variety of factors were evaluated for each match in either database. For all matches, peak alignment and integration were manually checked using an MS-Dial-integrated tool, and features with considerable errors were eliminated. The quality of MS/MS spectra was assessed within the respective MS-Dial interface and matches were disregarded if the spectra were of low quality due to factors such as low intensity, excessive noise, or consisting only of the precursor ion. MS/MS spectra matches with the external database were also checked within MS-Dial and evaluated for numbers of counts, ion intensities and ion intensity ratios. For the in-house database matches, this evaluation was performed using the software PeakView (Sciex). In some cases, additional spectra from the MassBank of North America (MoNA) database were considered as well. Furthermore, the plausibility of the match was evaluated by considering the likelihood that the observed feature corresponded to an isomer or isobar rather than the suggested metabolite. This step relies heavily on the analytical experience regarding the chemical class of the putative metabolite. The primary tools used for evaluation were the in-house library of standards that had already been measured with the method, as well as whether MS-Dial found multiple putative matches. Lastly, literature and databases (LOTUS [170], FooDB [171], ChEBI [172], and KEGG compound [173]) were searched to determine if the putative analyte had been reliably detected in potatoes or other Solanaceae species before. This step served to assess the biological plausibility of the annotation.

The goal of identification was to assign a confidence level for each annotation. The levels established by the MSI were used as a reference [56]. The original definitions and how they were applied in this work are given in Table 1. It should be noted that only compounds identified via the in-house library were allocated to MSI Level 1. As the process above shows, metabolite identification depends greatly on the person performing it. Therefore, the maxim was adopted that, in any disputable cases, the lower of the considered levels was chosen to preserve the meaning of higher identification levels. Also, all of the steps of the identification process were documented to allow for all decisions to be traced back later.

Table 1: Definitions of MSI Levels as originally proposed [56] and summary of their application in this work. Formal definitions are taken via direct quotes from the cited work. FT, Feature; RT, Retention time.

Level	Expression	Formal definition	Application
1	Identified compounds	„ [Identified using] a minimum of two independent and orthogonal data relative to an authentic compound analyzed under identical experimental conditions. “	FT identified via in-house database using the RT, exact mass of the precursor ion and the MS/MS spectrum.
2	Putatively annotated compounds	„[Identified] without chemical reference standards, based upon physicochemical properties and/or spectral similarity with public/commercial spectral libraries. “	FT identified via in-house database but without sufficient MS/MS match or FT identified via external database but based on their precursor mass and MS/MS-spectrum only. RT falls within the range expected for the compound class.
3	Putatively characterized compound classes	„Based upon characteristic physicochemical properties of a chemical class of compounds, or by spectral similarity to known compounds of a chemical class.”	Compound class of FT identified based on MS/MS match, but cannot be distinguished from known isomers. RT falls within the range expected for the compound class.
4	Unknown compounds	“Unidentified or unclassified”	Not included in the lists of identifiable FT.

3.1.4.8.2 Identification of in-house Database Matches in Stem, Leaf and Root Samples

In a separate annotation process, the features from the stem, leaf, and root sample datasets were annotated using only the in-house database. This semi-targeted approach aimed to consider all metabolic pathways equally, rather than solely focusing on the most altered ones.

Unlike the initial processing of the stem and leaf samples, the root samples were processed directly with an updated version of the in-house database that now included MS/MS spectra. This enabled direct comparison of the acquired MS/MS spectra with those of authentic reference standards, which were measured using the same instrument and method. This led to a high level of identification reliability. To harmonize procedures between the three datasets (or six, when considering the two polarities), the leaf and stem measurement data were reprocessed, this time also using an updated version of the in-house MS/MS spectral library. However, not all of the previously analyzed standards had been integrated into the new in-house library at the time this work was conducted. Therefore, manual comparisons of spectra had to be performed on occasion using PeakView. Apart from the identification step, parameters were kept the same as those described for the data processing in Chapter 3.1.4.1.

One additional alteration was made while handling the MS-Dial processed data. Other than what was described in Chapter 3.1.4.3, no filtering was performed based on the missing rate of QC samples. For the wide-scope approach taken here, this was considered too strict. However, the subgroup filter was applied as described. The individual data tables, split by tissue and polarity, were imputed as detailed in Chapter 3.1.4.4, and statistical tests as well as FC calculations were performed and saved with the data as outlined in Chapter 3.1.4.6. Identification followed the framework summarized in Chapter 3.1.4.8.1., though it did not include matches with external databases and used a less strict filter for in-house database matches. Only the $\Delta RT < 0.1$ min criterion was still applied, m/z deviations were factored into the overall evaluation instead.

3.2 Establishment of a UHPLC-MS/MS Targeted Phytohormone Analysis Method

A targeted phytohormone analysis method was established to complement the untargeted metabolomics methods in plant metabolism research. The following chapters summarize the important steps in adapting and optimizing the method. It should be noted that comprehensibility was prioritized over chronological order.

Since no broad-scope phytohormone analyses had been performed in-house prior to this work, the process was initiated by defining criteria for a targeted analysis method. Next, literature was searched for published methods that could be adapted, and a UHPLC-MS/MS method was chosen as the basis for adaptation (Chapter 3.2.1). Afterwards, relevant analytes were selected for inclusion in the scope of the method, and their authentic reference substances were acquired. These standards were then analyzed to optimize their detection and separation (Chapter 3.2.2). After all analytes were included in the method, chromatographic conditions were optimized (Chapter 3.2.3). Then, attempts were made to improve the sample preparation workflow (Chapter 3.2.4) and a modular R workflow was developed to facilitate quick and thorough data evaluation after acquisition (Chapter 3.2.5). The method validation and its applications to plant samples are presented in the subsequent chapters.

3.2.1 Definition of Method Requirements and Literature Research

In order to effectively investigate hormonal crosstalk, as described in Chapter 1.4, the selected analytical method had to fulfill several technical requirements. These considerations are briefly summarized below, as they served as benchmarks throughout the method development process and are referenced throughout this work.

Primarily, the method required sufficient sensitivity to reliably detect low-abundance phytohormones in plant tissues. It also needed to be capable of detecting multiple phytohormone classes in parallel, while maintaining sufficient specificity to resolve chemically similar analytes.

Versatility across different plant matrices, such as fruits, leaves, stems and roots, was essential to ensure wide applicability in plant research. Furthermore, the method needed to operate with small sample amounts to facilitate sampling of small tissues as well as tissue sub-sampling. Ideally, these amounts would not exceed the requirements of established in-house metabolomics methods (approximately 10-100 mg of freeze-dried powder). Efficiency was also a priority, although it was not held up to specific benchmarks. The workflow required short sample preparation and analysis times to support a high throughput and facilitate the analysis of large study sets. Despite this emphasis on efficiency, the method had to remain robust and deliver reproducible results. Lastly, the method had to function with pulverized freeze-dried plant samples, which both established in-house methods also use. Harmonization with these methods was desired to enable the use of all methods together in multi-omics projects.

As described in Chapter 1.4.3, the targeted method currently offering the highest coverage of phytohormones is one published by Šimura et al. in 2018 [126], which uses a UHPLC coupled with ESI and a triple quadrupole MS. Analytes are measured in multiple reaction monitoring (MRM) mode. This method uses low sample amounts, a relatively simple sample preparation workflow and can detect and even quantify 101 phytohormone-related metabolites. As this method served as the backbone for the subsequent method adaptation, its capabilities are highlighted here.

First, the biggest advantage of the chosen method is its ability to absolutely quantify its analytes. Originally, the goal of this work was mainly relative quantification, equal to the in-house metabolomics methods. Second, the method provides precise and sensitive detection of a wide range of analytes, offering good coverage of the phytohormone classes of cytokinins, auxins, brassinosteroids, gibberellins, jasmonates, salicylates, and abscisates. Of the phytohormone classes of interest, only ethylene metabolites and strigolactones are not included in this method. Third, the required sample amounts are low. In an exemplary application on salt-stressed *Arabidopsis thaliana* seedlings, 20 mg of plant FW are used. Fourth, technologically comparable instruments and equipment were available in-house, enabling an adaptation without major changes. Lastly, analysis times were short, and the sample preparation workflow was simple. To summarize, the workflow involves extracting samples using an extraction volume of 1 mL, purifying extracts with a single-step SPE, and evaporating the SPE flow-through under nitrogen. To reconstitute the evaporated samples, 40 μ L of solvent is used, resulting in a 25-fold concentration of analytes. Two UHPLC-measurements are performed, one in positive and one in negative ESI mode. The injection volume was 10 μ L of reconstituted extract and the runtime of each analysis was under 20 min.

In contrast, the method also has several drawbacks, primarily analytical ones. Since these were more directly relevant to the work presented, they are addressed in the results section (see Chapter 4.2). Other minor drawbacks of the original method and the adaptations made in response are covered in the Chapters 3.2.2.2 to 3.2.4.

3.2.2 Selection and Optimization of Analytes and MRM Transitions

After a method was chosen as the base for an adaptation, analytes to be included in the adapted method had to be selected. This selection process was performed independently of the analyte scope of the original method. The final MRM method is presented in the Appendix (Table A-19), along with the instrumental setup (Table A-6). The successive developments leading to its final version are detailed in the following chapters.

3.2.2.1 Literature Research and Acquisition

Literature research was conducted for each phytohormone class considered for inclusion in the targeted method. It was aimed to include crucial compounds of all metabolic branches of each phytohormone class. Besides the bioactive hormone forms, precursors and inactivation products were researched as well as intermediate forms, such as transport or storage compounds. Specific attention was paid to rate-limiting steps in the metabolism or compounds that might accumulate. Where it was possible, branching points in the metabolism were investigated, too. As an example, a large number of strigolactones has been identified to date, and including many of them into the method was not feasible due to a lack of available reference standards. However, strigolactones often share the catabolite 3-hydroxy-5-methylbutenolide (see Chapter 1.4.2.8), which was consequently chosen to be included in the method.

The phytohormone classes considered were auxins, abscisates, brassinosteroids, cytokinins, gibberellins, jasmonates, strigolactones, salicylates, and ethylene metabolites (see Chapter 1.4.2). As mentioned previously, the terms abscisates, jasmonates, and salicylates are used here as biological group names, whereas chemically, they refer specifically to the conjugate bases or esters of abscisic, jasmonic, and salicylic acid. This functional grouping extends to the other classes as well, which encompass metabolites that are biologically significant to the respective class, even though they fall outside the traditional structural scope of the group name.

A list of 118 phytohormone metabolites 15 metabolite groups of interest, was compiled from these research results. A summary of this list is presented in Table A-16 in the Appendix. Then, priority categories were assigned to each metabolite or metabolite group, and the availability and pricing of standards were investigated. Category 0 was assigned to metabolites for which standards were found out to be available in-house. Category 1 metabolites were considered crucial to the

respective phytohormone metabolism and were therefore highly prioritized during the acquisition. Metabolites allocated to Category 2 were researched for availability, but standards were only acquired when their cost was sufficiently low. During this further research into the analytes, if it was determined that analysis of a compound would not be feasible, it was sorted into Category 3 and not further considered for acquisition. Most often, the reason was that the substances were too volatile or chemically unstable for analysis to be feasible. Of the 118 considered metabolites, 29 metabolites were allocated to Category 0, 44 to Category 1, 39 to Category 2, and six to Category 3. Out of the 15 metabolite groups, which summarized larger groups of substances, 13 were allocated to Category 2, and two to Category 3.

The availability and pricing of authentic reference standards for these compounds were investigated online and via trusted vendors. Ultimately, 53 reference substances were purchased, in addition to the Category 0 reference standards that were already available in-house. All substances intended for inclusion in the method are listed together in Table A-16. Importantly, the abbreviations given in that table will be used hereafter to avoid wordy introductions.

Furthermore, 10 internal standards (IS) were acquired, which are listed in Table A-18. 9 out of these 10 IS were isotope-labeled standards of bioactive phytohormones, one was the synthetic Strigolactone GR24, which does not occur in nature. KIN and KIN-R, were originally intended to serve as IS as well, since they were thought not to occur in nature. However, these were later detected in tomato samples (Chapter 4.4.3) and are therefore not included as IS in the final method. Two labelled standards for IAA were purchased (d2-IAA and d5-IAA), the latter of which was bought to replace the former due to low analytical performance. Since most isotope-labelled phytohormone standards are very costly, a careful selection was made, and IS were only acquired for these crucial analytes. These were also the analytes intended for quantification.

3.2.2.2 Optimization of MS Conditions and Parameters

Systematic product ion scans were performed for all 82 prospective analytes and 10 IS to identify optimal fragment ions for MRM transitions. Additionally, MS parameters were optimized for the precursor and fragment ions of each analyte.

First, stock solutions of all analytes were prepared. For some of the standards already available in-house, previously prepared stock solutions were used. To prepare the stock solutions, the standard substances were allowed to warm to room temperature. Then, approximately 1 mg was weighed in a glass vial using an analytical balance (precision 0.01 mg) and the mass was recorded. Immediately after weighing, the vial was closed and placed on ice until the solvent was added. For some reference standards, only small quantities were obtained, often due to high

costs. When the acquired mass of a substance was ≤ 1 mg, no weighing was performed and the solvent was added directly to the original container of the substance. Consequently, the resulting concentrations were known not to be exact for these standards. This step was only necessary for some analytes, none of which were intended for quantification.

The solvent volumes were calculated based on the weighed mass of the substances. The purity of each substance was also factored in. The choice of solvent relied primarily on specifications provided by the manufacturers, which were aggregated for each standard. When information on solubility was unavailable, the logP value of the molecule was calculated using a built-in ChemDraw tool. For logP values between -2 and 4, methanol (MeOH) was chosen as the initial solvent. Acetonitrile (ACN) was chosen for higher logP values, and water for lower logP values. The target concentration for stock solutions was 10 mM, unless adaptations were necessary due to low amounts of substances. If the standards did not dissolve, the standard dispersions were mildly heated to 40°C, placed in an ultrasonic bath for 1 min, or further diluted. If the substance still did not dissolve after these steps, the solvent was evaporated under a stream of nitrogen, and a different solvent was applied. All steps were performed in laboratories with blacked-out windows and only artificial light sources in order to minimize the risk of light-induced degradation. The final solvents and concentrations for all stock solutions are listed in Table A-17 in the Appendix.

To optimize the MS parameters, diluted analyte solutions were prepared using ACN/H₂O (50:50, v/v) as the solvent, hereafter referred to as 50% ACN. From each stock solution, diluted solutions at concentrations of 0.1 μ M, 1 μ M, 10 μ M and 100 μ M solutions were prepared. The solutions were injected directly into the MS using an integrated syringe pump port. To do so, they were manually loaded into a 1-mL glass syringe, which was then placed into the pump port and connected to the ESI source. Solvent blanks were typically injected first to check for analyte signals present in the solvent. Then, optimization began with the lowest analyte concentration. If no signal was detected at a given concentration, the next higher concentration was used until a signal within the optimization range required by the instrument was detected. If no signal was detected at a concentration of 100 μ M, the preparation process was checked for errors or repeated. If the process was repeated carefully and still yielded no signals, the analyte was deemed not suitable for MS detection. For most of components, optimization was performed in both positive and negative ESI polarity.

The Compound Optimization Module in the MS software Analyst was run on each injected solution. This module automatically selects the most intense product ions for MRM transitions and screens substance specific parameters-ranges to find the value that provides the highest

precursor or product ion intensity. The general settings and the parameter ranges for these measurements are provided in Table A-20 in the Appendix.

First, the precursor selection in Quadrupole 1 (Q1) was optimized by determining the ideal declustering potential (DP) for each analyte. This was performed three times to obtain an average value. Next, a product ion scan was performed at a range of increasing collision energies (CEs) and the six most intense product ions and their optimal CEs were automatically selected. Then, the cell exit potential (CXP) was optimized for these precursor-to-product ion transitions. Product ion selection and MS/MS optimization were performed three times. Since the MS/MS optimization also includes a Q1 DP optimization, six values were obtained for the precursor DP and up to three values were obtained for product ion mass, CE and CXP. Automatically generated reports for all of these steps were saved and a summary of the four best transitions per analyte was manually aggregated. Product ions that were possibly isotopes of another fragment ion (rounded $\Delta m/z = 1$) were disregarded if their signal intensity matched the expected intensity based on the standard isotope distribution. The transition candidates were then compared with the blank measurements to confirm that the selected product ions were analyte fragment ions.

Additionally, manual precursor and product ion scans were performed using the optimized DP. This was done for all analytes and the corresponding blank solvent injections. Summed scans (sums of 100 individual scans) were saved as graphs in order to serve as visual references for the obtained optimization results. If additional promising product ion candidates were identified through visual evaluation, they were manually selected for optimization, and the above-described process was applied. Table A-17 highlights the analytes that were discarded due to no candidate transitions being identified. In all but one case, the reason was that no analyte signal was detected, even at high concentrations ($\geq 100 \mu\text{M}$). For eKA, an analyte signal was detected in negative polarity, but no fragments with an $m/z > 50$ were observed at any of the tested CEs (-5 to -130 V).

3.2.2.3 UHPLC Measurements of Candidate Transitions

After potential transitions per analyte were obtained through direct infusion of standards into the MS, these transitions were measured via UHPLC-MS/MS. A gradient closely adapted from the original method [126] was used, which is listed as Gradient 1 in Table 3. The goal was to ultimately identify the two best-performing transitions per analyte, which were then included in the final method. Except for some test experiments, all versions of the UHPLC-MS/MS method were scheduled MRM-methods, meaning RT windows were allocated to all transitions. Transitions were only measured during their respective windows to minimize the number of transitions measured simultaneously and therefore maximize dwell time per transition.

All potential transitions were measured at various analyte concentrations. First, they were measured in mixtures of standard substances and then, if any signs of coelution or contamination occurred, as single analytes. The acquired signals of individual transitions were evaluated regarding their intensity, their signal-to-noise (S/N) ratios, and the presence of interfering peaks. No harmonized approach was taken, and the handling of different phytohormone classes and individual standards varied. Some standards required many individual tests, while others yielded satisfactory results after only a few measurements. Providing full details on the handling of every analyte would exceed the scope of this work. However, Table A-17 notes analytes that were deemed unsuitable due to their chromatographic performance. The reasons were mostly that no identifiable analyte signal was detected (IC, IPY, CN-Ala, and SAM) or that the analyte signal was detected only at unrealistically high concentrations (IAA-Glu, GA4-ME, and 2,5-DHBA). In one case, coelution with a more important and likely more concentrated analyte occurred (epiBL) and in another case, interfering signals of unknown substances were present (CHO).

3.2.2.4 Sample Preparation of Prospective Analytes

In addition to analyzing candidate MRM transitions in diluted standard solutions, all analytes were also measured after sample preparation. The workflow used in these experiments was adapted from the original method and is described briefly here. It should be noted that improvements to the workflow were devised based on experience gained from these applications. Therefore, the final sample preparation method presented in Chapter 3.2.4 differs from the one used for these experiments. As in the previous chapter, experiments for the various analyte classes or individual standard substances were not fully harmonized. The main differences were in the measurement concentrations and how many times the analytes were prepared using this workflow. Therefore, only the general proceedings are summarized here.

Reference standard solutions were prepared in 1.5-mL microcentrifuge tubes using 50%ACN and cooled to 4°C in a laboratory refrigerator. Then, the cooled solutions were placed in an ice-cooled ultrasonic bath for 3 min and subsequently in a thermomixer (4°C, 1,400 rpm, 30 min). Afterwards, the tubes were centrifuged (4°C, 16,100 g, 10 min) and placed on ice. The centrifuged extracts were then purified via SPE. For this step, SPE columns were placed atop an SPE vacuum chamber and first washed and pre-conditioned with 1 mL of methanol, followed by 1 mL of ultrapure water, and 1 mL of 50%ACN. After each solvent addition, a vacuum was applied to the respective chamber until no liquid remained in the columns. The flow-through of all these steps was discarded and a rack containing 4-mL brown glass vials was placed under the column outlets. Then, the analyte extracts were loaded onto the columns and a vacuum was applied until no liquid remained in any column. 1 mL of 30%ACN was added to each column, and subsequently drained

by applying a vacuum. The flow-through from both additions was collected in the brown glass vials, which were placed in an evaporation rack. Pipette tips connected to a nitrogen valve were lowered into the vials, and the sample solutions were fully evaporated under a nitrogen stream. Once no liquid was observed in any vial, the nitrogen flow was stopped, and 80 μ L of 30%ACN was added to each vial. The vials were then vortexed for 30 s and the reconstituted extracts were transferred to glass inserts in 1.5-mL brown glass vials. These vials were then placed in the cooled (4°C) autosampler of the UHPLC until analysis. In general, the extracts were analyzed on the same day that the samples were prepared.

As with the non-prepared analyte solutions (see Chapter 3.2.2.3), the intensity, S/N ratio, and presence of interfering compounds were evaluated for each candidate transition. Additionally, recovery after sample preparation was calculated, and baselines compared between prepared and non-prepared analyte solutions. If analytical inadequacies were present, additional transitions determined in the product ion selection process (see Chapter 3.2.2.2) were sought out and tested. If no suitable fragment ions for an MRM transition produced acceptable results, the analyte was excluded from the method, which is highlighted in Table A-17. Two analytes (ACC and MTA) were removed in this step. In the case of one analyte (PAA), only one transition was deemed sufficiently well performing. This analyte was not of major importance, so a workaround was used, where the same fragment ion was measured twice at different CEs (11 V and 25 V). For two pairs of analytes (cZ/tZ and IAA-Leu/IAA-Ile), joint transitions are used for analysis due to a low difference in RT.

The final MRM method with all transitions and their optimized parameters is listed in Table A-19 in the Appendix. It spans 63 analytes and 8 IS.

3.2.3 Optimization of UHPLC Conditions and Parameters

After including all transitions in the final method, alternative UHPLC eluents and gradients were tested to improve overall method performance. The UHPLC-MS instrumental setup is listed in Table A-6, and the respective parameters applied to all measurements are listed in Table A-21 in the Appendix.

Early method applications had shown that auxins specifically exhibited unstable signal intensities during and between measurement series. This was attributed to pH instability of the column. Thus, various eluent compositions were tested to determine if auxin detection could be improved without impairing the detection of other analytes. Table 2 shows the eluents tested. The evaluation of eluent combination 9 was limited by technical failures during negative ESI polarity measurements.

Table 2: Eluent combinations tested during method development. All water used was ultrapure water, FA percentages are volume percentages (%v/v). ACN, Acetonitrile; FA, Formic acid; NH₄FA, Ammonium formate.

Eluent combination	Eluent A	Eluent B
1	0.01% FA in water	0.01% FA in ACN
2	0.05% FA in water	0.05% FA in ACN
3	0.1% FA in water	0.1% FA in ACN
4	0.03% FA in water	0.03% FA in ACN
5	0.01% FA, 5mM NH ₄ FA in water	0.01% FA in ACN
6	0.05% FA, 5mM NH ₄ FA in water	0.05% FA, 5mM NH ₄ FA in ACN
7	0.01% FA, 5mM NH ₄ FA in water	0.01% FA, 5mM NH ₄ FA in ACN
8	0.03% FA, 2mM NH ₄ FA in water	0.03% FA, 2mM NH ₄ FA in ACN
9	0.03% FA, 2mM NH ₄ FA in water	ACN
10	0.03% FA in water	ACN

Afterwards, gradient variations were tested to see if analysis times could be shortened without affecting peak separation. The best-performing eluents (Eluent Combination 10) were used for all gradient tests. Table 3 shows the tested gradients. Gradient 1 closely resembles the method published by Šimura et al. [126], and was applied in Chapters 3.2.2.3 and 3.2.2.4; Gradient 5 is used in the final method. All gradients were first tested in five separate methods on different analyte classes to obtain their RTs for a scheduled MRM method. Gradients 2 and 4 were not tested further than these initial tests due to poor peak separation.

Table 3: UHPLC gradients used during method development. Only the gradients for measurements in positive ESI polarity are given. Negative gradients were 1 min longer for all gradients except for Gradient 5. B, Fraction of Eluent B in the eluent mix.

Gradient 1		Gradient 2		Gradient 3		Gradient 4		Gradient 5	
Time (min)	B (%)	Time (min)	B (%)	Time (min)	B (%)	Time (min)	B (%)	Time (min)	B (%)
0.0	5	0.0	5	0.0	5	0.0	5	0.0	5
5.0	5	2.0	5	2.0	5	0.5	5	0.5	5
15.0	65	16.0	75	16.0	89	2.5	11	11.5	71
15.5	100	16.5	100	16.5	100	14.0	80	12.0	100
18.0	100	18.0	100	18.0	100	14.5	100	14.5	100
18.5	5	18.5	5	18.5	5	17.5	100	15.0	5
22.0	5	22.0	5	22.0	5	18.0	5	18.5	5
						22.0	5		

For each eluent combination and each gradient tested, the same analyte mixes were used. These mixes were prepared by analyte class, their base concentrations and mix concentrations are given as Mix 1 in the Appendix (Table A-22). In these experiments, a 2-fold concentration of the base

concentration given in the table was used. To ensure equal and stable conditions, the column was equilibrated with a 1% FA solution for 1 h (total flow 0.3 mL/min, 25% Eluent B). Additionally, the MS was equilibrated by measuring 16 mix samples and then evaluating only the last six.

The acquired data was integrated via MultiQuant and peak lists exported as text files. These files were then imported into R and processed with specifically written scripts. Means, standard deviations (SD) and coefficients of variations (CVs) were calculated for each analyte in each experiment group, i.e. each eluent combination or gradient tested. The experiment group was also used as the factor for an ANOVA and subsequent paired Tukey test. Statistical tests were not performed for all groups together, but in three separate evaluations. First, various FA percentages in Eluent A were compared (Eluent combinations 1 – 4, and 10), where Eluent Combination 1 served as the reference group. Second, the use of additives in the eluents was evaluated by comparing eluent combinations with and without additives (Eluent combinations 1, 2, 4 and 10; as well as 5 - 8). Eluent combination 1 served as the reference group again. Third, Gradients 1, 3 and 5 were compared, using the data obtained with Gradient 1 as the reference. In addition to statistical tests, this grouping was also used for FC calculations. FC and Log₂ FC were calculated for each group's mean analyte signal intensity against the reference group mean signal intensity.

As the basis for evaluation, bar plots were created for each analyte as well as a heatmap overview for all analytes. Bar plots showed the mean signal intensity ($\pm$ SD) and the significance letters exported from the Tukey Post-Hoc test ($p < 0.05$ for pairs with different letters). The heatmaps visualized the log₂ FC between analyte signal intensities obtained with a gradient or eluent combination and the respective reference group. Two heatmaps, one for each measurement polarity, were created for each of the three evaluations described above.

Ultimately, Eluent Combination 10 (A: Water + 0.03% FA, B: ACN) was deemed to be the best-performing of the eluent combinations and Gradient 5 to be the best-performing gradient. These were used for the subsequent method optimization and validation, with the exception of the analyte stability analysis (see Chapter 3.3.1).

3.2.4 Optimization of Sample Preparation

Based on insights from compound optimization experiments (see Chapter 3.2.2.4), the initial sample preparation workflow was improved in various aspects. Chapter 3.2.4.1 outlines the final protocol. Additionally, Chapter 3.2.4.2 details an alternative sample preparation method using a dispersive SPE technique, and Chapter 3.2.4.3 presents a small-scale experiment comparing the two sample preparation workflows. The use of freeze-dried material instead of fresh plant material is only briefly addressed here, as it will be discussed in more detail in Chapter 3.4.1.

3.2.4.1 Finalized Sample Preparation and Data Acquisition Protocols

The following sections detail the finalized sample preparation protocol. They describe general considerations, as well as sample weighing, sample extraction, extract purification, extract evaporation, extract reconstitution, and sample analysis. Changes to the original method [126] are described at the beginning of each subsection to clearly indicate own contributions to the work. These contributions are also summarized and discussed in Chapter 4.2.2.1. This method was later used for method validation and for the application to tomato plant samples (see Chapters 3.3 and 3.4.2).

Sample Types and Numbers

In addition to the study samples (StS), up to three other types of samples were processed using the following workflow. Control samples contained only solvent and no analyte or IS mixes. Similarly, blank samples contained only solvent and IS mix and no analytes. While both types of controls were only prepared on the day of extraction, their microcentrifuge tubes were pre-arranged and stored alongside the StS to ensure consistent handling across the experimental batch.

Depending on the experimental design, quality control (QC) samples were also prepared. This process was analogous to the metabolomics analysis (see Chapter 3.1.3). These samples contained the same type of sample material as the respective StS, but from a pooled sample. QC samples were used to evaluate matrix effects on signal intensities and RT of the IS, to monitor signal intensities throughout a measurement series, to equilibrate the LC-MS system, and to evaluate analyte RT and peak shapes in matrix. Unless otherwise noted, QC samples were treated the same as StS in the sample preparation process.

The number of samples that could be prepared at once was limited to 24 by the number of cartridge positions of the SPE chambers and the nitrogen evaporation racks. This aligned well with the analysis time needed to measure 24 samples in both ESI polarities, when accounting for additional necessary runs, e.g. for system equilibration, calibration curves, and analysis of unprepared substance mixes.

Sample Weighing

The sample weighing process was substantially modified from the original method, since freeze-dried, ball-milled plant material was used instead of fresh plant tissue. The amount weighed depended on the sample material. Between 4 and 10 mg of freeze-dried powder were used in the experiments in this work. However, preliminary tests (not presented in this work) were conducted with sample masses up to 50 mg. Weighings were carried out on an analytical balance with a precision of 0.01 mg. All weighings were recorded so that the analysis results could be related to the respective sample masses.

Samples were weighed at room temperature. Immediately after weighing, the weighed aliquots and the sample container were placed on ice. They were both overlaid with argon and stored at -80°C as soon as possible. Sources of natural light were consistently blocked out to prevent analyte degradation.

Extraction

The extraction protocol followed the one described in the original method, but was adapted to the available laboratory equipment. No major optimizations were attempted for this step.

Before extraction, the samples were placed in a laboratory refrigerator to warm them to 4°C. Then, 990 µL of ice-cold 50%ACN was added to each StS, followed by 10 µL of IS mix. The samples were vortexed for 10 s, and then placed in an ultrasonic bath cooled to approximately 0°C with ice for 3 min. The samples were then placed in a thermomixer for 30 min (4°C, 1,400 rpm), before being centrifuged for 10 min (16,100 g, 4°C). The extracts remained in the centrifuge at 4°C until SPE loading.

SPE Purification

SPE is used in this workflow for the removal of unwanted substances, mainly chlorophylls, as per the authors of the original method. This is achieved using an SPE cartridge (Oasis HLB 1 cc Vac, 30 mg, 30 µm, Waters) that binds chlorophylls, but does not strongly retain most analytes. Therefore, all eluates from the step of sample addition are collected together, while the unwanted substances remain bound to the column and are discarded. The authors chose a reverse-phase sorbent composed of *N*-vinylpyrrolidone and divinylbenzene monomers. This mixture is referred to as Hydrophilic-Lipophilic-Balanced (HLB) by the manufacturer. Since the HLB cartridges provided sufficient recovery and matrix removal in preliminary tests, the sorbent chemistry was maintained as in the original method. In contrast to the original method, improvements were made

to standardize vacuum application, and a standardized pre-cleaning step was introduced. Both alterations aimed to increase robustness and reproducibility of the SPE purification step.

General considerations

Up to two chambers, each with 12 cartridge positions, were used at once. Each chamber was connected to a vacuum pump. When the vacuum pump was activated, internal pressure could be regulated via an adjustment ring on the vacuum port that partly covered a bleed valve. A gauge displayed the vacuum intensity in negative mbar units. For most steps, -10 mbar was the target pressure on the gauge scale. When the gauge indicator needle oscillated, the midpoint of the oscillation was used as the reference point. When a temporary increase in vacuum pressure was required, the bleed valve on the adjustment ring was manually occluded. Vacuum was usually applied until no liquid remained in any cartridge of the chamber. According to the manufacturer's specifications, the SPE columns used in this work could "run dry", i.e., the taps on the cartridge connectors did not need to be closed.

Pre-cleaning

Before placing the SPE cartridges on the connectors, the connectors and outlets were cleaned in a standardized manner. A vacuum was applied throughout the entire process (-10 mbar). First, isopropanol was added to each connector opening via a laboratory wash bottle for 2-3 s. Then, 1 mL of methanol was added, followed by 1 mL of ultrapure water. The flow-through was collected and discarded.

Cartridge conditioning

SPE cartridges were placed in each connector position. When a position was not used, its tap was closed. Each cartridge was treated with 1 mL of methanol, followed by 1 mL of water. Lastly, the cartridges were equilibrated with 1 mL of 50%ACN. After each solvent addition, a vacuum (-10 mbar) was applied until no liquid remained in any cartridge. After the final addition, the bleed valve on the vacuum port adjustment ring was occluded until the gauge reached a pressure of -40 mbar, and then opened again. This step was repeated three times to ensure that all liquid had left the connectors and outlets before proceeding to the next step. All flow-through fractions were collected and discarded.

Sample loading and elution

After all cleaning and conditioning steps, racks containing 5-mL microcentrifuge tubes for each sample was placed in the SPE chambers. It was ensured that the outlets aligned with the correct

tubes. Then, the sample centrifugates were transferred to the corresponding SPE cartridges, and a vacuum was applied (-10 mbar) until all cartridges were fully drained. Next, 1 mL of 30%ACN was added to each cartridge and a vacuum was applied again (-10 mbar). Once all cartridges were fully drained, the bleed valve was occluded until the pressure gauge displayed -40 mbar. Equivalent to the conditioning step, this process was repeated three times. The eluate from these two steps was collected in 5-mL centrifuge tubes, which were then transferred into evaporation racks.

Evaporation

As with the extraction, the evaporation process closely followed the original method, with adjustments to available equipment. The evaporation setup used in this work consisted of two custom-built sample racks, each offering 12 positions. Both racks were connected to the same nitrogen gas valve. Replaceable pipette tips served as the nitrogen outlets and were adjustable in height. At the start of the evaporation process, the outlets were placed less than 1 cm below the opening of the sample tubes, and the nitrogen valve was slowly opened. Throughout the evaporation process, the outlet height and nitrogen flow were adjusted to ensure constant agitation of the liquid, while avoiding overly strong turbulence that could lead to splashing.

Evaporation was considered finished for each rack five minutes after no liquid was observed in any microcentrifuge tube. This process usually took between 3 and 4 hours. Finally, the nitrogen flow was switched off and the microcentrifuge tubes were closed until reconstitution.

Extract Reconstitution

The approach to extract reconstitution was modified from the original method. Due to technical necessity, more than the 40 μ L of reconstitution solvent was used. Experience has shown that, with the vial and insert combination used for analysis, at least 60 μ L of liquid must be present to ensure stable injections. To stay above that level with two injections of 10 μ L, a reconstitution volume of 80 μ L was targeted. This was later increased to 100 μ L after an additional centrifugation step was introduced. This was done because reconstituting an evaporated extract might result in solids that do not redissolve. Additionally, steps were introduced to keep analytes cooled during and in between steps.

Before evaporation was complete, brown glass vials were labeled and placed in a covered vial box without inserts or lids. Then, the box was put in a -20°C freezer. Once evaporation was finished, 100 μ L of 30%ACN was added to each 5-mL microcentrifuge tube. Each tube was vortexed for 30 s and placed on ice. Then, the tubes were centrifuged for 10 min (10,000 g, 0°C). Next, 80 μ L of the centrifugate was carefully transferred to a room-temperature insert placed in

one of the prepared, precooled brown glass vials (-20°C). The vials were placed in a cooled autosampler (4°C) until analysis.

Sample Analysis

The UHPLC-MS/MS measurements of samples varied between experiments and are described in the respective chapters of this work. Therefore, only the general measurement procedures are described here. The instrumental setup (Table A-6), generally relevant parameters (Table A-21), and the MRM transitions of the final method (Table A-19) are listed in the Appendix. The processing of acquired sample data is described in Chapter 3.2.5.

Before conditioning the system for sample analysis at the UHPLC-MS starting parameters, the column was equilibrated with a 1% FA solution for 1 h (total flow 0.3 mL/min, 25% Eluent B, oven temperature 50°C). Equal sample batches were created for measurements in positive and negative ESI polarity. All measurements of one polarity, typically positive, were performed first, followed by all measurements of the other polarity. Extracts from the same vial were injected for the measurements in both polarities. For most experiments, data acquisition batches started with a variety of samples (8-12), which served to equilibrate the MS system as well as the UHPLC column. In experiments where only reference substances were analyzed, mixes containing all analytes were used. When plant material was analyzed, plant extract samples were used. Next, control and blank samples were measured, followed by non-prepared standard substance solutions and calibration curve solutions. StS were measured as last in a measurement block. Each analysis took 18.5 min, plus approximately one additional minute for the autosampler to ready the next injection. Therefore, 74 samples could be injected and analyzed in one day (24 h).

3.2.4.2 Testing of an Alternative Sample Preparation Workflow

An alternative sample purification strategy to the standard SPE of the original method was tried out [127]. This method used a dispersive solid phase extraction strategy, similar to the QuEChERS (quick, easy, cheap, effective, rugged, and safe) approaches often applied in pesticide analyses. Besides the different approach to sample preparation, the authors also used different solvents for extraction and analysis. Since all previous optimizations to the method were made using specific solvents, these were not changed for these experiments. The adapted workflow was applied to analyte standard solutions without plant matrix.

Prior to the extraction, GCB aliquots of 10 mg ($\pm$ 1 mg) were weighed in 1.5-mL microcentrifuge tubes using an analytical balance (precision 0.1 mg). Analyte solutions were prepared in 50% ACN. Extraction was performed equal to the standard sample preparation workflow (3 min ultrasonic bath with ice, 30 min thermomixer at 4°C and 1,400 rpm, 10 min centrifugation at 4°C

and 16,100 g). The extracts were then transferred into the GCB-containing microcentrifuge tubes, placed in the thermomixer again for 3 min, and centrifuged again for 10 min. The supernatants were transferred to 2-mL microcentrifuge tubes and evaporated under nitrogen flow. The residues were dissolved in 100 μ L 30%ACN and thoroughly vortexed for 30 s. The tubes were then centrifuged (4°C, 5 min, 16,100 g) and the supernatant transferred to inserts in brown glass vials. Data acquisition followed the standard sample preparation workflow, as presented in the previous chapter.

3.2.4.3 Comparison of Sample Preparation Workflows

A small-scale experiment was devised to test the two sample preparation workflows against one another. The analyte mixes listed as Mix 1 in Table A-22 were used for this experiment. Three samples at base concentration and three samples at 5-fold base concentration were prepared with each workflow, together with three controls (no analytes, no IS) and three blanks (no analytes).

The acquired data was integrated via MultiQuant and the peak lists exported as text files. Further data analyses were performed in R. The signal intensity means and CVs for all prepared and non-prepared analyte mixes were calculated. Due to large signal intensity losses in some prepared samples, only the higher concentrated mixes were considered for recovery calculation. Recoveries were calculated as the percentage ratio between the mean signal intensities in prepared samples and in non-prepared samples.

3.2.5 Development of a Data Analysis Pipeline in R

The final method included a data analysis pipeline, which consisted of custom written R scripts. This chapter outlines the individual modules of this workflow. In this work, the complete code was applied only to the analysis of tomato plant samples (see Chapter 3.4.2). Parts of the final code were used for data analysis in various experiments, particularly for method validation. The respective chapters will discuss these proceedings and reference the workflow described here, where applicable.

It is important to note that peak detection and integration were not part of the coding workflow. The pipeline began with the peak tables generated by integrating chromatographic peaks for each transition in the MultiQuant software. Although this integration process was automated, it required manual corrections for many peaks and remained a bottleneck for data analysis, even after the development of the data analysis pipeline.

The pipeline requires the peak tables exported from MultiQuant as well as four additional files as input. These files provide additional transition information, sample masses, sample dry mass

contents, and the known qualifier/quantifier intensity ratios for each analyte. The code uses a master script that executes a total of 11 other scripts. Their functions are summarized in Table 7 (Chapter 4.2.2.3). Relevant details are outlined and discussed in the following chapters.

3.2.5.1 Master Script

One of the primary objectives in developing a data analysis pipeline was to ensure its ease of use and universality. Therefore, a centralized master script was developed to orchestrate the pipeline. This script contains a variety of core functions and includes marked fields where user input is necessary. It also executes all other scripts.

The master script begins by clearing the current workspace and loading all dependencies (project packages). Here, the *p_load* function from the R package *pacman* is used so that any packages not yet installed by the user are automatically installed and loaded. Then, conflict preferences are set using the *conflicts_prefer* function from the R package *conflicted*. Next, the current date and time are saved as a character string. These will later form the basis of all file names of exported files.

After that, the user specifies the output folder and a variety of project-specific variables. These include the number and names of matrices, as well as the subgroup names and factors. Additionally, the names of the input text files are provided, as well as the IDs of the blank samples and a non-prepared IS mix sample. Other inputs related to specific scripts are required, which are marked and thoroughly commented to guide prospective users through the script. Overall, the script is concise, comprising fewer than 80 lines of executable code.

3.2.5.2 Data Export Structure

Under a specified output folder name, a data folder structure is created in the Windows file system. All exported files and graphs are saved in a user-friendly manner in one of the eight folders created. The data structure is organized by evaluation type and includes a folder each for IS, calibration curves, quantification results, transition data tables, heat maps, identification tables, graphical summaries, and exploratory data analysis (PCA).

3.2.5.3 Data Import

Data tables are imported from the specified files. A custom function, *create_allAnalytes_table*, is used to generate complete data tables for each matrix. This table includes the supplemental information for each transition, which was provided as a text file. Subgroup identifiers and names are automatically extracted from sample names. This step is project-specific and must be adapted

for each project. Further improvements to this pipeline could be made by providing sample data through a supplemental data file instead.

As part of the data import script, a table of the average signal intensities of all analytes in the specified control samples is created. These values will later be subtracted from the measured signal intensities of the samples.

3.2.5.4 Creation of Calibration Curves

Calibration curve samples are identified by their sample names, including their concentration factor. The base concentration at the lowest calibration level is provided in the transition data input file, and is multiplied by the concentration factors to obtain the level concentrations. The user provides the names of analytes for quantification and calibration in the master script. Additionally, the user can specify calibration levels that should be excluded.

As described in Chapter 3.3.3, calibration curves are calculated for each analyte using linear regression and a weight of $1/x$. The response ratio (the signal intensity ratio between analyte and IS) is used as the dependent variable, and the concentration factor is used as the independent variable. Regression parameters, such as the linear equation, the coefficient of determination (R^2), and the root mean square error (RMSE), are saved. The RMSE is calculated as the square root of the mean of squared residuals and was further divided by the mean response ratio of the curve to obtain a relative value. Calibration-point-specific determiners, such as residuals (standardized and non-standardized), are extracted, and calibration level concentrations are back-calculated. These back-calculated concentrations are then used to calculate the percentage bias for each calibration point. Summaries of these bias analyses are saved with the regression parameters. Most notably, flags are created to indicate the number of calibration levels with biases greater than 15%, 20% and 25%. All of these evaluation criteria are exported to an xlsx table. Graphs are exported as a pdf, with one page for each calibration curve of an analyte. These graphs also show some quality parameters, such as the R^2 .

The architecture of the script facilitates an iterative approach to calibration. The exported files are designed so that calibration curves can be evaluated quickly. If it is deemed necessary, calibration points can then be removed before running the script again. This allows for a fast, iterative evaluation of calibration curves until a satisfactory calibration curve is obtained for each analyte.

3.2.5.5 Quantification Results

Absolute concentrations are calculated using the calibration curves and the response ratio of the analytes in the samples. Initially obtained analyte concentrations in the extract (in nM) are converted into substance amounts (in nmol), and then into analyte content of the sample (in fmol/mg), using the sample masses. Summaries for each subgroup are created and exported together with the sample results as two sheets in an xlsx file.

Additionally, the percentage deviation from the subgroup mean is calculated for each sample. This information is exported to a separate sheet in the same xlsx file and can be used to identify sample outliers in the data.

3.2.5.6 Analyte Data Tables

All transition data from all samples are summarized in one data table. Then, several corrections are made to the signal intensities of each transition in each sample. First, the signal intensities of all analytes detected in the control samples (prepared alongside the StS) are corrected by subtracting the mean signal intensity of the control samples from each sample's intensity. This background subtraction procedure is consistent with the methodology of the original method. Second, all signal intensities are normalized according to the mass of the respective sample. The target mass at weighing is used as the reference point. This reduces variance introduced solely through sample weighing. Third, all analytes that are quantified, and therefore have an allocated IS, are normalized using the IS intensity in the sample. The IS signal in a non-prepared IS mix sample is used as the reference value. These normalized intensities were used for subsequent steps, for both non-quantified analytes and quantified analytes. Response ratios were employed for quantification only. The corrected signal intensity data for each transition in the StS and QC samples is summarized, and the summary and individual sample values are exported as sheets in an xlsx file.

Then, a subgroup filter is applied in order to select only reproducibly detected analytes for subsequent steps. Only analytes detected in enough samples of at least one subgroup (either transition) are kept. The threshold can be specified by the user. For six samples per subgroup, a threshold of 66% was chosen, meaning an analyte must be detected in at least four out of six samples in one subgroup. Summaries are created for both detected and non-detected analytes. These summaries are exported as sheets to the same xlsx file. Formatting and conditional formatting are automatically applied to the summary sheet for detected analytes (*Summ_DetectedAnal*). This was done to easily identify whether quantifier and qualifier transitions should be interchanged. An input in the master script allows the user to manually designate the

qualifier as the primary transition, e.g. if only the qualifier has been detected. For all subsequent steps, only quantifier transitions are used, making this an important step.

Lastly, a table with the signal intensities of the IS is created, spanning all samples. Separate tables are generated for the StS and QC samples. Relative calculations are performed, and additional tables generated to show the signal intensity changes of the IS through sample preparation and matrix effects. Unprepared and prepared IS mixes (blank samples) are used to calculate intensity loss through sample preparation. Prepared QC samples and blank samples are used to calculate signal intensity changes in the presence of matrix. All of these tables are exported as an xlsx file to the IS folder.

3.2.5.7 Heatmap Generation

The quantifier transition data of reproducibly detected analytes are used to create heatmaps. This is done separately for each matrix. The heatmaps show the Log2 FC for the subgroup means of each quantifier signal intensity. QC samples are used as the reference value for FC calculation. To simplify visual comparisons, a consistent color palette is applied to the heatmaps of different matrices. Heatmaps are exported as pdf and svg files.

3.2.5.8 Statistical Tests

Statistical tests are performed on the quantifier signal intensities of each reproducibly detected analyte. All tests were performed separately for each matrix. A one-factor ANOVA is performed, as well as the subsequent Tukey tests. When two factors are available, an additional two-factor ANOVA is performed. The one-factor ANOVA uses the subgroup as its factor, which also applies to the Tukey tests. The CLD labels from the pairwise Tukey tests are saved for graph generation. Two subgroups with different letters have a statistically significant mean difference ($p < 0.05$). The two-factor ANOVA uses the user-specified subgroup factors and provides a p-value for each factor and for the interaction between the factors. All statistical test results are saved.

3.2.5.9 Identification

Identification is performed on reproducibly detected analytes by comparing the Quantifier/Qualifier signal intensity ratios from the analysis to known value ranges. This is referred to as the “common range” in the code. The common range values were obtained by measuring analytes at various concentrations across their dynamic range (Chapter 3.2.2.2) and expanding the recorded maximum and minimum values by 25%.

A Boolean flag (true/false) is set for each analyte in each sample, showing whether the sample has a Quantifier/Qualifier ratio within the common range. Then, analyte flags are allocated based

on the ratio of detected samples with a Quantifier/Qualifier ratio within the common range. If the ratio has a value of 0, or if a ratio cannot be calculated, the analyte is flagged as “not identifiable”. If the ratio is ≥ 0.5 , the analyte is flagged as “identified”. Analytes with a ratio between 0 and 0.5 are flagged as “manual check required”. However, it is recommended in the documentation to manually evaluate every identification.

Multiple tables summarizing the identification process are created and exported as an xlsx file. Conditional formatting is automatically applied to highlight the identification flags in Excel.

3.2.5.10 Graphical Summaries

One-page pdf summaries are created for each reproducibly detected analyte and exported into a single pdf file. These graphical summaries provide an overview of several key aspects of data analysis. Example pages for two analytes from the tomato analysis dataset (Chapter 3.4.2) are provided in the results section (Figure 34).

First, bar plots showing the mean corrected signal intensities in all subgroups and matrices are presented, with respective SD as error bars. A custom color scheme for the plots can be specified in the master script. These plots also show the number of samples in each subgroup in which the analyte was detected, as well as the CLD labels from the Tukey tests. Importantly, in a two-factor experiment, these letters provide an indication of significant differences, but should not be interpreted statistically. This is why the second element of the report is the two-factor ANOVA p-value table. These tables provide the p-value and the corresponding significance star code for each matrix. Only these tables should serve as the basis for actual statistical interpretation. Third, another graph shows the signal intensities of the StS and QC samples throughout the measurement series. This graph includes the CV of signal intensities in the QC samples. These graphs can be used to evaluate signal drifts over the course of the data acquisition, or to identify sample outliers. Although it has not yet been included in the code pipeline, signal drift correction based on this evaluation could be performed using the QC samples. Fourth, identification flags, along with brief explanations of their meanings, are displayed at the bottom of each page to indicate whether an analyte has been identified.

In addition to the graphical report generated for each detected analyte, an additional report with the same layout is automatically created for each IS. The one-page pdf summaries for each IS are also exported together as one pdf file into the output folder designated for IS evaluation.

3.2.5.11 Principal Component Analysis

Similar to the metabolomics data analysis, PCA is performed on all reproducibly detected analytes in each matrix. Missing values, resulting from an analyte not being detected in a sample, are imputed by a fixed-value substitution. Specifically, they are replaced with half of the lowest detected analyte signal intensity. The signal intensities are $\log_{10}$ -transformed and Z-transformed. Two safeguards are executed and reported to the user in text form. First, the number and ratio of analytes with over 30% missing values are displayed. If this ratio is too high, it can distort the results of the PCA, and the user is informed of this. Second, analytes with non-finite values or zero variance are removed and this is reported to the user. Such analytes should not be present in the PCA dataset, but are evaluated nonetheless since PCA cannot be performed if any are present.

The PCA itself is performed by converting the PCA data into a matrix, and then applying the *prcomp* function from the base R *stats* package. Scores and loadings are extracted from the resulting object for the first five PCs. 2D score and loading plots are created using the data from the first two PCs. Subgroup samples and QC samples are marked and labeled in the 2D score plots. The score and loading plots for each matrix are exported as pdf and svg files.

3.3 Validation

The final UHPLC-MS/MS method was validated separately for the phytohormone analytes intended for relative and absolute quantification, respectively.

Chapter 3.3.1 describes the initial stability tests performed on all analytes. Chapter 3.3.2 details the validation protocol for analytes measured via relative quantification, which are hereafter referred to as semi-quantified analytes. Absolute quantification was strictly reserved for analytes with corresponding IS (ABA, IAA, GA1, GA4, JA, SA, iP, and tZ). Validation of these analytes was performed according to the SANTE 11312/2021 guidelines [174] and is detailed in Chapter 3.3.3. The ICH (International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use) for the validation of analytical procedures [175] were additionally consulted in some aspects. These guideline specifications were also applied to the evaluation of the semi-quantified analytes wherever possible. Following these validations without matrix, validations were performed for two distinct tomato tissues. The details of these in-matrix validations are described in Chapter 3.3.4.

3.3.1 Analyte Stability

According to the SANTE 11312/2021 guidelines [174], analyte stability in extracts should be evaluated during method validation. This step was performed early in the method adaptation process to avoid erroneous results due to analyte degradation.

The analyte mixes listed in the “Mix 1” column of Table A-22 were used in this experiment. 30%ACN was used as the solvent. A mix of all analytes at a 10-fold base concentration was created to account for potential reactions between all analytes. The analyte mixture was diluted to the base concentration and distributed into different vials. All vials were placed in the cooled (4°C) UHPLC autosampler. The mixes were measured on four different days: the day of preparation (Day 0), after 24 h (Day 1), after 48 h (Day 2), and after seven days (Day 7). Three replicates were measured per day and polarity. Gradient 1 (see Chapter 3.2.3, Table 3) and a slightly altered version of the final MRM method (Table A-19) were used for these measurements.

After data acquisition, peak detection and integration were performed using the software MultiQuant. The peak tables were exported, and further data processing and analysis were done in R. The mean signal intensities over the four measurement days were plotted in line graphs with SD error bars. Statistical tests were performed on each transition in the form of a one-factor ANOVA and pairwise Tukey tests, using measurement day as factor. While triplicate measurements provide limited statistical power, these tests were primarily used as a preliminary assessment of signal intensity trends. The results were only used to provide an orientation regarding the magnitude of signal intensity changes. The p-values of the pairwise tests between Day 0 and each of the other measurement days were saved as a table. Two pdf files, one for each polarity, were created and exported. These files contained the graphs and p-value tables for each analyte and served as the basis for data evaluation.

3.3.2 Semi-quantified Analytes

Semi-quantified analytes were subjected to a thorough evaluation in several aspects. First, the limits of detection and quantification were determined for both transitions (Chapter 3.3.2.1). Using these levels for each analyte, new concentration mixes with comparable analyte levels were prepared. These mixes were then measured at various concentrations to determine analyte response linearity and precision at different levels (Chapter 3.3.2.2). Lastly, analyte solutions were prepared multiple times using the established sample preparation workflow, and their recoveries and interday precisions were calculated (Chapter 3.3.2.3).

3.3.2.1 Limits of Detection and Quantification

The sensitivity of the analyses was evaluated, and the limits of detection (LOD) and lower limits of quantification (LLOQ) were calculated for each transition.

Previous analyte mixes ("Mix 1", Table A-22) were used for this experiment. One mix containing all analytes at a 10-fold concentration of the base concentration was prepared. From this primary mixture, four successive 10-fold serial dilutions were prepared. Each of these decimal levels, including the primary stock solution, was further diluted to create five equidistant points per order of magnitude. Three additional dilutions were made to cover one more order of magnitude. These steps resulted in 28 analyte dilutions spanning a concentration range from 0.00001- to 10-fold of the base concentration. The diluted mixtures were measured using the finalized UHPLC method (see Chapter 3.2.3, Table 3, Gradient 5) and the same MRM method used for the stability measurements (see Chapter 3.3.1). Only one injection per sample solution was analyzed.

While most previous peak integrations were performed using the software MultiQuant, the S/N ratio calculated by the software uses the subtracted peak height divided by the standard deviation of the noise region. This approach often overestimates S/N ratios. Therefore, the software Analyst was used instead, as it allows for calculation of peak-to-peak S/N ratios, which divide the subtracted peak height by the amplitude of the noise region. In Analyst, peaks were manually integrated for each transition in each of the 28 samples. The S/N ratio values were manually entered into an Excel file and served as the basis for evaluation.

The LOD was defined as the lowest measured concentration where the S/N ratio was greater than 3, provided that all higher concentrations also exhibited values greater than 3. Similarly, the LLOQ was the lowest measured concentration with an S/N ratio greater than 10, provided that all higher concentrations had an S/N ratio above 10. This approach was chosen to align with the published original method that was adapted [126], and corresponds to the ICH Q2(R2) guidelines [175] as well as common praxis in LC-MS analysis [176]. LOD and LLOQ were determined for each transition. For each analyte, the transition with the lower LLOQ was designated the quantifier, and the other transition was designated the qualifier. The transition names were adapted accordingly, resulting in the final transition names listed in Table A-19. Additionally, new analyte mixes were prepared for each class, where the base concentration of each analyte corresponded to its qualifier LLOQ. These are listed in Table A-22 as Mix 2.

3.3.2.2 Instrumental Linearity and Precision

Analyte mixes were measured at different concentrations to evaluate analyte response linearity and precision at each concentration. This was done without sample preparation of the analyte solutions to review the instruments' performance.

The LLOQ-based mixes were used (Table A-22, "Mix 2"), where the base concentrations at analysis corresponded to the LLOQs of the analytes' qualifier transitions. To evaluate the quantifiers as well, additional mixes were prepared where the base concentration at analysis corresponded to the LLOQ of the analytes' quantifier transitions. These mixes were then measured at 1-, 5-, 10-, 25-, 50- and 100-fold LLOQ concentrations. If higher mix concentrations were available, the mixes were also measured at 500- and 1,000-fold concentrations. Each mix was measured in positive and negative ESI polarity, unless no analyte of a polarity was present in the mix. The analyte IAA-Asp was measured separately from the analyte mixes. Eight replicates were measured per concentration level. The measurements were performed in several batches, as the total measurement time exceeded one week. Data acquisition was performed using the final UHPLC-MS/MS method (see Chapter 3.2.3) and MRM transitions (see Appendix, Table A-19). As described in Chapter 3.2.4.1, the column was equilibrated with a 1% FA solution before each analysis batch and the MS system was equilibrated by measuring mixes containing each analyte at the base concentration 10-fold at the start of each batch.

Peak detection and integration were carried out in MultiQuant. Further processing and analysis of all peak data were performed in R using custom scripts. A linear regression with a weight of $1/x$ was performed for each analyte transition. The regression quality parameters were saved, and plots displaying signal intensity against analyte concentration were created. These plots included the regression lines and their R^2 . Additionally, a table summarizing the mean signal intensity, SD, and CV for each concentration level was generated for each analyte transition. One pdf page displaying the respective plot and table was created for each transition and exported as one pdf file per ESI polarity. These files served as the basis for data evaluation. Furthermore, the signal mean and precision tables generated for the individual transitions were used to visualize CV and R^2 distributions in the form of faceted histograms.

3.3.2.3 Method Precision and Absolute Recoveries

After evaluating the instrument precision, the precision of the entire method was validated. This was done on three separate days to review intraday and interday precision. Five replicates were tested per day, using concentrations close to the LLOQ (Table A-22, "Mix 2", 2.5-fold LLOQ concentrations). The samples were prepared using the sample preparation and data acquisition

workflow detailed in Chapter 3.2.4.1. Unprepared analyte mixes were analyzed alongside the prepared samples each day to calculate recoveries.

Peak detection and integration were performed in MultiQuant. The exported peak lists were imported into R and processed via custom scripts. One outlier sample (Day 3, positive polarity, Replicate 1) was removed, since the sample was known to have been mishandled during preparation. Mean signal intensities, SDs, CVs and absolute recoveries were calculated for each day, as well as for all days combined. Tables summarizing the data were exported as an xlsx file. Additionally, faceted histogram plots were created to visualize the intraday and interday precisions (CVs).

3.3.3 Quantified Analytes

Validation of the quantified analytes adhered more closely to the SANTE 11312/2021 guidelines [174] than that of the semi-quantified analytes described in the previous section. This is because these guidelines were developed specifically for methods using absolute quantification. However, they are still not perfectly applicable to this work because they focus on pesticide residues in food. For instance, the guidelines often refer to “reporting limits” based on the maximum legal residue levels of pesticides, a concept that does not apply to natural plant hormones. Therefore, the SANTE 11312/2021 guidelines were used as a general reference wherever possible, and any specific deviations are noted and discussed. The following chapter outlines the validation of the analytes using reference standard solutions. Further validation steps in matrix are presented in Chapter 3.3.4.

The quantified analytes are listed in Table 4, including the respective IS used for quantification. It was originally intended to quantify the cytokinin tZ by using the rarely naturally occurring KIN as an IS until KIN was detected in tomato samples (see Chapter 4.4.3). In consequence, quantifying tZ with the isotope-labeled standard for the cytokinin iP (d6-iP) was attempted as an alternative. Following the SANTE 11312/2021 guideline specifications, the response ratio was used instead of the signal intensity to create calibration curves and perform subsequent quantifications. The response ratio is the ratio between analyte and IS signal intensities in the same sample. Mathematically, this is equivalent to normalizing the raw analyte signal by a correction factor, calculated as the ratio of the IS in a reference sample to the IS in the current sample.

Table 4: Quantified analytes in the final targeted phytohormone analysis method. ID, abbreviated analyte identifier (see Table A-17); IS, internal standard; ESI, electrospray ionization.

Analyte	ID	IS ID	Phytohormone class	ESI polarity
(+)-Abscisic acid	ABA	d6-ABA	Abscisates	Negative
Indole-3-acetic acid	IAA	d5-IAA	Auxins	Positive
<i>N</i> 6-Isopentenyladenine	iP	d6-iP	Cytokinins	Positive
<i>trans</i> -Zeatin	tZ	d6-iP / KIN	Cytokinins	Positive
Gibberellin A1	GA1	d4-GA1	Gibberellins	Negative
Gibberellin A4	GA4	d2-GA4	Gibberellins	Negative
(±)-Jasmonic acid	JA	d5-JA	Jasmonates	Negative
Salicylic acid	SA	d4-SA	Salicylates	Negative

3.3.3.1 Limits of Detection and Quantification

The limits of detection and quantification were initially determined in the same experiment that was performed for the semi-quantified analytes (see Chapter 3.3.2.1). However, before method validation, the quadrupoles of the MS system underwent technical maintenance and cleaning by a Sciex technician. As this procedure typically influences signal sensitivity, the LOD and LLOQ values were redetermined to ensure the accuracy of the validation data for the quantified analytes. Analyte mixes were prepared at concentrations adapted from the LLOQ-based mixes used previously (Table A-22, “Mix 2”). Diluted mixes spanning three orders of magnitude (0.01-fold to 10-fold concentrations) were prepared, with 5 concentration steps per order of magnitude. Each solution was injected once. As in the procedure for the semi-quantified analytes (Chapter 3.3.2.1), S/N ratios were evaluated via Analyst.

New mixes of quantified analytes were prepared with concentrations corresponding to the newly determined LLOQs. For all analytes but JA, LLOQs of qualifier and quantifier transitions were close to each other and the qualifier LLOQ was chosen as the respective base concentration. Since the quantifier and qualifier transitions of JA differed by two orders of magnitude, the quantifier LLOQ was chosen as the base concentration of JA. Initially, separate analyte mixes were created for the analytes measured in positive and negative ESI polarity. In later experiments, one mix was created for all analytes. In the concentrated stock mixes, analytes were present at 2,000-fold concentrations of their LLOQs. IS mixes were prepared based on their LLOQs (as determined in Chapter 3.3.2.1). The concentration of these mixes was intended to be 25-fold the concentration of their LLOQs, though some adaptations were made for certain IS. The analyte and IS concentrations in the respective mixes are given as “Mix 1” in Table A-23 in the Appendix.

3.3.3.2 Calibration Curves

Calibration curves were generally created by measuring analyte mixtures starting at LLOQ concentration and increasing by specific factors while keeping IS concentrations at the same level. Linear regression fits were then calculated in R, as described in Chapter 3.2.5.4, using the response ratio and LLOQ-concentration factors as variables. The calibration levels typically included 1-, 5-, 10-, 25-, 50-, 100-, 250-, 500-, and 1,000-fold concentrations of the LLOQ, although the 250-fold concentration level was not used in all experiments. As explained in the previous chapter, the qualifier LLOQ was used for all analytes but JA. The solution at LLOQ concentration was always measured twice and the mean of the two signals used to reduce random variance at this level.

To evaluate the model fitness of each calibration, the back-calculated concentrations of the standards were compared to their true concentrations at each calibration level. Following the SANTE 11312/2021 guidelines, a deviation (bias) of less than $\pm 20\%$ was set as a target [174]. While the SANTE guidelines require linearity to be reviewed, they do not specify how to do so. Here, linearity was further assessed using R^2 values, RMSE, and visual inspection of the graphs. While an R^2 value of >0.999 was generally targeted, it was not used as a rigid threshold.

An initial experiment was conducted to assess the calibrations for the quantified analytes, in which three analyte calibration curves were separately prepared, measured, and processed as described above. The 250-fold concentration level was not used. The iterative approach described in Chapter 3.2.5.4 was applied until calibration curves met the set criteria. The calibrations were then evaluated regarding their concordance. Additionally, potential detector saturation and isotopic effects were checked at high-concentration levels. Specifically, it was examined whether high levels of an analyte produced a signal in the IS transitions. To assess potential interference from the IS themselves, the IS mix was analyzed at concentrations up to the maximum concentration given in Table A-23 in the Appendix ("Mix 1"), to ensure that no false-positive signals were present, e.g. due to impure standards.

3.3.3.3 Method Precision and Apparent Recoveries

The method precision and analyte recoveries were evaluated in the same manner as for the semi-quantified analytes (see Chapter 3.3.2.3), except with more concentration levels. Analyte solutions were prepared at three concentration levels: 2.5-fold, 75-fold and 400-fold concentration of the LLOQ. These levels were chosen to evaluate method performance across the entire dynamic range, without being too close to any calibration point. The analyte mix provided in Table A-23

and the IS mix provided as “Mix 2” in the same table were used for this experiment. GA4 was measured at all levels but only the lower two levels were evaluated.

Five replicates were prepared and analyzed at each concentration level, following the workflow detailed in Chapter 3.2.4.1. This was done three times on separate days, leading to a total of 15 replicates analyzed per concentration level. One sample of the third experiment day (2.5-fold LLOQ concentration) was removed from the dataset due to mishandling in the preparation workflow, which produced outlier results in the data evaluation. Calibration followed the protocol outlined in the previous chapter and included a 250-fold LLOQ calibration level. Peak detection and integration were performed in MultiQuant, and the peak lists were processed and analyzed in R via custom scripts. The mean extract concentration, and precision (SDs and CVs) were calculated for each concentration level and summarized for each day (intraday) and for the entire three-day experiment (interday). Method performance was evaluated in terms of trueness, which was calculated by relating the quantification results to the known analyte concentrations. The precision and recovery data were exported as an xlsx file and served as the basis for data evaluation.

3.3.4 In-matrix Validation

In-matrix validations were performed for tomato leaf and fruit tissues by assessing the analyte recoveries and the precision in spiked matrix extracts. First, endogenous analyte concentrations were determined in pooled tomato leaf and pooled tomato fruit samples (Chapter 3.3.4.1). Then, samples were spiked at various concentrations (Chapter 3.3.4.2). The tomato samples were obtained independently of the experiment described in Chapter 3.4.2.

This approach aligns with the SANTE 11312/2021 guidelines. According to these guidelines, blank material is preferable for determining in-matrix analyte recoveries. Blank material is sample material that is equal to the analyzed material, but without analytes present. If no such blank material is available, recoveries should be determined by spiking analytes at ≥ 3 times the level present [174]. It is improbable that any plant material contains none of the phytohormone analytes included in this method. Furthermore, it is unrealistic to create a blank material by removing the analytes from a plant sample without drastically altering its matrix composition. Thus, the approach presented here is therefore intended as a framework for all future method applications.

3.3.4.1 Preparation and Analysis of Tomato Plant Material

Two cultivars of organic tomatoes were purchased at a supermarket for creating a pooled tomato fruit sample. One was a cherry tomato cultivar, and the other was a medium-sized vine tomato cultivar. The cultivars' names were not listed on the respective packaging. Two tomato cultivars,

'Tiny Tim' and 'Tumbling Tom', were grown in-house in soil and hydroculture, respectively, for the creation of a pooled tomato leaf sample. The leaves were harvested approximately five to six weeks after germination.

The tomato fruits and harvested leaves were shock-frozen in liquid nitrogen. The larger tomato fruits were quartered immediately before being placed in the liquid nitrogen bath; the cherry tomatoes were not cut. The samples were submerged in liquid nitrogen until no more nitrogen was evaporating. Then, they were transferred to large glass containers designed for freeze-drying. The samples were freeze-dried for 72 h (condenser temperature -80°C , $p < 1$ mbar) with light-blocking bags over each glass container. The samples were then stored at -80°C until ball milling. Ball milling was performed in 50-mL and 125-mL sample containers containing eight balls each (diameter 12 mm or 20 mm, respectively), depending on sample volume. The containers were cooled to 0°C by submerging them in an ice-filled water bath while they were packed in plastic bags. The frequency was 30 Hz for both sample types, but the duration varied (20 s for leaf and 30 s for fruit samples). The pulverized samples of each matrix type were combined into one sample pool per matrix. Only these pooled samples were subsequently used for validation analyses.

Samples were weighed at three masses for each sample type (3 mg, 10 mg, 30 mg) using an analytical balance with a precision of 0.01 mg. Three replicates were weighed per sample type and target mass. However, the 30 mg leaf samples were ultimately not measured. The samples were prepared and analyzed using the final phytohormone analysis method described in Chapters 3.2.3 and 3.2.4, including the final MRM method (Table A-19). The analyte mixes listed in Table A-23 were used for calibration. The IS mix listed in the "Mix 1" column of the same table was used, but at half of the listed concentrations.

The acquired data were integrated, processed, and evaluated using a slightly altered version of the R data analysis pipeline described in Chapter 3.2.5. The statistical tests, heatmap generation, and PCA were not performed. The analysis results were evaluated separately for each matrix. The absolute concentration means of the different sample masses were evaluated for concordance. Additionally, the numbers of identified analytes at each sample mass were compared.

3.3.4.2 Analyte Precision and Recovery in Spiked Samples

Analyte mixes were added during the sample preparation process to determine in-matrix recoveries and precision. The endogenous concentrations determined in the previous chapter

were used to set the spiking levels. Spiking experiments were performed separately for each tissue type.

The goal was to spike each analyte at two concentrations, once at the endogenous level and once at five times the endogenous level. However, this approach was applied only to analytes detected between the LLOQ and 50-fold LLOQ concentration. When an analyte was not detected, or detected below the LLOQ, it was spiked at 2.5-fold and 12.5-fold concentrations of the respective LLOQ. Likewise, when an analyte was detected at concentrations greater than the 50-fold LLOQ concentration, it was spiked at 50-fold and 250-fold LLOQ concentrations to avoid exceeding the calibration range. This approach was based on the SANTE 11312/2021 guidelines, but did not follow them strictly. The guidelines only require an analyte to be spiked at ≥ 3 times the endogenous level [174]. Furthermore, the analytes were spiked at two different timepoints in the workflow, once during extraction and once during reconstitution.

Analyte mixes were prepared separately for each tissue type, depending on the endogenous analyte levels. The analyte mix concentrations, spiked extract concentrations at the time of analysis, and fold concentrations relative to the LLOQ (“LLOQ factors”) are provided in Table A-24 in the Appendix. 23 aliquots of freeze-dried, pulverized material were weighed for each tissue type. An analytical balance with a precision of 0.01 mg was used. The target mass was 6 mg (± 0.2 mg) for leaf samples and 10 mg (± 0.3 mg) for fruit samples. The weighed samples were divided into five groups depending on the spiked analyte level and the spiking timepoint. First, five replicates were not spiked and were used to determine endogenous levels. The second and third groups consisted of five replicates each that were spiked during extraction at lower and higher concentration levels, respectively. The fourth and fifth groups consisted of four replicates each that were spiked after evaporation of the samples by adding the analytes to the reconstitution solution. In addition to these samples, one blank sample was prepared alongside the matrix-containing samples. The IS were used at the concentrations listed in Table A-23 (“Mix 2”). The IS were added during extraction, except for the samples spiked during reconstitution. In those cases, they were also added to the reconstitution solution.

Other than analyte spiking, the samples were prepared and analyzed as described in the previous Chapter 3.3.4.1. The R data analysis pipeline (Chapter 3.2.5.) was applied as well, with modifications made for recovery and precision calculations. The mean extract concentrations were calculated for each concentration level. The extract concentrations were converted into analyte amounts (in fmol) and then into sample contents (in fmol per mg of sample) by factoring in sample target masses. Recoveries of spiked analytes were calculated by subtracting the mean content in the non-spiked samples from the content of spiked samples. The subtracted analyte amount was

then related to the known added analyte amounts and expressed as a percentage. Precision was calculated as the CV of the spiked sample amounts. Tables listing the recoveries and precision were exported as one xlsx file per matrix.

3.4 Application of the Established Phytohormone Analysis Method

The UHPLC-MS/MS phytohormone analysis method was applied to plant samples at various stages of its development. It was applied to the potato plant samples, which were described in Chapter 3.1, as well as rice plant samples, before all analytes were incorporated into the method scope. These preliminary analyses are detailed in Chapter 3.4.1. The application of the finalized, validated method to tomato plants is described in Chapter 3.4.2.

3.4.1 Preliminary Applications

Samples of two distinct plant species were analyzed, each at a different stage of method development. The potato leaves and roots of the experiment on fungal effector proteins (see Chapter 3.1) were analyzed when the method only included gibberellins, strigolactones, and auxins. These experiments are outlined briefly in Chapter 3.4.1.1. Rice plant samples were analyzed after more metabolites were included, mainly stress-relevant salicylates, jasmonates, and abscisates. These experiments included a comparative analysis of freshly prepared and freeze-dried material, as well as an assessment of the effects of ball milling during sample preparation. The detailed proceedings are presented in Chapter 3.4.1.2. The phytohormone analyses of rice plants were performed and published as a Master's thesis within this project. Therefore, only aspects relevant to method development are summarized, and work contributions are stated clearly.

3.4.1.1 Analysis of Gibberellins, Auxins, and Strigolactones in Potato Plants

The cultivation, sample acquisition, and sample processing of potato plants were described in detail in Chapter 3.1. Since phytohormones were identified as metabolites of interest during data interpretation, targeted phytohormone analysis was applied to the potato samples. As these results did not contribute strongly to further method development, the description of these experiments is kept brief.

All 36 leaf samples (six per plant line) and 18 root samples (six per EV-2, SP7-7, and SP7-10 plant lines) were analyzed. Root sample analysis was limited due to low sample amounts in the other groups. 20 mg ($\pm$ 0.2 mg) was weighed for each leaf sample and 15 mg ($\pm$ 0.1 mg) was weighed for each root sample. While these sample masses were high in comparison to those used by the original authors (20 mg fresh plant tissue) of the method [126], they yielded the most promising results in preliminary experiments (not detailed in this work). Samples were then prepared

following the protocol described in Chapter 3.2.2.4. The following IS were added to each sample during the extraction: d2-GA4 (1 μ M), d4-GA1 (10 μ M), d2-IAA (10 μ M), and GR24 (0,1 μ M). The samples were measured using the UHPLC method listed as Gradient 1 in Chapter 3.2.3, as well as an early version of the MRM method listed in Table A-19. This version of the MRM method included only auxin, gibberellin, and strigolactone analytes (36 total analytes). Absolute quantification was not attempted.

Peak detection and integration were performed in MultiQuant, and the generated peak lists were exported. The performance of the IS was evaluated in Excel. The signal intensities of analytes for which both transitions were detected were evaluated in JMP. Statistical analysis was performed as an ANOVA with subsequent Tukey tests, using the plant line as the univariate factor.

3.4.1.2 Analysis of Stress-relevant Phytohormones in Rice Plants

Rice plants were cultivated in collaboration with the Molecular Cell Biology group at the KIT Botanical Institute, headed by Prof. Dr. Peter Nick. The phytohormone analyses described in this chapter were performed and evaluated as a part of the Master's thesis of student Thérèse Dreßler, which was supervised by Prof. Dr. Peter Nick and Prof. Dr. Mirko Bunzel (Department of Food Chemistry and Phytochemistry). The experimental design was devised as a part of this work. The plant cultivation and harvesting were a collaborative effort. The metabolome data of the rice plants, which will be described in Chapter 3.5.1, were analyzed solely as a part of this work.

Two experiments were designed, both with parallel cultivation and stress application processes. Young rice plants were grown in boxes filled with a culture medium. One hour before harvesting, half of the rice plants were exposed to wounding stress by pinching them with tweezers, while the other half were not exposed. The common goal of both experiments was to validate the suitability of the phytohormone analysis method for detecting short-term stress in plants. The experiments are illustrated in Figure 14, and relevant details are described below.

In the first experiment, the plants were harvested and immediately frozen in liquid nitrogen. All plants of one cultivation box constituted one biological replicate. They were subsequently freeze-dried and ball-milled, as described in Chapters 3.1.2 and 3.1.3 for potato plants. The parameters used for ball milling differed from those used for processing potato plant samples (Retsch MM500, cup volume 25 mL, 4 balls, ball diameter 12 mm, frequency 30 Hz). The duration of processing differed slightly between samples (30-45 s) due to some samples not appearing to be fully homogenized; this is considered a drawback of this study. The ball milling process temperature was intentionally varied as a factor of interest. Specifically, sample containers were either left at room temperature or cooled to 0°C in an ice bath before each milling process. Thus, two factors

of interest (stress application and ball-milling temperature) with two levels each were assessed. Four to five replicates were produced for each combination of factor levels, which are referred to as subgroups. The freeze-dried, pulverized samples were then weighed (8 mg), and analyzed together with QC samples and controls. The samples of this experiment were also used for metabolome analysis, which is described in Chapter 3.5.1.

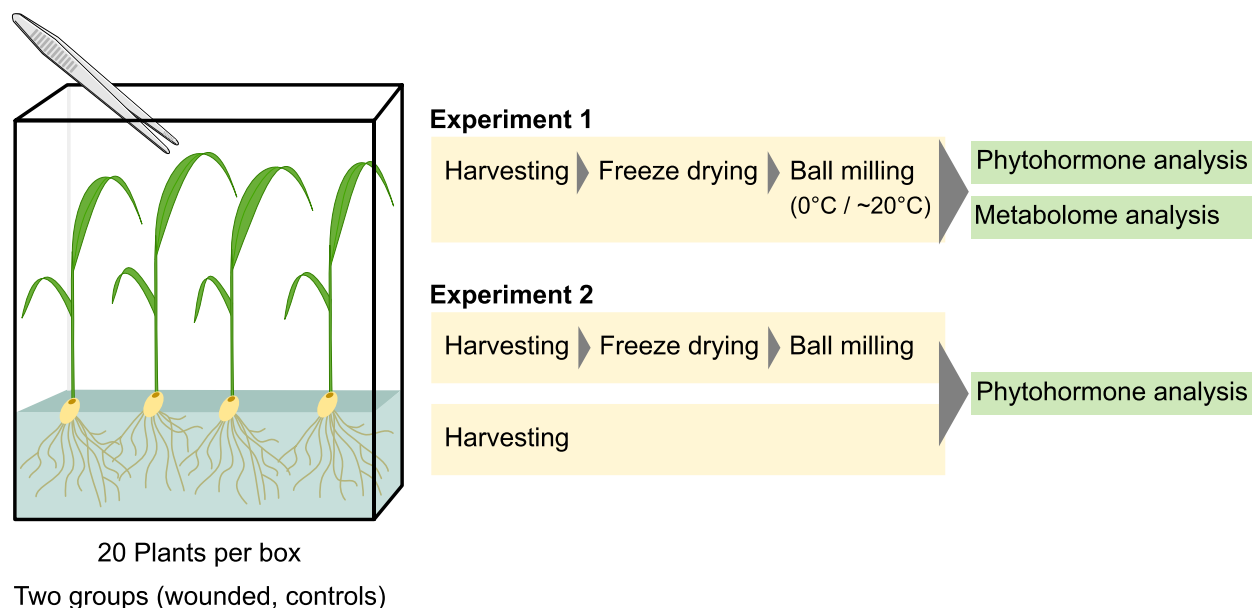


Figure 14: Schematic cultivation of rice plants and key point differentiating the two experiments regarding sample processing (yellow) and sample analysis (green). Further details are provided in the body text.

In the second experiment, the plant seedlings grown in each cultivation box were divided into two groups. One group was intended for freeze-drying, and the other for fresh preparation. All but one of the seedlings were processed as described above. The seedlings were frozen in liquid nitrogen, freeze-dried, and ball-milled in sample containers cooled to 0°C for 40 s. However, one seedling of each box was weighed in a sample tube containing a zirconium oxide bead. The mass of the individual seedlings ranged between 55 and 85 mg. The tubes were then shock-frozen with liquid nitrogen. Sample preparation differed for these two groups. The freeze-dried, pulverized samples were weighed (8 mg) and prepared as in the prior experiment, while the freshly frozen samples were prepared using a workflow that followed the original, adapted method more closely [126]. 50%ACN and IS mix (total volume 1 mL) were added to each tube containing a fresh plant sample. The tubes, which each contained a zirconium oxide bead, were then placed in a homogenizer (speed 6.5 m/s, duration 20 s). Further sample preparation was equal to the workflow for the freeze-dried material, beginning with ultrasonic treatment. As in the first experiment, two factors of interest (stress application and sample material type), each with two levels, were assessed.

Eight replicates were produced for each subgroup. All samples were analyzed in one measurement series, together with QC samples (from freeze-dried material) and controls.

Phytohormone analysis followed the procedure described in Chapter 3.4.1.1 for potato plants, differing only in the MRM method used. This version of the MRM method included auxin, gibberellin, and strigolactone analytes, as well as abscisate, salicylate, and jasmonate analytes, which were added to the method since then. A corresponding IS mix was prepared for these analytes. Absolute quantification was not attempted. The phytohormone analysis, the data integration, and the data analysis were carried out as part of the aforementioned Master's thesis.

3.4.2 Full Application to Tomato Plant Samples

The finalized targeted phytohormone analysis method was applied to a study of tomato leaves and fruits. An experiment was designed that captured multiple biological influencing factors simultaneously. The goal was to assess the method's applicability to complex plant research studies, with a focus on analytical performance. The following chapters provide the experimental design, the plant cultivation, the sample acquisition and processing, and the data acquisition and analysis.

3.4.2.1 Experimental Design

Following the short-term stress application to rice plant seedlings (Chapter 3.4.1.2), the goal of this validation study was to apply long-term stress to the plants. After successful testing in the model plant rice, the focus shifted to analyzing nightshade plants again since they were the focus of this work. Additionally, this experiment included fungus-plant interactions because phytohormones were previously shown to be relevant in this context through the metabolome analysis of potato plants. For this cultivation, which was performed in-house, all biological aspects were recorded rigorously to meet the standards set by the MSI for reporting biological context in metabolome studies [57].

The experimental setup ultimately involved growing tomato plants in two climate chambers under nearly identical conditions (Figure 15). After an initial growth period, the tomato plants in one chamber were subjected to heat stress, while those in the other chamber were cultivated under optimal conditions. Temperatures of 25/20°C (day/night) are widely reported as optimal for tomato growth [177-179]. Although heat stress tolerance depends heavily on the tomato genotype, temperatures above 30°C are generally considered significantly detrimental to growth [180, 181]. In this study, temperatures of 32/25°C were chosen to induce heat stress in the plants, and 25/20°C, which are reportedly optimal, were selected for the control group. These plants are referred to as low-temperature (LT) or high-temperature (HT) plants. Some studies have shown

that inoculating plants with *Trichoderma* cultures can have beneficial effects under water-deficiency stress [182, 183]. Therefore, the plants in this experiment were inoculated twice with a *T. afroharzianum* spore suspension early in their cultivation.

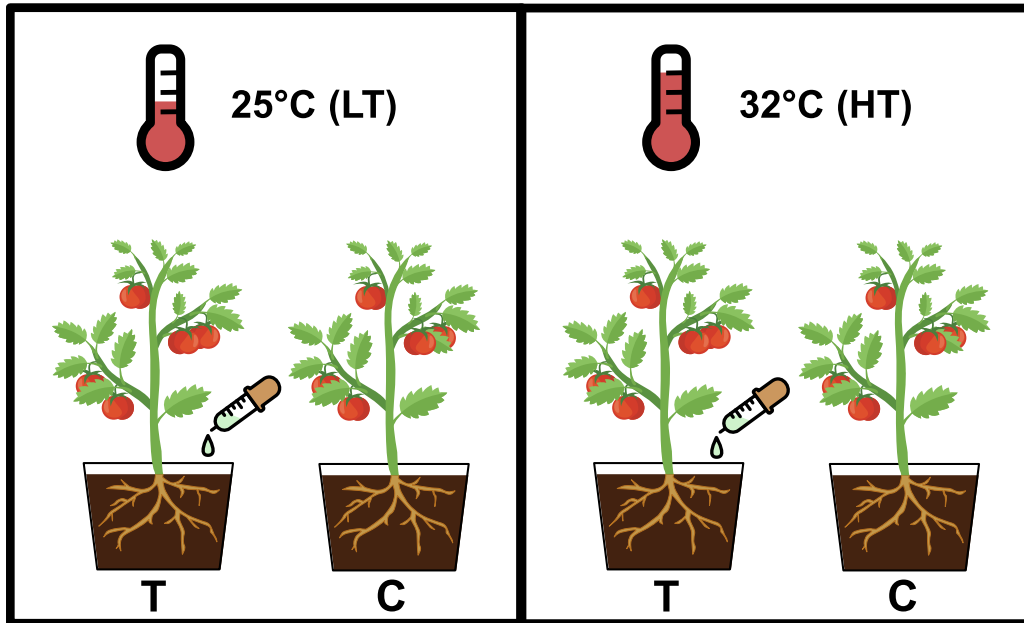


Figure 15: Influencing factors and resulting subgroups of the tomato cultivation experiment. Plants were grown in two climate chambers set to either high or low temperatures (HT/LT). Half of the plants in each growth chamber were inoculated three times with a *Trichoderma* spore suspension (T), while the other half served as controls (C). One plant was one biological replicate; six replicates were cultivated per subgroup (24 total plants).

3.4.2.2 Plant Cultivation

Tomato plants were cultivated in-house for 12 weeks. A micro-tomato cultivar ‘Tiny Tom’ (Garten Magie, Maxhütte-Haidhof, DE) was used in this experiment (see [184]). The growth systems and substrate used during the cultivation are listed in Table A-4 in the Appendix. Table 5 outlines the timeline of plant cultivation, including major events and the plants’ development stages.

Initially, seeds were planted in 250-mL pots filled with substrate. The plants were placed in a controlled climate chamber and grown under LED lights, which emitted light at a photosynthetic photon flux density (PPFD) of 100 $\mu\text{mol}/\text{m}^2/\text{s}$. PPFD is defined as the number of photons emitted in the photosynthetically active light range (400-700 nm) per unit of time and area. The temperature was set to 23°C during the day and 18°C during the night, with a photoperiod of 16 h. The relative humidity was set to 65%, which was not changed throughout the entire cultivation. After germination, the 24 plants with the best growth were selected and repotted into 5-L pots

containing 1.75 kg of substrate. The plants were returned to the climate chamber and grown together. The temperature was set to alternate between 25°C during the day and 20°C at night, the photoperiod remained at 16 h. The plants were grown under these conditions until they all reached the flowering stage. Half of the plants were then transferred to a different growth chamber, which was powered by sodium vapor lights. The temperature in the second chamber was set to 32°C during the day and 25°C at night. The photoperiod in both chambers remained at 16 h. but the light intensity in both chambers was raised to a PPFD of 600 $\mu\text{mol}/\text{m}^2/\text{s}$. Temperature and humidity were controlled via internal logging systems and additional loggers, which were placed among the pots in both chambers.

Table 5: Timeline of tomato plant cultivation. Development stage is marked on the day that all plants had entered the given stage. LT/HT, low/high temperature in cultivation chamber.

Days after sowing	Event	Development stage
14	1st inoculation with <i>T. afroharzianum</i> spore suspension	Pre-flowering
24	Repotting to 5L pots, transfer to climate chamber	
24	2nd inoculation with <i>T. afroharzianum</i> spore suspension	
24	Fertilization (Nutrient solution 1)	
33	Fertilization (Nutrient solution 1)	Flowering
40	Fertilization (Nutrient solution 2)	
42	Separation of LT/HT plants, begin of stress experiment	
45	Fertilization (Nutrient solution 2)	
47	Fertilization (Nutrient solution 2)	Fruit bud formation
48	3rd inoculation with <i>T. afroharzianum</i> spore suspension	
54	Fertilization (Nutrient solution 2)	Fruit formation
55	Fertilization (Nutrient solution 3)	
61	Brief removal from growth chambers, harvest of leaves	
75	Brief removal from growth chambers, harvest of HT fruits	
84	Brief removal from growth chambers, harvest of LT fruits	

The pots were inoculated three times with a *Trichoderma* spore suspension. The experiment used a *Trichoderma afroharzianum* strain isolated in-house (internal ID 359). Spore solutions were prepared separately for each inoculation. To prepare the spore suspensions, the fungus was cultivated on potato dextrose agar plates. After several days of cultivation, a small amount of NaCl-Tween solution was added to each plate. The top layer was then removed with a flame-sterilized inoculation loop and added to NaCl-Tween solution (0.9% NaCl, 0.1% Tween). This process was repeated for several plates, and the spores from all plates were collected in one container. The resulting spore suspension was then diluted with more NaCl-Tween solution, thoroughly mixed, and the spore concentration determined in a counting chamber. Counting was performed in a meandering pattern. The final spore concentrations in the three suspensions were 1.8×10^7 ,

$1,5 \times 10^6$ and $5,8 \times 10^6$ spores/mL, respectively. 10 mL of the first suspension was added to each pot 14 days after sowing, when all plants had germinated. 100 mL of the second suspension was added to each pot 24 days after sowing, immediately after replanting the plants. 100 mL of the third suspension was added to each pot 48 days after sowing, when most of the plants' flowers had turned into fruit buds. For each of these inoculations, the specified volume of spore suspension was carefully added to the substrate surface, avoiding direct contact with the plant stems. It was ensured that the substrate was sufficiently moist to absorb the spore suspensions, but not so moist that the solution flowed out of the pots.

A nutrient solution designed for the growth of tomatoes in greenhouses [185] was used for fertilization. This solution was adjusted multiple times throughout the cultivation process to accommodate the plants' developmental stage. The nutrient solution concentrations are listed in the Appendix (Table A-25). The plants were supplied with nutrient solution at seven timepoints, as listed in Table 5. During each fertilization, 2.5 L of nutrient solution were applied to each pot. Plants were additionally watered in sufficient amounts, and the substrate did not fully dry out throughout the cultivation.

3.4.2.3 Sample Acquisition and Processing

Tomato leaves and fruits were harvested separately. Ultimately, 24 samples of each tissue type were obtained. At each harvest timepoint, photos were taken of each harvested plant using equal lighting conditions.

The plants' leaves were sampled 19 days after the start of the stress treatment. Each pot was briefly removed from its growth chamber and approximately five to six leaves (total fresh mass 8-10 g) were cut from different parts of the plant. Photos were taken of each plant before leaf harvesting. The leaves were collectively weighed, and their masses were recorded. They were then swiftly frozen in liquid nitrogen and further processed as described in Chapter 3.3.4.1. After submersion in liquid nitrogen, all leaves of a plant were combined in a 50-mL centrifuge tube. They constituted one pooled sample for the plant during all further steps. The tubes were weighed before and after freeze-drying to determine water loss. After leaf removal, each pot was returned to its chamber for further cultivation.

The fruits were harvested at two different times for the HT and LT plants because the HT plants' fruits ripened earlier. Harvesting the fruits at two different times allowed for obtaining fruits at a similar point of ripeness. At least six fruits, with a total mass of at least 65 g, were harvested from each plant. The fruits were washed before being submerged in liquid nitrogen, but were not cut. Additionally, the stem scar, i.e. the former point of attachment to the vine, was not removed

because the tomatoes were small and were therefore treated like cherry tomatoes. Shock freezing and further processing were performed as detailed in Chapter 3.3.4.1. Unlike the procedure described there, the tomatoes were placed in freezer bags inside the lyophilization glasses. This was done so that the bags could be closed immediately after freeze drying had finished. Then, the bags could be weighed without the hygroscopic samples absorbing moisture from the ambient air. Following this first harvest of tomato fruits, which was used for the phytohormone analysis, tomatoes from all plants were harvested whenever they were ripe and then weighed. Their masses were noted and added together for each plant to compare total harvest amounts.

After ball milling, separate pooled samples were prepared for the leaf and fruit material to serve as QC samples during sample acquisition. For this purpose, several spatulas of each tissue sample were combined into 30-mL sample containers. The sample containers were filled with argon before and after collecting the pooled samples. The sample container lids were sealed with Parafilm, and the containers were stored at -80°C until weighing.

3.4.2.4 Data Acquisition and Analysis

The freeze-dried, ball-milled tomato fruit and leaf samples were analyzed using the final UHPLC-MS/MS method. The UHPLC gradient of the method is listed in Chapter 3.2.3, Table 3. Further UHPLC and MS parameters (Table A-21), and the MRM method (Table A-19) are provided in the Appendix.

Sample preparation and data acquisition followed the workflow detailed in Chapter 3.2.4.1. The IS mix listed in Table A-23 ("Mix 2" column) was used for this experiment, while the analyte mixes from Table A-23 were used to create calibration curves for the quantified analytes. Additionally, the analyte mixes provided in Table A-22 were used to overspike select samples at 5-fold and 25-fold base concentrations.

Optimal sample masses were determined in a brief preliminary analysis of QC samples, which is not detailed in this work. The target masses were 10 mg (± 0.5 mg) for fruit samples and 8 mg (± 0.5 mg) for leaf samples. In addition to the 24 StS of each tissue type, 15 QC samples were weighed. All sample weights were recorded. Measurements were performed separately for fruit and leaf samples. The samples of each tissue were prepared and measured on two consecutive days. On each day, the respective samples were prepared and subsequently measured, first in positive polarity and then in negative polarity. Since only one extract is prepared per sample, injections for both polarities were taken from the same vial.

The injection order was based on the structure of the metabolome analysis (see Chapter 3.1.3), and remained consistent throughout each measurement block. First, the equilibration samples (10

QC extract injections) were analyzed, followed by a solvent control, the control samples, and the blank samples. Next, the non-prepared analyte mixes were measured, specifically the IS mix and the mixes of semi-quantified analytes, followed by the calibration samples of the quantified analytes. Then, three overspiked QC samples were measured, two of which had been spiked with the analyte mix at a 5-fold base concentration, and one that had been spiked at a 25-fold base concentration. These overspiked samples were later used to aid in the identification of analytes. Lastly, the StS and QC samples were measured. This was done in a block design, where two QC samples were measured, followed by six StS, two QC samples, six StS, and two QC samples. Thus, 6 QC samples and 12 StS were measured in each polarity per analysis day.

The acquired data were processed and analyzed via the established R data analysis pipeline, which is detailed in Chapter 3.2.5. Analytes were identified by evaluating the ion ratio, as described there, as well as by comparing peak shapes and retention times in the samples and the overspiked samples. This approach follows the recommendations of the SANTE 11312/2021 guidelines [174]. Tomato yields were compared via a two-factor ANOVA, which was performed in the software JMP (version 18.1). Additionally, FCs were calculated for each biological factor by comparing treated plants (HT or T subgroups) with their respective control plants (LT or C subgroups, respectively). This resulted in two FC values for each factor and tissue, which were subsequently visualized in heatmaps. These calculations and the generation of heatmaps were also performed in R, but were not part of the standard data analysis pipeline.

3.5 Combined Phytohormone and Metabolome Analyses

The UHPLC-MS metabolomics method presented in Chapter 3.1 was used to measure two sample sets that were previously analyzed with the targeted phytohormone method. These analyses aimed to compare the performance of the two methods regarding the detection and identification of phytohormones. Metabolome analysis was performed on the rice plants exposed to short-term wounding stress, and on the tomato plants exposed to long-term heat stress. The proceedings are described in the following Chapters 3.5.1 and 3.5.2, respectively.

3.5.1 Metabolome Analysis of Rice Plant Samples

The samples from the first rice plant experiment (see Chapter 3.4.1.2) were used for metabolome analysis. The samples from the second experiment were not analyzed with the metabolomics method because no sample preparation protocol has been established for fresh plant material.

Sample preparation and data acquisition closely followed the procedures described in Chapter 3.1.3. All 18 samples from the experiment were analyzed. Sample masses of 20 mg and an

extraction volume of 400 μ L were used. This ratio was determined to be optimal in a preliminary experiment (data not presented here).

Initial data processing was performed as detailed in Chapter 3.1.4, but only the in-house database was used for annotation. This version of the in-house database included all the phytohormone metabolites within the scope of the phytohormone analysis method. Sample outlier evaluation and missing value imputation were also performed as described in Chapter 3.1.4. Feature statistics were also calculated in JMP, but using a non-FDR corrected two-factor ANOVA instead of univariate feature statistics. Similarly, signal FCs were calculated only between each subgroup and its control (e.g. cold-milled stress samples vs. cold-milled control samples). For calculation of stress FCs, the FCs previously calculated for the cold-milled and room temperature-milled samples were averaged. Subsequent steps differed from the workflow applied to the potato plant data. Feature outliers were not eliminated, and no QC filters were applied. No exploratory data analysis was performed, and metabolite patterns were not visualized.

Only metabolites with annotated matches in the in-house database were evaluated for feature identification. This was done in two steps. First, the features annotated as phytohormone metabolites were evaluated, and identifications were confirmed or rejected by following the steps outlined in Chapter 3.1.4.8. Second, metabolites with highly significant changes in signal intensity ($\text{FDR-p} < 0.01$) between wounded and non-wounded plants were evaluated in the same manner, and their annotations were accepted or rejected. In both cases, no rigid filter criteria were applied, as the number of matches was low, making a manual evaluation feasible.

3.5.2 Metabolome Analysis of Tomato Plant Samples

Metabolome analysis was performed on the tomato plant samples exposed to heat stress and inoculated with *Trichoderma* (see Chapter 3.4.2). Since tomato samples had previously been analyzed with this method in other projects, sample masses and extraction solvent volumes were based on those experiences. Fruit and leaf samples were weighed in 50 mg aliquots. QC pools were prepared separately for each tissue, and QC aliquots were prepared in the same manner. 500 μ L of solvent was used to extract the metabolites of the samples. The further sample preparation and data acquisition were performed as described in Chapter 3.1.3, with separate measurement series for each tissue type.

The acquired data were processed as detailed in Chapter 3.1.4, but a newer version of the MS-Dial software was used (v5.5.250627), which allowed for processing of the data with multiple reference libraries simultaneously. However, for the evaluation presented in this work, only annotations with the in-house library were considered. This evaluation followed the reduced approach described in

the previous chapter for the rice plants. The only filtering step employed was a subgroup detection filter in MS-Dial, which required features to be detected in at least four out of the six samples of one subgroup. Statistical analysis (two-factor ANOVA) and FC calculations were performed in R for the identified analytes only. FCs were calculated for each treatment factor and visualized in heatmaps, as described in Chapter 3.4.2.4 for the targeted analysis.

4 Results and Discussion

4.1 Metabolome Analysis of Potato plants Expressing Fungal Effectors

To evaluate the performance of an established in-house metabolomics method in the analysis of Solanaceae plants, the method was applied to investigate metabolic shifts in transgenic potato lines. The primary aim was to determine the contribution that the metabolomics approach can make to deciphering plant responses to external influencing factors. To this end, two experiments on potato plants were conducted. Leaf and root samples were obtained from the first experiment and root samples from the second experiment. The experimental design, sample acquisition, data acquisition and subsequent data processing and analysis are outlined in Chapter 3.1.

The examined potato plants were transgenically modified to express effector proteins from the soil fungus *Rhizophagus irregularis*, an arbuscular mycorrhizal (AM) fungus (see Chapter 1.5). These effector proteins are typically secreted by the fungus and absorbed into plant cells, where they then modulate plant metabolism [162, 165]. Here, the genes encoding these effectors were introduced into the plants using an overexpression vector. By overexpressing the effectors directly *in planta*, their influence on plant metabolism could be studied independently of the fungal colonization process. The aim was to identify the role of these proteins in the interaction between fungi and plants, which is often considered beneficial for plant nutrient uptake or stress resilience. Six potato plant lines were used in the experiments: two SP5 expressing lines (SP5-6 and SP5-8), two SP7-expressing lines (SP7-7 and SP7-10), and two empty vector (EV)-lines (EV-1 and EV-2), which served as the control lines.

4.1.1 Data Pattern Evaluation and Visualization

The untargeted metabolomics approach generated extensive datasets. For each experiment, a large number of metabolite signals, referred to as features (see Chapter 3.1.4.1), were obtained.

Following the initial processing and subtraction of blank features, each dataset comprised more than 20,000 features. Several filtering steps were applied to retain only those features detected reliably and reproducibly (see Chapter 3.1.4.3). This reduction resulted in approximately 8,300 features (negative | positive polarity: 3,411 | 4,922) for the leaf samples, 6,000 features (negative | positive polarity: 2,450 | 3,502) for the stem samples, and 12,000 features (negative | positive polarity: 4,660 | 7,560) for the root samples.

Initial data analysis focused on identifying broad patterns within these filtered datasets. Structural metabolite identification of individual features was not the focus at this stage, since only a small percentage of features can be reliably identified (typically 5-10% for the employed method). Therefore, restricting the interpretation solely to identified compounds could introduce bias and

misrepresent the overall metabolic response. Patterns in the data were investigated using two complementary methods. First, principal component analysis (PCA) was performed as an exploratory data analysis to assess sample clustering. Second, heatmaps of the most highly significant features were created to visualize metabolic shifts across the plant lines relative to the controls. The results are presented and discussed in the following chapters.

4.1.1.1 Principal Component Analysis

PCA is a common method of exploratory data analysis which examines large datasets without any prior categorization of samples and can help identify trends and outliers. It is often employed as the first step of data analysis in metabolomics studies [186, 187]. Here, PCA was performed separately for each tissue and ESI polarity, as detailed in Chapter 3.1.4.5.

The 2D score plots for the leaf sample dataset are presented in Figure 16. Since all six PCAs yielded highly similar results, the score plots for the stem and root datasets are provided in the Appendix (Figure A-1).

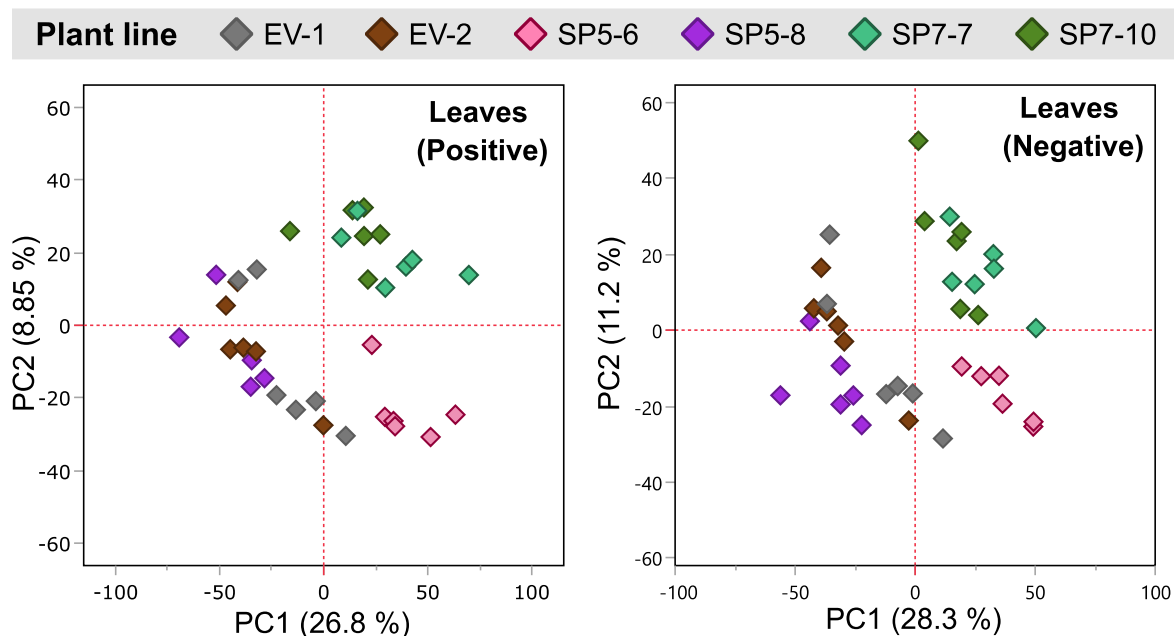


Figure 16: Principal component analysis of potato leaf samples by measurement polarity. Percentage of total variance explained by each PC is provided in parentheses. PC1/2, Principal component 1/2.

First, the clustering of samples of each plant line was assessed, and the plots were evaluated for potential sample outliers. The variances between the samples of each plant line differed between the datasets, resulting in varying degrees of overlap or separation between samples of different plant lines. The root dataset had higher variance than the leaf and stem datasets, especially in negative polarity. However, the overall variance within plant line groups was low enough to

evaluate trends between plant lines across all score plots. One sample was excluded due to a confirmed labeling error (see Chapter 3.1.4.2), and the PCA was repeated. Apart from this error in sample handling, no unusual clustering of samples was observed.

Similar trends were observed for all datasets when evaluating the first two principal components (PC1 and PC2). The samples were separated into the two groups along PC1, which explained 20.4 - 28.3% of the total variance. The samples from both SP7-expressing plant lines and the SP5-6 plant line were separated from EV line samples and the SP5-8 samples. This was consistently the dominant effect in all analyses; SP5-8 samples substantially overlapped with the EV samples in all score plots. Along PC2, which accounted for 8.9 - 15.7% of the total variance, the SP7 samples were separated from the SP5-6 samples. No clear effect on the EV samples and the SP5-8 samples was observed along PC2.

Higher PCs were subsequently evaluated to assess the separation between the SP5-8 and EV-1/2 samples. While these samples were not separated along any PC (up to PC8) in either leaf or root datasets, separation was observed in the stem datasets in both negative and positive polarity (PC3 and PC4), where the respective PC explained 5.6 and 7.3% of the total variance.

Overall, the PCA results indicated that the metabolism of the plant lines SP5-6, SP7-7, and SP7-10 was substantially altered compared to the control lines EV-1 and EV-2. In contrast, metabolic changes in the SP5-8 plant line seemed much less pronounced, with differences to the control lines only apparent under closer investigation of higher PCs. The SP5 expression levels in these plant lines had not been determined, so it was unclear whether these effects resulted from different SP5 expression levels. However, metabolic changes in plant line SP5-6 seemed to correlate with those in the SP7-expressing plant lines to a certain extent, indicating potentially similar effects on potato metabolism. Hence, metabolite patterns were subsequently evaluated further to elucidate these findings.

4.1.1.2 Metabolite Pattern Visualization

After the PCA revealed discrepancies in the metabolite signals of the putative SP5-expressing lines, an additional approach was applied to highlight metabolite patterns. Fold change (FC) heatmaps were created for the 100 features with the most significant changes in signal intensity (lowest FDR-corrected ANOVA p-values). This approach was intended to complement the PCA and examine metabolite signatures at the feature level rather than the sample level. Since the root samples had not been generated at this stage, heatmaps were created using only the stem and leaf datasets, following the procedures described in Chapter 3.1.4.7. The heatmaps of all four datasets are shown in Figure 17.

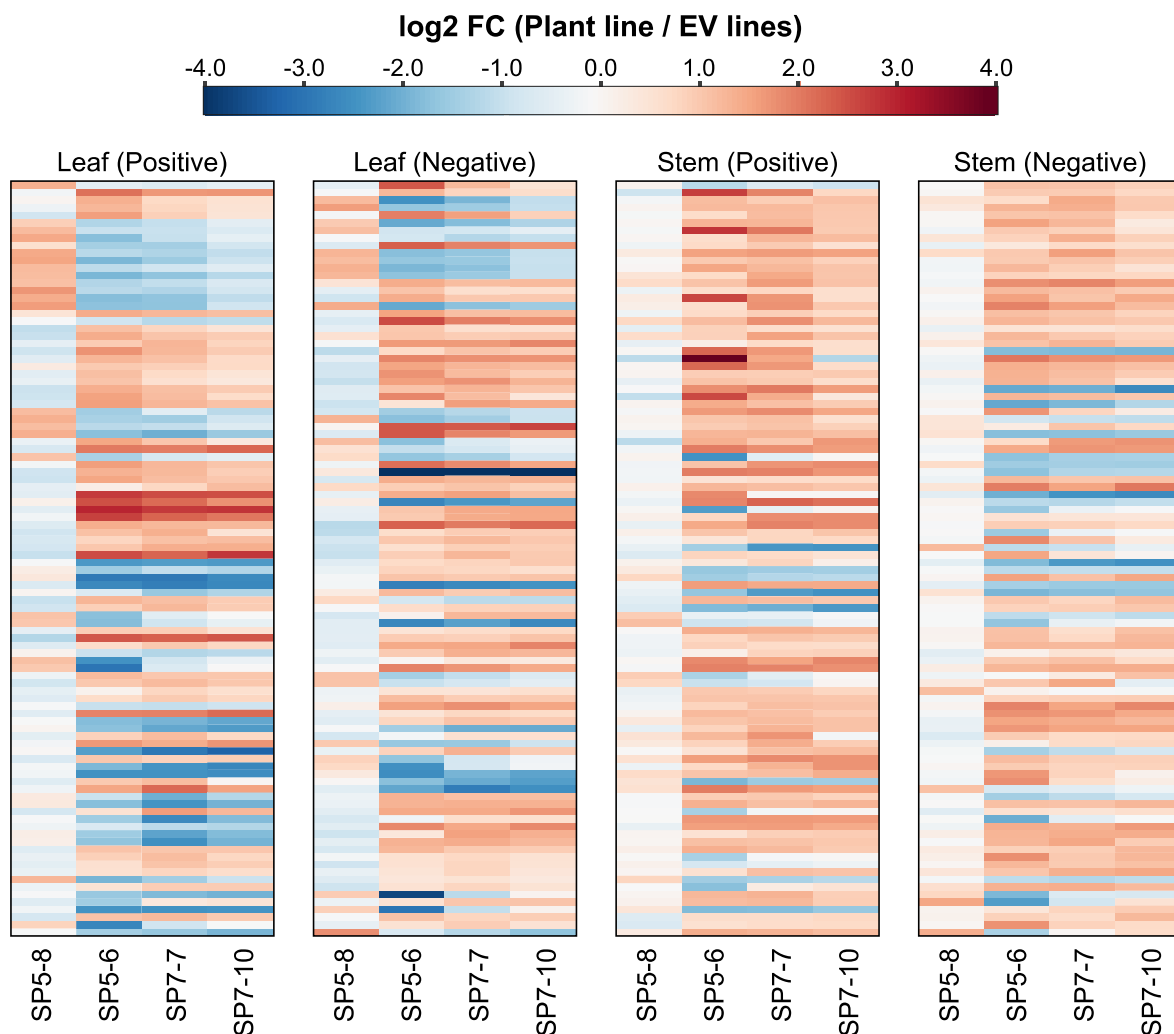


Figure 17: Heatmap visualization of the 100 features with the highest statistically significant signal intensity changes (FDR-corrected ANOVA) per plant line, separated by tissue and measurement polarity. Each row in each dataset corresponds to one metabolite feature. The log₂ FC for each plant line is color coded (blue, positive values; red, negative values). The color intensity maximums and minimums are fixed to the values +4 and -4, respectively, so that some higher FCs are limited to these values. The mean signal intensity of both EV (control) lines was used as the reference for FC calculation. ANOVA, Analysis of variance; FC, Fold change; FDR, False discovery rate.

The heatmaps were visually evaluated for patterns. Most notably, the FCs of the SP5-8 line differed substantially from those of the other three lines. The FCs of this line were generally closer to zero, and therefore the signal intensities more similar to the control lines. Furthermore, the leaf datasets, where SP5-8 FC were overall higher than in the stem datasets, revealed metabolic responses opposite to that of the other three plant lines. In contrast, the FC patterns for most features were highly similar between the SP5-6, SP7-7, and SP7-10 plant lines. Strong deviations in their metabolic response were limited to a few features and were only slightly more pronounced for the SP5-6 line than for the SP7 lines.

These observations underlined the findings of the previously performed PCAs. The plant line SP5-8 showed only a minor metabolic difference to the control lines and differed strongly from plant line SP5-6, suggesting that different SP5 expression levels were likely the cause of this discrepancy. However, this could not be confirmed at the time due to complications in analyzing SP5 expression. In contrast, SP7 expression in the respective plant lines had already been verified by analyses conducted at the KIT Molecular Phytopathology Department, which are not presented in this work.

Due to the contrasting metabolic responses of the putative SP5-expressing plant lines, subsequent evaluations focused solely on the SP7-expressing plant lines. Nevertheless, the similarities observed between the SP7 lines and the SP5-6 line imply that SP5 and SP7 have a similar effect on potato metabolism. Therefore, future investigations into the effector protein SP5 could be highly valuable in elucidating the role of different SP7-like effector proteins in plant-fungus interactions.

4.1.2 Untargeted Analysis of Leaves and Stems

After evaluating patterns in the data and excluding the SP5-expressing plant lines as a result, individual features of the SP7-expressing plant lines were examined. The leaf and stem samples of these plant lines were assessed in an untargeted identification process to identify the metabolites most relevant to the expression of the fungal effector protein. This process is described in detail in Chapter 3.1.4.8.1, including a summary of the MSI levels used to indicate identification confidence (Table 1). As stated in the previous chapter, root samples were generated at a later stage of the project and were thus only considered in the subsequent identification step (see Chapter 4.1.3).

Since identifying large numbers of features is labor-intensive, a stringent pre-selection of highly relevant features was performed. Features were considered highly relevant when their signal intensity change was highly significant and reproducible. Specifically, the mean signal intensities of both SP7-expressing lines had to be changed with high significance ($p < 0.01$) compared to the means of both EV plant lines (Tukey tests: SP7-7 vs. EV-1, SP7-7 vs. EV-2, SP7-10 vs. EV-1, SP7-10 vs. EV-2).

This process resulted in a total of 140 selected features. All of these features were annotated during the data processing through a match with either the in-house database or an external database. Features that fulfilled the relevance criteria but had no match in either database (“unknown” features) were removed. Database matches were manually evaluated and either accepted or rejected. Ultimately, 32 features were identified at MSI levels 1 to 3. An overview on

the numbers and confidence levels of identified features in each dataset is given in Table 6. The list of identified metabolites is provided in the Appendix (Table A-26). It should be noted that sugar moieties can often not be differentiated in the MS/MS spectra. Thus, the name given to identified glycosylated metabolites reflects the maximum level of information obtainable for these compounds.

Table 6: Numbers of highly relevant and identified features across plant tissues and polarities. MSI levels (see Table 1) are provided in parentheses. Overlapping features between tissues were removed for total counts. MSI, Metabolomics Standards Initiative.

	Relevant		Identified			
	Positive	Negative	Positive	MSI level (1/2/3)	Negative	MSI level (1/2/3)
Leaf	44	21	5	(1 / 4 / 0)	7	(1 / 5 / 1)
Stem	62	24	15	(2 / 10 / 3)	9	(1 / 4 / 4)
Total	99	41	17	(2 / 12 / 3)	14	(2 / 7 / 5)

Overall, four metabolites were identified at MSI level 1. This level was assigned only to metabolites confirmed using the in-house database comprised of authentic reference standards that were measured using the same analytical method and instrument. The following subsection presents all identified metabolites, including those putatively identified (MSI level 2) and those identified at the substance class level (MSI level 3). The individual metabolites will then be placed into a common context in the subsequent subsection.

Identified Metabolites

N-trans-Feruloyltyramine, often referred to as feruloyltyramine, was the most abundant compound identified at MSI level 1. It has been widely associated with cell wall reinforcement and cell wall polymers, such as lignin and suberin, in potato and other Solanaceae species [188-192], though suberin is predominantly found in the subterranean parts of potato plants. The signal intensity of feruloyltyramine was reduced by 75% in both SP7 lines compared to the control plants. Considering its abundance, this constitutes a substantial decrease of signal intensity. Besides feruloyltyramine, two tyrosol glycosides were putatively identified. These compounds are related to tyramine metabolism and have been proposed to be the result of an alternative metabolic pathway to feruloyltyramine formation, which could serve as a means for plants to store tyramine [193]. Unlike feruloyltyramine, both tyrosol glycosides exhibited higher signal intensities in the SP7 samples than in the EV samples (FC 1.96 – 2.35).

The flavonol quercetin-3,4'-*O*-diglucoside was the only flavonoid identified at MSI level 1. It has previously been detected in tomato plant leaves [194], though no specific functional role in

Solanaceae species was identified. Its signal intensity was more than doubled in the SP7-expressing lines when compared to the control lines (FC SP7-7 | SP7-10: 2.56 | 2.24).

In addition to the identified quercetin diglucoside, two other quercetin glycosides, a quercetin disaccharide and a quercetin trisaccharide, were putatively identified. Furthermore, several other flavonol glycosides were putatively identified, such as isorhamnetin rutinoside, kaempferol sophoroside, and an unknown kaempferol disaccharide. Outside of the flavonol class, two hexosides of the dihydroflavonol dihydroquercetin (taxifolin) were putatively identified. Three features were only identified as flavonoid hexosides and one as a likely isoflavone conjugate. These substances correspond well to the flavonoids identified in previous analyses of potato plants [158, 195, 196]. Except for the quercetin trisaccharide (FC SP7-7 | SP7-10: 0.52 | 0.52), all flavonoids and putative flavonoids had higher signal intensities in the SP7 samples than in the EV samples (FC 1.83 – 3.39).

Betaine, also referred to as glycine betaine, was the only identified metabolite (MSI level 1) selected as highly relevant in both leaf and stem samples. It is a derivative of the amino acid glycine and a common metabolite in many plant species. The formation of betaine is often increased under abiotic stress conditions, but it is generally involved in many stress-related processes in plants, interacting with multiple phytohormone signaling pathways [197-199]. Interestingly, its signal intensity was doubled in the leaf samples (FC SP7-7 | SP7-10: 2.01 | 2.15), but halved in the stem samples (FC SP7-7 | SP7-10: 0.55 | 0.54) compared to the control lines.

Vanillic acid glucoside was also identified at MSI level 1 in the leaf samples. It has previously been identified as a phenolic compound in potato plants [200] and occurs in plants as a metabolic intermediate between ferulic acid and vanillin [201]. Its non-glycosylated form, vanillic acid, is one of the major phenolic compounds in potatoes [195], and it has been described in relation to cell wall polymer formation as an unconventional lignin monomer [191]. Its signal intensity was higher in the SP7 lines than in the control lines (FC SP7-7 | SP7-10: 1.82 | 1.42). Two more vanillic acid derivatives were putatively identified, both comprised of vanillic acid linked to a sulfated (deoxy)hexose. Similar to vanillic acid glucoside, both showed increased signal intensities in SP7 samples (FC 1.46 – 2.71). However, the likelihood of these annotations could not be confirmed. Although some sulfated vanillic acid compounds have been reported in plants [202], these compounds are not identical to those identified here, and their role in plant metabolism is not well understood.

Several other metabolites of the phenylpropanoid pathway were identified at lower levels of confidence. Coumaric acid *O*-hexoside was putatively identified in the stem samples, where its

signal intensity was higher in SP7 lines than in control lines (FC SP7-7 | SP7-10: 2.76 | 2.44). *p*-Coumaric acid is a key metabolite in the phenylpropanoid metabolism, because it is the common precursor to lignin and flavonoid biosynthesis [190, 203, 204]. Glycosylation of *p*-coumaric acid is an important mechanism by which plants control the allocation of resources between these two metabolic pathways [204, 205]. Two other putative metabolites of this pathway were identified: an unknown conjugate of ferulic acid and an unknown conjugate of caffeic acid. While the acid components were identified with high confidence, the type of conjugate could not be determined due to ESI in-source fragmentation. Both of these conjugates showed increased signal intensities in the SP7 samples (FC 1.63 - 2.44).

Pyridoxine and two pyridoxine hexosides were putatively identified. The signal intensities of pyridoxine and one of its glycosides were reduced in the leaf samples (FC 0.34 – 0.50), while the signal intensities of both hexosides were elevated in the stem samples (FC 1.91 – 3.35). Pyridoxine can readily be converted to pyridoxal and then to pyridoxal phosphate (PLP), which is an important cofactor in the metabolism of all life forms, especially for amino acid metabolism [206, 207]. All three compounds belong to the group of vitamin B6 vitamers, i.e. structurally related compounds that exhibit vitamin B6 activity. In plants, pyridoxine and pyridoxal are often present at comparable concentrations and are often glycosylated [206]. Pyridoxine glucosides have been reported to be the predominant form of vitamin B6 in many plants and plant-derived foods [208].

Phosphocholine, the key precursor of all phosphatidylcholine-type phospholipids, was putatively identified in the leaf samples, along with lysophosphatidylcholine 16:0. These two compounds exhibited opposite changes in signal intensity in response to SP7 expression. The signal intensity of phosphocholine was higher in the SP7 lines than in the controls (FC SP7-7 | SP7-10: 2.42 | 2.34), whereas the signal intensity of lysophosphatidylcholine 16:0 was lower in the SP7 samples (FC SP7-7 | SP7-10: 0.56 | 0.66).

Homogentisic acid was also putatively identified in the leaf samples, where its signal intensity was higher in the SP7 plant lines than in the EV lines (FC SP7-7 | SP7-10: 2.00 | 1.51). In plants, homogentisic acid can be synthesized from chorismate or formed through the degradation of tyrosine [209, 210]. It is a key precursor in tocopherol and tocotrienol biosynthesis, and its glucoside has been described as being involved in plant defense processes in the Solanaceae species *Nicotiana attenuata* [211].

Several other metabolites could only be putatively identified at the substance class level. One such compound was a phenethyl disaccharide that was putatively identified in leaf samples, where it was substantially induced in SP7-expressing plants (FC SP7-7 | SP7-10: 4.01 | 3.41). However,

although phenethyl and phenylethanoid glycosides occur widely in many plant families with great chemical diversity, their biological roles are not well understood [212, 213]. A furofuranoid lignan glycoside was putatively identified in the leaf samples, where its signal intensity was also induced in SP7 samples (FC SP7-7 | SP7-10: 2.21 | 1.91). Lignans are not components of lignin but are also derived from phenylpropanoid precursors, specifically cinnamic acids [214]. Their biological role has not been fully elucidated, but it is known that they can function as phytoalexins. In this role, their biosynthesis is often induced following fungal attack [215].

Conclusions

Of the identified compounds, the majority are part of the plant's phenylpropanoid metabolism, which is derived from the PAL pathway (see Chapter 1.4.2.7) and branches into the lignin and flavonoid metabolisms. Substantial changes in signal intensity were primarily observed for the identified flavonoids, which comprised one-third of the identified features. Of these 16 flavonoid features, all but one exhibited higher signal intensity levels in the SP7 plants than in the control plants. In contrast, the signal intensity of feruloyltyramine, a common lignin monomer specifically in Solanaceae species, was strongly decreased in the SP7-expressing plants. Considerable signal intensity changes were also observed for the putatively identified *p*-coumaric acid hexoside. The glycosylation of coumaric acid and other cinnamic acids is known to play an important role in directing phenylpropanoid metabolism. Therefore, it is hypothesized that metabolic resources from the phenylpropanoid pathway are redirected from lignin biosynthesis to flavonoid formation.

Altered levels of vanillic acid glucoside and tyrosol glycosides further hint at changes in lignin metabolism but comprise a lower level of evidence because their roles in plant metabolism are not fully understood. Signal intensity changes of phosphocholine and a lysophosphatidylcholine suggest cell membranes could be undergoing compositional changes under SP7 expression. Furthermore, multiple metabolites indicate that major metabolic shifts have occurred in the SP7-expressing plants. The metabolites pyridoxine and two pyridine hexosides were putatively identified. These compounds are directly linked to the cofactor PLP, which is crucial for amino acid metabolism in plants. Additionally, the metabolite betaine was identified, which is involved in a wide array of stress-related metabolic processes. These metabolites show contrasting signal intensity changes in leaf and stem samples, indicating a tissue-specific response of plants to SP7 expression. Besides betaine, the putatively identified homogentisic acid and a furofuranoid lignan glycoside have also been linked to metabolic adaptations to stress. Although the role of these compounds in plant metabolism has not been fully established, these findings suggest that plant stress responses are activated.

In summary, the untargeted analysis of metabolites with high relevance to SP7 expression indicates that major metabolic reorganization is taking place in the plants. The phenylpropanoid metabolism was identified as the most altered metabolic pathway, with increased signal intensities of flavonoids as a major trend. This indicates a substantial role for flavonoid metabolites in the plant's response to the fungal effector protein SP7. While changes to other metabolic pathways were observed as well, their interpretation was often limited by a low number of identified compounds and by lower identification confidence levels.

The implications of these findings for the interaction between the plants and the SP7-expressing fungi will be discussed more thoroughly in Chapter 4.1.4, along with the results of a more extensive semi-targeted identification process.

4.1.3 Semi-targeted Analysis of Leaves, Stems and Roots

A more thorough identification process was performed that focused solely on the features that could be identified with the in-house library. As described in the previous chapter, this in-house database is comprised of analytical metabolite data (RTs, m/z of quasi-molecular ions and known adducts, MS/MS spectra) obtained from the measurement of authentic reference standards. Since this approach limits potential identifications to the metabolites included in the library, it is considered semi-targeted.

The goal of this approach was to provide metabolite identifications with a high level of confidence that could be interpreted alongside the results of the untargeted identification process (see previous chapter). In the preceding identification process, the flavonoid metabolism was identified as the metabolism most altered by SP7 expression *in planta*. Consequently, the in-house database was evaluated for its coverage of the flavonoid biosynthetic pathway. Standard substances of missing key metabolites (e.g. *p*-coumaroyl-CoA, leucocyanidin) were acquired and added to the in-house database. Then, the data were reprocessed with the updated in-house database, as detailed in Chapter 3.1.4.8.2. In this identification process, the samples of both experiments and all three tissue types (leaves, stems, and roots) were examined.

Using this approach, 124 features corresponding to 85 individual metabolites or metabolite groups were identified. As intended, the overall confidence level of the annotations was high. Of the 124 identified features, 109 were identified at MSI level 1, while 15 were identified at the substance class level (MSI level 3). Figure 18 provides an overview of the differences in identification between tissues in the form of Venn diagrams.

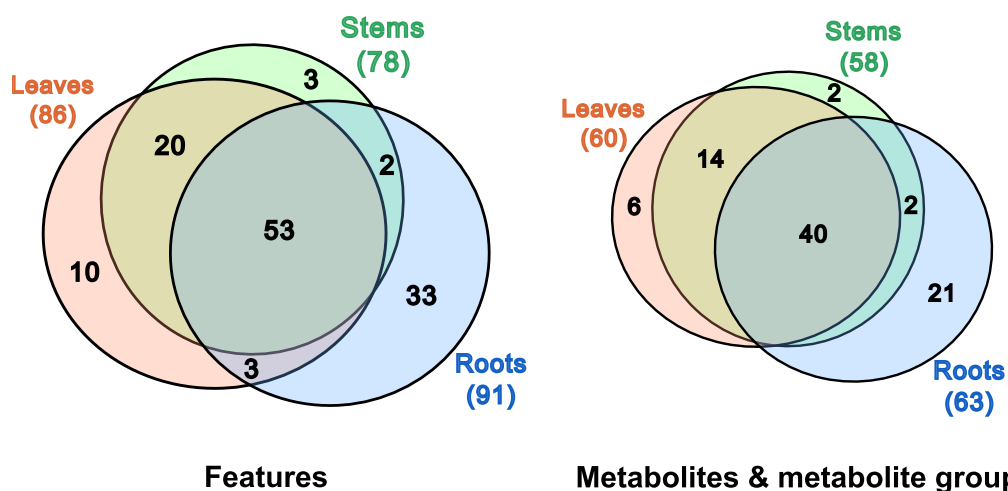


Figure 18: Venn diagrams displaying the numbers of features and metabolites identified in the different potato tissues with the in-house database. Total numbers for each tissue are given in parentheses.

Leaf and stem samples shared more identified metabolites with each other than with the root samples. Similarly, the number of metabolites identified in only one tissue was much higher for roots (21) than for leaves (6) or stems (2). Since the leaf and stem samples were harvested from the same plants in one experiment, as opposed to the root samples, it is unclear whether these observations reflect metabolic differences between tissues or differences between experiments. However, the overall number of identified metabolites was very similar between tissues (58 – 63), which suggests comparable analytical coverage.

The identified metabolites were sorted into eight different classes: flavonoids, other phenylpropanoids, amino acids and derivatives, other nitrogen compounds, sugars and derivatives, benzoic acids and derivatives, alkaloids, and organic acids. This categorization was chosen to facilitate a more coherent data interpretation, but it is acknowledged that these classes do not have strictly defined limits and some compounds may bridge multiple classes. The metabolite groups are presented individually in the subsequent subsections, after which they are discussed together.

Flavonoids

The $\log_2$ FCs for all identified flavonoids are visualized in Figure 19. For leaf and stem samples, the flavonoid signal intensity changes follow the trends observed in the untargeted identification process and are closely related to the previously identified flavonoids (see previous chapter). Most flavonoid signal intensities were higher in the SP7-expressing plants than in the control plants, particularly in stem samples. In the leaf samples, dihydrokaempferol (aromadendrin), a

dihydroflavonol aglycone, was the only flavonoid metabolite with consistently lower intensities in the SP7 samples than in the controls (FC 0.55 – 0.70).

Conversely, signal intensity reductions were observed in the SP7 root samples for three out of the four identified glycosylated flavonoids (FC 0.41 – 0.97), while an unknown quercetin disaccharide exhibited inconsistent metabolic responses between the SP7 lines. Only the dihydroflavonol aglycones dihydrokaempferol and dihydroquercetin (taxifolin) exhibited substantially higher signal intensities in the SP7 samples (FC 2.34 – 3.78).

Other Phenylpropanoids

Alongside flavonoid metabolites, Figure 19 also displays the $\log_2$ FCs for all non-flavonoid phenylpropanoid metabolites. For leaf and stem samples, many of the identified metabolites show inconsistent FCs between the two SP7 lines with very few consistent, noteworthy signal intensity changes between SP7 plants and control plants. Besides the already identified and discussed feruloyltyramine, the most notable finding is the increased signal intensity of neochlorogenic acid in SP7 stem samples (FC 1.39 – 2.53). The metabolite *p*-coumaric acid was identified in both of these tissues, but its signal intensity changes are not of the same magnitude as those of the putative coumaric acid glycoside (see previous chapter). Its signal intensity was slightly lower in the SP7 stem samples than in the SP7 EV samples (FC SP7-7 | SP7-10: 0.59 | 0.76), but changed only marginally in leaf samples (FC SP7-7 | SP7-10: 1.20 | 1.05).

As with the flavonoid metabolites, overall metabolic shifts were much more pronounced in the root samples. Additionally, the observed signal intensity changes were often opposite to those observed in the stem and leaf samples. The signal intensities of the metabolites ferulic acid glucoside (FC 0.10 – 0.24), neochlorogenic acid (FC 0.42 - 0.61), chlorogenic acid (FC 0.30 - 0.64), and sinapic acid (FC SP7-7 | SP7-10: 0.60 | 0.46) were all lower in the SP7 root samples than in the EV root samples. These metabolites are all important in lignin biosynthesis [189, 190, 203] and chlorogenic acid(s) also play a role in plant defenses against oxidative stress [196]. In contrast, the signal intensities of sinapine were substantially higher in the SP7 samples (FC SP7-7 | SP7-10: 2.70 | 2.98). Sinapine, an ester of sinapic acid and choline, is primarily found in Brassicaceae species. It is present in high concentrations in the seeds of these species and can accumulate in response to stress [216-218]. Other phenylpropanoid metabolites, such as caffeic acid, its 3-*O*-glucoside, and *N*-*trans*-feruloyltyramine, showed only marginal changes in signal intensity, while *N*-*trans*-coumaroyltyramine showed inconsistent changes between SP7 plant lines.

4 - Results and Discussion

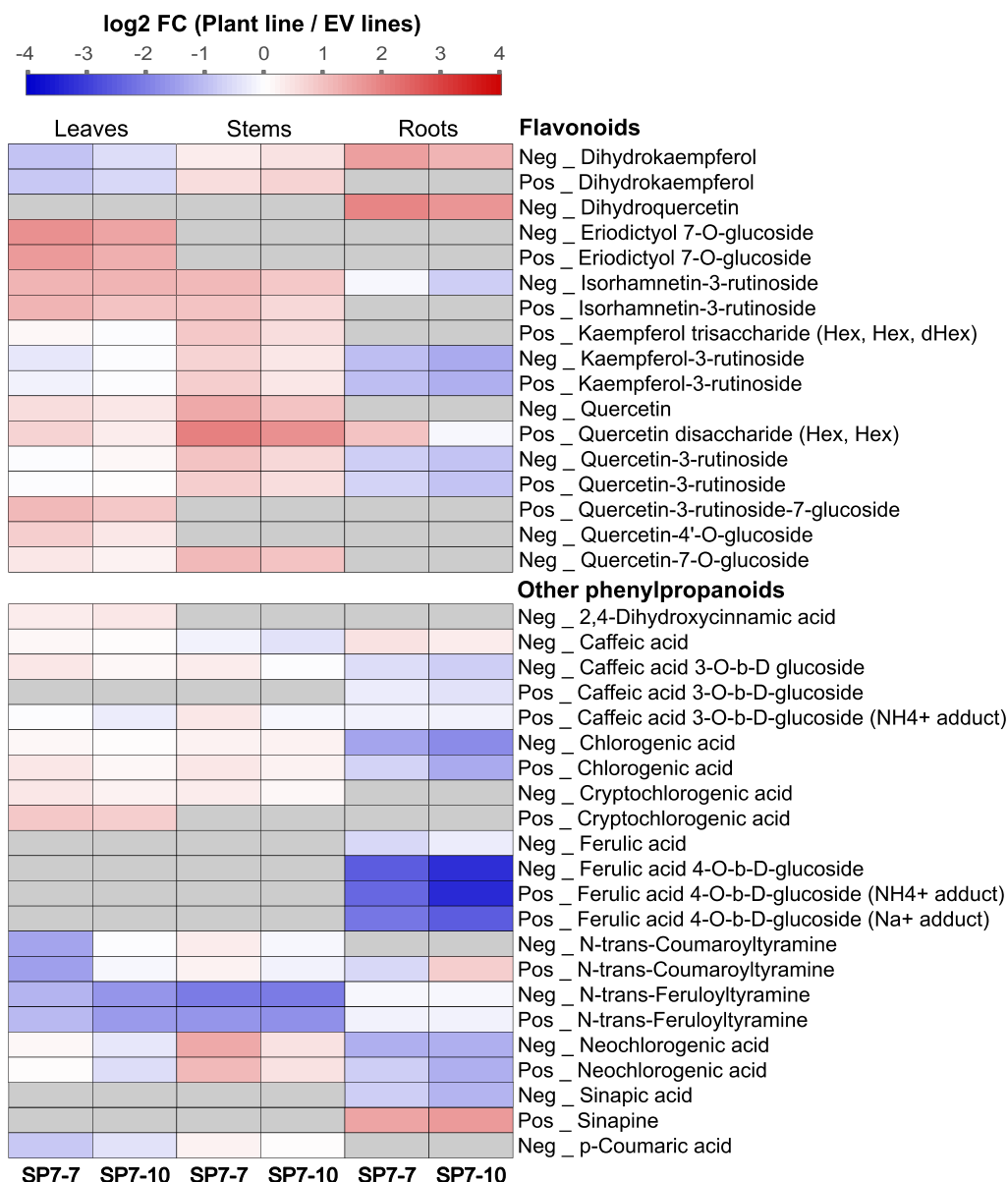


Figure 19: Heatmap visualization of the log₂ FC of identified flavonoid and phenylpropanoid features in the three analyzed potato tissues. Non-detected features are marked in gray. The mean signal intensity of both EV lines was used as the reference for FC calculation. FC, Fold change; EV lines, Empty vector (control) lines; Pos/Neg, Positive/negative ionization; (d)Hex, (Deoxy)hexose.

Amino Acids and Other Nitrogen Compounds

Many of the identified compounds had nitrogen-carrying functional groups, including numerous proteinogenic amino acids and their derivatives. Figure 20 shows the log₂ FCs of these metabolites, which are separated into amino acids and non-amino acid nitrogen compounds.

As with the flavonoid and phenylpropanoid metabolites, clear differences between tissues were observed for these metabolites as well. However, here the stem samples show the largest

deviation from the other two tissues. Several amino acids showed lower signal intensities in the SP7 stem samples, including L-arginine, L-histidine, L-glutamine, L-isoleucine, L-leucine, L-threonine, and L-valine (FC 0.32 – 0.68). The signal intensity of the amino acid L-proline was reduced substantially in both SP7 leaf and stem samples (FC 0.22 – 0.40).

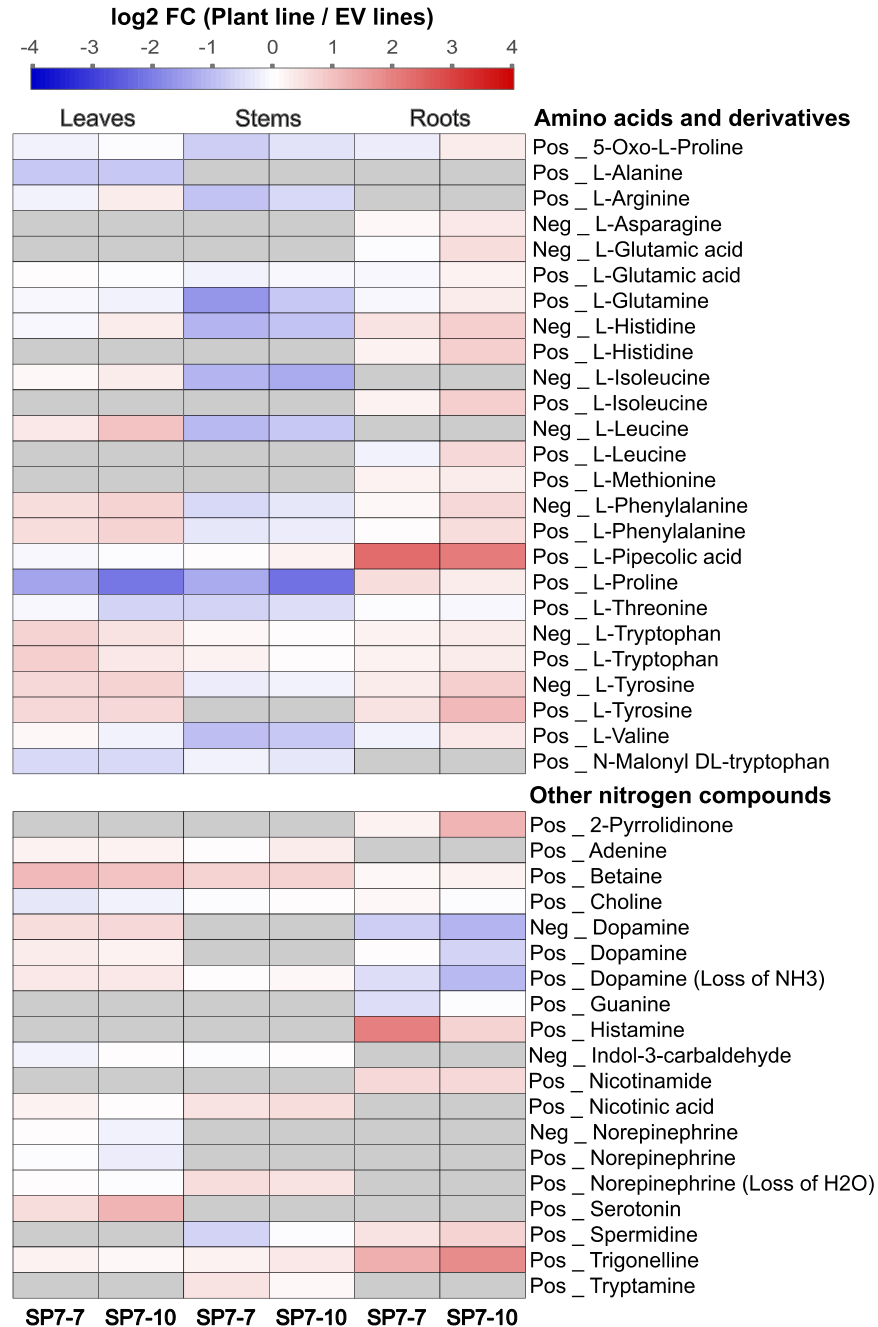


Figure 20: Heatmap visualization of the log₂ FC of identified amino acid and other nitrogen features in the three analyzed potato tissues. Non-detected features are marked in gray. The mean signal intensity of both EV lines was used as the reference for FC calculation. FC, Fold change; EV lines, Empty vector (control) lines; Pos/Neg, Positive/negative ionization.

In the SP7 leaf samples, the signal intensities of the amino acids L-leucine, L-phenylalanine, L-tyrosine, and L-tryptophan were increased compared to the control samples (FC 1.31 – 1.97), whereas log FCs for the other proteinogenic amino acids were low. This is notable because the three aromatic amino acids are all products of the shikimate pathway and are important precursors to many plant biosynthetic pathways [219], including phenylpropanoid biosynthesis via L-phenylalanine.

Evaluating amino acid intensity shifts in the root samples was difficult due to the different responses of the two plant lines to SP7 expression. The SP7-10 samples consistently showed higher signal intensities than the SP7-7 samples, resulting in divergent FCs when compared to the control plants. In summary, signal intensities of the proteinogenic amino acids showed a slight trend of being increased in the roots under SP7 expression, but detailed conclusions were not drawn. However, L-pipecolic acid signal intensity levels were strongly elevated in SP7 root samples relative to the intensity in EV lines, but not in leaf or stem samples. Pipecolic acid is converted to NHPA in plants. The latter is a major signaling molecule in the mobilization of plant defense systems (see Chapters 1.4.2.7 and 1.5), while pipecolic acid itself is key to its regulation and can accumulate in this role [23, 108, 220, 221].

Most of the identified non-amino acid nitrogen compounds belonged to the catecholamines or biogenic amines, which are known regulators of growth and stress adaptation processes in all plants [222-224], as well as in potato plants specifically [225]. The analyzed tissues showed differential signal intensity changes for these compounds between SP7 plants and control plants. In the leaf samples, the signal intensities of dopamine and serotonin were increased in the SP7 samples (FC 1.19 – 2.34), while the signal intensities of norepinephrine did not differ greatly between SP7 and control samples (FC 0.84 - 1.08). Only one dopamine feature (FC SP7-7 | SP7-10: 1.04 | 1.14) and one norepinephrine feature (FC SP7-7 | SP7-10: 1.48 | 1.39) were detected in the stem samples. Similarly, only dopamine was identified in the root samples, where its signal intensity was lowered, but not consistently across both lines and all three detected features (FC 0.48 - 1.06). Two biogenic amines, spermidine and histamine, were identified. Both exhibited higher signal intensities in the SP7 root samples than in the EV root samples (FC 1.41 – 4.08), though spermidine exhibited only a slight change in signal intensity in the SP7-7 stem samples (FC SP7-7 | SP7-10: 0.64 | 1.00).

Nicotinamide, nicotinic acid, and trigonelline (the *N*-methylated form of nicotinic acid) were identified, but only trigonelline was detected in all three tissues. These metabolites are part of the metabolic pathway centered on the cofactors nicotinamide adenine dinucleotide (NAD) and NAD phosphate (NADP), which serve as electron carriers in a numerous enzymatic processes [226,

227]. Nicotinamide, which was only detected in the root samples, had higher signal intensities in the SP7 lines than in the EV lines (FC SP7-7 | SP7-10: 1.57 | 1.57). Trigonelline signal intensities were also strongly elevated in the SP7 root samples (FC SP7-7 | SP7-10: 2.51 | 3.53), but not in the SP7 leaf or stem samples (FC 1.09 - 1.30). Nicotinic acid signal intensities were slightly increased in the SP7 leaf samples (FC SP7-7 | SP7-10: 1.44 | 1.51) but less so in the SP7 stem samples (FC SP7-7 | SP7-10: 1.05 | 1.18).

Glycoalkaloids

Several metabolites that were grouped as alkaloids were identified in the samples. All of these were glycoalkaloids, except for the spermine alkaloid kukoamine A. However, since the latter showed contrasting metabolic responses between the SP7-7 and SP7-10 plant lines, it was not considered for further interpretation. The log₂ FCs of all identified alkaloids are provided in the Appendix (Figure A-2).

The interpretation of the glycoalkaloids is hindered by their high signal intensities, which caused many features to reach the detector saturation limit. Thus, the major features of α -solanine and α -chaconine, the two primary glycoalkaloids in potato plants [228], could not be evaluated and were removed from the dataset. Only the α -chaconine features measured in negative polarity were included. Overall, a tendency toward higher glycoalkaloid signal intensities was observed in leaf and stem samples. Root samples showed a more diverse metabolic response, with higher signal intensities of solanidine (the aglycone of α -solanine and α -chaconine) and α -chaconine in the SP7 samples compared to the controls. However, the signal intensities of four lesser glycoalkaloids were decreased in the SP7 root samples.

Phenolic Acids and Derivatives

Multiple phenolic acids were identified, most of which were benzoic acids or reduced forms thereof. Their log₂ FC heatmaps are shown in Figure A-2 in the Appendix. Their interpretation is kept brief because their FCs are low, and most have only been detected in root samples, where they show contrasting metabolic responses between the SP7-7 and SP7-10 lines. Most of the identified benzoic acids and derivatives are related to vanillic acid metabolism (vanillic acid, vanillic acid glucoside, vanillin, 4-hydroxybenzaldehyde, 3,4-dihydroxybenzaldehyde, and vanillyl alcohol). The only compound exhibiting notable FCs is vanillic acid glucoside, which was discussed in the previous chapter.

Additionally, salicylic acid, a major signaling compound in numerous pathways, was identified in leaf and stem samples. One of its central roles is its involvement in the plant's response to biotic stress after the detection of pathogens (see Chapters 1.4.2.7 and 1.5), and SP7 is known to

interact with salicylic acid-mediated pathways [149]. Here, the signal intensities of salicylic acid were higher in the SP7-expressing plants than in the EV plants. This shift was more pronounced in the stem samples (FC 1.48 - 1.99) than in the leaf samples (FC SP7-7 | SP7-10: 1.22 | 1.31).

Sugars and Derivatives

The metabolomics method used in this project cannot distinguish between isomers of highly polar compounds because they are not chromatographically separated. Therefore, sugars can only be evaluated as sum parameters. Multiple features each were identified as sums of hexoses, sucrose-like disaccharides and C18 trisaccharides. Their $\log_2$ FCs are visualized in Figure A-2 in the Appendix.

Although the individual sugars cannot be evaluated, the FCs of sugars provide an indication of whether the sugar metabolism is affected by metabolic changes. Here, only low FC were observed for all features, indicating stable total sugar levels in all tissues under SP7 expression. However, further interpretations, e.g. regarding the interconversion of sugars, could not be made for these features.

Organic Acids

Eight features of seven metabolites were not allocated to any of the previously described categories and were grouped as organic acids. Changes in their signal intensity between SP7 samples and EV samples are shown in the Appendix (Figure A-2).

Four of the seven organic acids (azelaic acid, maleic acid, malic acid and pimelic acid) are dicarboxylic acids. Despite their structural similarities, however, these metabolites serve different roles in the plant. Malic acid is a part of the citric acid cycle and can accumulate in vacuoles as a form of stored energy, among other functions [229]. Azelaic acid is functionally tied to salicylic acid and plays a role in plant immune responses, primarily as a signaling molecule [230]. Pimelic acid is a biosynthetic precursor to biotin, a cofactor for crucial carboxylase enzymes found in all living organisms [231]. In the SP7 root samples, the signal intensities of all three of these acids were consistently reduced (FC 0.62 – 0.83). Only malic acid was detected in the stem and leaf samples as well, where its signal intensity changes were inconsistent between the SP7 lines, in both leaf (FC SP7-7 | SP7-10: 0.93 | 0.72) and stem samples (FC SP7-7 | SP7-10: 1.49 | 1.03). The same applied to maleic acid, an organic acid associated with plant interactions with soil microorganisms, which has been reported to be a component of potato root exudates [232, 233]. Maleic acid signal intensities were inconsistently lowered in SP7 leaf samples (FC SP7-7 | SP7-10: 0.88 | 0.65) and inconsistently raised in SP7 stem samples (FC SP7-7 | SP7-10: 1.49 | 1.03), respectively.

In addition to pimelic acid, pantothenic acid was identified as another precursor to a ubiquitous cofactor because the metabolite is necessary for coenzyme A (CoA) biosynthesis [234]. While pantothenic acid showed only marginal changes in signal intensity in roots, its signal intensities in SP7 leaf and stem samples were consistently higher than in the control samples (FC 1.27 – 1.52).

Quinic acid, the alcohol component of chlorogenic acid and neochlorogenic acid, was identified in all three tissue types. Its FCs were inconsistent in the SP7 stem samples (FC SP7-7 | SP7-10: 1.20 | 0.78), but it showed a slight intensity increase in the SP7 leaf samples (FC SP7-7 | SP7-10: 1.25 | 1.17) and the SP7 root samples (FC SP7-7 | SP7-10: 1.31 | 1.45). Lastly, the jasmonic acid precursor OPDA (see Chapter 1.4.2.6) was identified in the root samples, but its signal intensity was only marginally lower in the SP7 plants than in the control plants (FC SP7-7 | SP7-10: 0.93 | 0.80).

Conclusions

Changes in flavonoid signal intensities were the most substantial changes observed, but were tissue-specific. For the SP7-expressing plant lines, all flavonoid signal intensities were increased in the stem samples, while most were increased in the leaf samples, except for the two dihydroflavonol aglycones detected. In contrast, these aglycones were the only metabolites with elevated signal intensities in the SP7 root samples. In these samples, the signal intensities of all glycosylated flavonoids (except for one quercetin disaccharide) were decreased compared to the EV control samples. These tissue-specific effects cannot be fully explained, but they suggest that increased flavonoid transport through the stem occurs. In roots, the strong contrast between flavonoid aglycone and glycoside FCs suggests that either flavonoid glycoside formation is hindered or followed by an efflux of the flavonoid glycosides. However, it is unclear which of these processes causes the observed trend, or whether a potential efflux redirects flavonoid glycosides to other plant parts or into the culture medium. Similarly, the metabolic changes observed in leaves could be caused by increased influx or increased local synthesis, but the lowered levels of flavonoid aglycones, as well as lowered levels of *p*-coumaric acid, in the SP7 samples suggest the latter.

Changes to the non-flavonoid phenylpropanoid metabolism were primarily observed in root samples. The exception is feruloyltyramine, which was also identified in the untargeted analysis and is discussed in the previous chapter. Several metabolites related to phenylpropanoid metabolism and lignin biosynthesis (ferulic acid glucoside, sinapic acid, chlorogenic acid, and neochlorogenic acid) were all detected at substantially reduced intensities in the SP7 root samples, compared to the EV samples. In contrast, levels of caffeic acid and quinic acid increased,

which could indicate a downregulation in the conversion of these monomers into their esters, chlorogenic acid and neochlorogenic acid. This conversion is part of one branch of lignin biosynthesis, which is a preferred route in many higher plant species [190]. This contributes to the conclusion that pathways related to lignin formation are likely altered in all three potato tissues, but to a higher degree in the roots. However, more importantly, the formation of chlorogenic acid via the enzyme hydroxycinnamoyl-CoA:quinate hydroxycinnamoyl transferase (HCT) has been demonstrated to control the redirection of the phenylpropanoid pathway in potato plants [196]. This supports the hypothesis, formed from the results of the untargeted identification (see previous chapter), that phenylpropanoid metabolism is downregulated in favor of flavonoid biosynthesis under SP7 expression.

Evaluating the signal intensities of proteinogenic amino acids provided insight into the overall metabolism of the three tissues. There was a clear trend of moderately increased amino acid signal intensities in the SP7 leaf and root samples, and moderately decreased amino acid signal intensities in the SP7 stem samples, compared to the control samples. The most notable intensity increases in the leaf samples were for the aromatic amino acids L-phenylalanine, L-tyrosine, and L-tryptophan, which are key metabolites in many pathways, including the phenylpropanoid metabolism. While changes in the intensity of proteinogenic amino acids can be seen as an indicator of overall metabolic activity, they cannot be reliably interpreted, since only free amino acids were identified. The conversion ratio of peptide-bound amino acids to free amino acids during sample preparation has not been extensively investigated for this metabolomics method. Nevertheless, these findings strongly emphasize the tissue specificity of metabolic changes and show that not all metabolic pathways are downregulated in the root tissue of SP7-expressing plants.

The magnitude of the metabolic changes in the different tissues was further emphasized by the signal intensity changes calculated for various enzyme cofactors in SP7 plant samples. Specifically, the CoA precursor pantothenic acid (increased in stems and leaves), the biotin precursor pimelic acid (decreased in roots), and the NAD precursors and metabolites nicotinamide, nicotinic acid, and trigonelline (increased in stems; strongly increased in roots) were identified. Additionally, multiple signaling molecules associated with plant responses to (biotic) external stressors were among the metabolites with the highest FCs between SP7 plants and EV plants. Notable metabolites include salicylic acid (increased in stems and leaves), spermidine and histamine (increased in roots), pipecolic acid (increased in roots), azelaic acid (decreased in roots), sinapine (increased in roots), proline (decreased in stems and leaves), as well as dopamine and serotonin (increased in leaves). While some of these metabolites did not exhibit substantial

FCs in other tissues, the overall signal intensity shifts demonstrate that potato plants undergo substantial metabolic adaptations during SP7 expression.

Other metabolite groups displayed fewer changes but gave additional insights into metabolic shifts. Sugar compound intensities were evaluated as sum parameters without differentiation between isomers. Overall, the FCs for these features were low, and no clear increases or decreases in signal intensity were observed in any tissue. Therefore, it was concluded that sugars are neither heavily accumulated nor metabolized under SP7 expression *in planta*. Two additional metabolite groups, alkaloids and phenolic acids (and their derivatives), were assessed, but yielded mixed results. No additional conclusions were drawn from the FCs of these metabolites.

These findings will be assessed in combination with the previous findings in the subsequent sections, where they are related to the processes that occur in plants when they interact with SP7-expressing fungi.

4.1.4 Summary of key findings

The metabolome analysis of potato plants expressing fungal effector proteins had two primary objectives. First, it aimed to improve our understanding of the interaction between fungi expressing SP7-like effector proteins and plants. Second, the study aimed to evaluate the effectiveness of the metabolomics method in elucidating plant metabolic pathways, and its overall applicability in plant research studies. The following chapters summarize the key findings in both of these aspects.

4.1.4.1 Relation of Metabolome Analysis Results to Plant-Fungi Interactions

As described in Chapter 1.5, when a plant detects the initiation of fungal colonization of its roots, it activates its defense mechanisms involving multiple phytohormone-mediated pathways. Simultaneously, the fungus secretes effector proteins, such as SP7, which the plant then takes up via its roots. In the case of SP7, its nuclear localization signal then prompts its transport into the cell's nucleus. There, it suppresses the plant's immune response and initiates further metabolic changes, many of which are not yet fully understood [149, 165, 166]. The following chapter relates these processes to the results and conclusions of the potato analysis, in which SP7 was transgenically introduced into the potato plant genome and overexpressed in all plant tissues.

By using a transgenic approach to express the effector protein SP7 *in planta*, this study provided a biologically effective way of analyzing its effect on plant metabolism. While this approach does not consider all interacting factors of a natural plant-fungus symbiosis, it served to overcome a drawback of previous metabolome studies, specifically those that aimed to analyze AM colonized

plant roots. Specifically, the issue is that in an AM symbiosis, the fungal material cannot be separated from plant roots. Hence, identified metabolites may belong to either organism and their interpretation could be potentially misleading [235].

Based on the magnitude of signal intensity shifts, the flavonoid metabolism was identified as the metabolic pathway most affected by SP7 expression in the plants. It was concluded that increased biosynthesis of flavonoid glycosides in the leaves was likely, while lowered signal intensities in the roots could not be definitely attributed to decreased biosynthesis or increased efflux to other plant parts or the culture medium. Flavonoid levels in the stems were substantially and consistently elevated, which was hypothesized to stem from increased transport. Meanwhile, the formation of phenylpropanoid lignin precursors was determined to be decreased, especially in the roots. It was hypothesized that the phenylpropanoid metabolism was redirected from lignin formation to flavonoid biosynthesis. This hypothesis was supported by strong signal intensity changes in metabolites involved in two individual control mechanisms for phenylpropanoid metabolite flux. First, a coumaric acid glucoside was putatively identified in the untargeted identification process. The glycosylation of coumaric acid has been shown to be a control mechanism for phenylpropanoid redirection in many plants [204, 205]. Second, the semi-targeted identification process revealed that the biosynthesis of chlorogenic acid and its isomers was likely hindered since the intensity levels of the precursors increased while the levels of the products decreased in SP7-expressing plants. The enzyme HCT, which catalyzes this reaction, has been shown to regulate the phenylpropanoid pathway in potato plants [196].

The reasons for the observed increase in flavonoid signal intensities were not fully elucidated. While an increased flavonoid content can result from an overall increased synthesis of antioxidants, increased flavonoid production can also occur to secrete flavonoids into the rhizosphere. Flavonoids have been shown to be exuded from AM-colonized plants and to modulate plant-microbe interactions in the rhizosphere [147, 236]. Since the culture medium was not analyzed in these experiments, the efflux of flavonoids into the medium could not be confirmed, but could be of interest to future studies.

As mentioned above, SP7 has only been reported to affect root cells. These studies constitute the first comprehensive metabolome analyses of plants that expressed SP7 in non-root tissues. A strong tissue specificity of SP7 expression effects on the plant metabolism was observed. For instance, major changes in the signal intensities of proteinogenic amino acids were observed between SP7 expressing plants and control plants. Similarly, the signal intensities of precursors to the cofactors biotin, CoA, NAD(P), and PLP also shifted, though not consistently, when comparing the three tissues. From these findings, it was concluded that each of the analyzed plant

tissues underwent major metabolic reprogramming under SP7 expression. While protein expression or distribution were a focus of this work, supplementary analyses performed by collaborators confirmed great changes in protein levels, which complements these results. The signal intensities of sugars, organic acids, glycoalkaloids, phenolic acids, and non-amino acid nitrogen compounds were also assessed comprehensively. These metabolic pathways showed fewer metabolic alterations, except for individual specialized metabolites, which will be discussed below. These observations suggest that, although plants adapt metabolically in many ways to SP7 expression, these changes effectively stabilize their overall metabolism.

These findings are consistent with the metabolic shifts described for plants after AM colonization, which has been studied in many plant species, including Solanaceae. The resulting symbiosis generally causes major alterations in the respective plant metabolism, including major changes in flavonoid and phenylpropanoid metabolism, as well as amino acid metabolism [159, 237, 238]. Thus, the results of this work mirror the general trends reported for plant-fungus interactions in soil. However, the specific metabolite classes and individual metabolites affected by AM root colonization vary greatly depending on the plant species and the fungal genotype [160, 237].

Although a recent meta-analysis highlighted that AM colonization generally benefits potato plants [164], comprehensive studies investigating the potato plant metabolome following AM colonization are scarce. A limited study of potato plant leaves identified eight phenylpropanoids and flavonoids [158], all of which were equal to or related to metabolites identified in this study (chlorogenic acids, kaempferol and quercetin glycosides). Interestingly, the AM colonization-induced concentration shifts of these metabolites differed drastically between different potato and AM fungus genotypes. This highlights the limited transferability of research results between studies. Thus, no further attempts were made to relate individual metabolite signal intensity changes from this work to previous studies.

The metabolome analyses performed here also identified a wide variety of signaling molecules related to plant defense mechanisms, which showed some of the most substantial FCs between SP7-expressing plants and EV plants. These included salicylic acid, spermidine, histamine, pipecolic acid, azelaic acid, dopamine, and serotonin. However, their detection and FCs were also often tissue-specific. These findings align with plant-fungus interaction in soil. The recognition of a fungus by a plant activates the plant's defense systems, even if a symbiosis is later established [149]. As described above, SP7 then actively suppresses the phytohormone-mediated defense systems that have been activated. The effects of SP7 expression on signaling pathways can only be speculated, as they have not yet been determined. It is assumed that the identified stress indicators reflect drastic changes in signaling pathways when SP7 is constitutively expressed and

not merely perceived by root cells. However, most of the identified compounds can only be considered minor signaling molecules, representing small parts of complex regulatory processes. Further contextualization is necessary to fully explain the adaptations occurring in the plant, which was not possible with the data obtained here. The lack of detected phytohormone metabolites especially hindered the elucidation of stress pathways.

In addition to these biotic stress markers, several compounds that typically accumulate under osmotic stress (e.g. drought or salinity) were identified, including betaine, L-proline, and sinapine. Similar to the other signaling metabolites, the signal intensity changes of these compounds between SP7 lines and EV lines were among the highest found in these studies. These findings reflect known processes in AM-colonized plants because symbioses between plants and AM fungi have also been shown to improve salt and drought tolerance. Relevant mechanisms include improved nutrient uptake from affected soils and modulation of ABA-mediated processes, but also include the increased synthesis of osmoprotective substances such as those identified here [161, 163]. These findings demonstrate that these metabolic adaptations, which are commonly described for AM-colonized plants, also occur when SP7 is expressed *in planta*.

In conclusion, the analyzed potato plants expressing SP7 exhibited metabolic shifts similar to those expected following colonization of their roots by AM fungi. New observations were made regarding the shift of phenylpropanoid metabolites from lignin biosynthesis to flavonoid biosynthesis, but their species- and effector-specificity need further investigation. By utilizing a transgenic system, this study ensured that the identified shifts were purely plant-derived, avoiding the confounded results of typical plant-fungus co-cultivation studies. The magnitude of metabolic reprogramming under SP7 expression was substantial in all three analyzed tissues. The specific alterations differed between these tissues, which matched expectations since SP7 typically only affects root cells. These observed effects on metabolism will contribute to revealing the extensive metabolic changes induced by this common effector protein.

4.1.4.2 Applicability of the Metabolomics Method to Plant Research Studies

The metabolome analyses conducted here provided a comprehensive view of the plant's response to fungal effectors. A wide range of primary and secondary metabolites spanning diverse chemical classes were successfully identified and placed in a biological context. A dual identification approach demonstrated the method's ability to selectively identify highly relevant metabolites as well as its ability to perform a comprehensive high-confidence metabolome characterization. These findings demonstrate that the method is not only capable of elucidating established mechanisms but also of identifying subtle, previously unobserved metabolic trends by comparing

plants exposed to stressors with control plants. Furthermore, many metabolites relevant to human nutrition were identified. These metabolites were not discussed here, but they could lead to practical conclusions when the method is applied on plant tissues intended for consumption, which is a key aspect when assessing food crops. In summary, the untargeted metabolomics method proved to be a promising tool that, when integrated with molecular biological analyses such as transcriptomics, could contribute to a more holistic understanding of physiological processes in plants.

However, despite the broad analytical coverage achieved, a significant gap remained in connecting the observed changes in different metabolic pathways. In many cases, changes in metabolisms were demonstrated, but it was not possible to prove how these changes initially arose, nor could the identified signaling compounds be adequately related to each other. Besides salicylic acid and the jasmonic acid precursor OPDA, no major phytohormones or their metabolites were identified in these analyses.

While this study focused solely on metabolome analysis of potato plants, the KIT Molecular Phytopathology Group performed gene expression analyses simultaneously. These results further suggested that phytohormone signaling pathways must be involved in the SP7-induced metabolic adaptations of the plants. Specifically, the plant hormone classes gibberellins, auxins, and strigolactones were suspected to be of high relevance. It was hypothesized that gibberellins in particular played a fundamental role by modulating the nuclear splicing machinery and thus the protein biosynthesis, the central element of the entire metabolism. However, this hypothesis could not be confirmed with the metabolome analyses because these metabolites had not been analytically detected. As described in Chapter 1.4, most phytohormones are present at very low concentrations in plants and are difficult to detect using wide-scope analytical methods, such as the metabolomics method employed here.

With regard to the general suitability of the in-house metabolomics method for plant research, it had to be assumed that lacking data on signaling processes would often present a missing link. Therefore, it was decided to develop a targeted phytohormone analysis method to be used in conjunction with the in-house metabolomics methods. Following this metabolome analysis, establishing and applying a phytohormone analysis method became the main focus of this work, as described in the following chapters.

4.2 Establishment of a UHPLC-MS/MS Targeted Phytohormone Analysis Method

Building on the findings of the metabolome analyses, which underscored the need for sensitive and specific phytohormone detection, this work aimed to establish a high-coverage targeted phytohormone analysis method. The objective was to cover not only the biologically active forms of each phytohormone class, but also a broad range of metabolites within respective pathways by including key precursors, inactivation products, regulatory metabolites, storage forms, and transport forms. Analytical and practical considerations and benchmarks were defined, and thorough literature research was performed. These steps are fully detailed in Chapter 3.2.1.

Ultimately, a UHPLC-MS/MS method intended for wide use in plant research was chosen for adaptation [126]. According to the authors, their “phytohormonomics” method provides precise and sensitive detection and absolute quantification of a wide scope of 101 analytes from the major phytohormone classes (cytokinins, auxins, brassinosteroids, gibberellins, jasmonates, salicylates, and abscisates), all while using low sample amounts (20 mg fresh mass per sample), a simple workflow for sample preparation, and short analysis times. However, several drawbacks were identified and addressed for the adaptation of the method. The advantages of the method are elaborated in Chapter 3.2.1, while key drawbacks that were subsequently addressed in this work are summarized below.

First, the original method used a single MRM transition for each analyte and therefore provided limited analytical confirmation of analyte identity. While the authors did not address this aspect explicitly, they did cross-validate their results with gene expression data in their analysis of *A. thaliana* samples. One goal set for the method adaptation was to develop the method into an independent analytical method by establishing two MRM transitions per analyte, as recommended by common method validation guidelines [174]. Second, using the method as published would require the acquisition of over 70 isotope-labeled IS, which are expensive and often difficult to obtain. To increase the accessibility of the method for standard laboratory settings, absolute quantification was limited to primary phytohormones, while robust and precise relative quantification was prioritized for the broader metabolite scope. Third, the method needed to be adapted to use freeze-dried, pulverized plant samples, to ensure compatibility with existing in-house metabolomics pipelines. The advantages of using freeze-dried materials were outlined in Chapter 1.4.3.

Several smaller adaptations were also performed. While not all of these are presented in this work, the key experiments and adaptations are summarized in Chapter 4.2.1. The final method is

presented and critically evaluated in Chapter 4.2.2. The validation of the final method and its application to plant studies are then presented in the subsequent chapters.

4.2.1 Method Optimizations

The following chapter outlines the systematic optimizations that led to the finalized phytohormone analysis protocol. Four key optimization experiments are presented: the assessment of analyte stability (Chapter 4.2.1.1), the refinement of eluent chemistry (Chapter 4.2.1.2), the optimization of the chromatographic gradient (Chapter 4.2.1.3), and the evaluation of an alternative sample purification strategy (Chapter 4.2.1.4).

The method adaptation process also involved the selection of analytes to be included in the scope of the method. Importantly, the selection of relevant metabolites was performed independently from the original method. Attempts were made to also include strigolactones and ethylene-related metabolites in the method, neither of which were included in the original method. Authentic reference standards were acquired for the analytes, if available. Each standard was then optimized for MS detection and chromatographic performance of its potential MRM transitions. The two best transitions were selected and designated quantifier and as qualifier transitions for further experiments. Of the 118 phytohormones identified as metabolites and metabolite groups of interest, 82 were tested, and ultimately, 63 analytes were included in the final MRM method. Additionally, ten IS were acquired, eight of which were included in the final MRM method. Chapter 3.2.2 details the entire process, including brief discussions of metabolites that were originally considered, but ultimately not included in the final method for various reasons. Table A-16 and Table A-17 (Appendix) list these metabolites, which may be of interest for future adaptations of the developed method.

4.2.1.1 Analyte Stability

In the context of high-throughput analysis, defining the stability of analytes over time is critical to ensuring that observed metabolic profiles remain reproducible across large experimental batches. While it is also an essential part of method validation [174], the assessment of analyte stability was explored early in the development process to ensure the validity and reproducibility of subsequent experiments. Since these results were relevant for further method optimization, they are summarized here rather than presented alongside the full method validation (see Chapter 4.3).

To account for the possibility of reactions between all analytes, only solutions containing all analytes were tested. Thus, one mixture of all analytes was distributed to separate vials and measured in triplicate on three consecutive days, as well as seven days after the initial measurements (Day 0, Day 1, Day 2, and Day 7). The experimental procedure and data analysis

are detailed in Chapter 3.3.1. As described there, a report was generated for each analyte using custom R code and was used as the basis for data interpretation. Observed signal intensity trends were considered relevant only if they were consistent for both analyte transitions. Figure 21 displays the relative signal intensities for each analyte transition by phytohormone class to highlight class differences found during data evaluation.

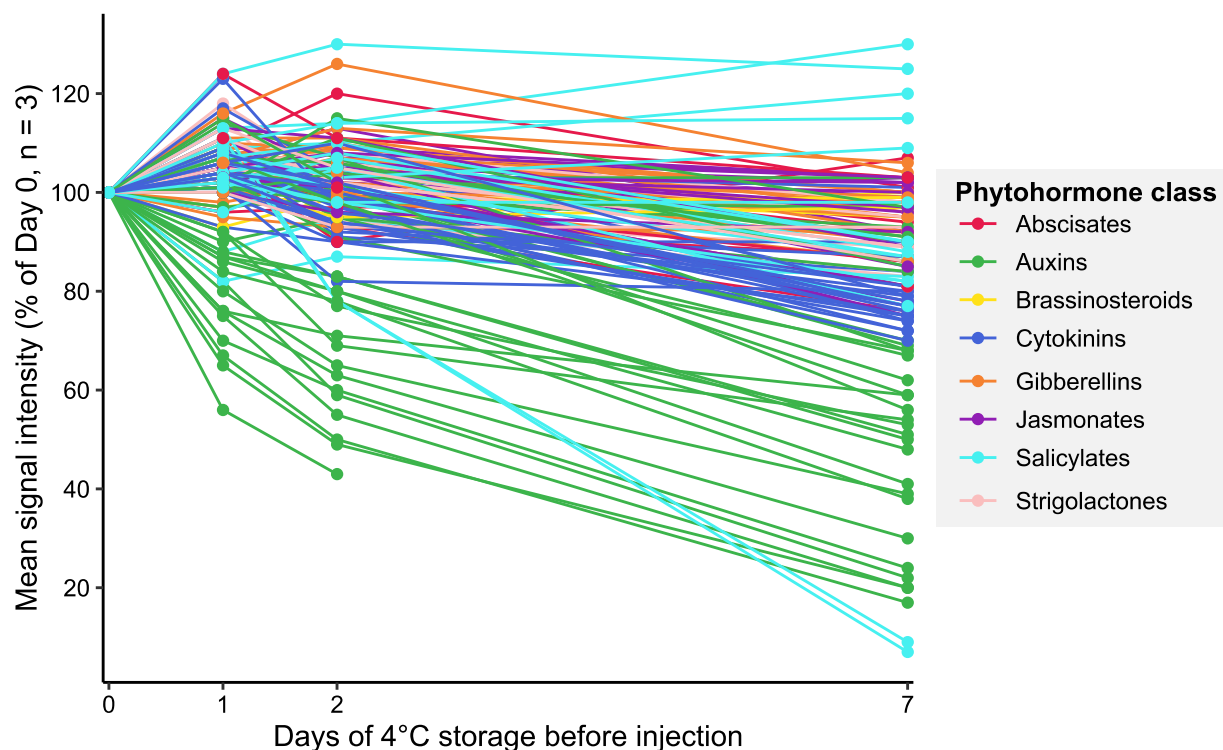


Figure 21: Stability line graphs of all analyte transitions. Each transition's signal intensity is displayed relative to the signal intensity on the first day of injections (Day 0). Measurements were performed in triplicate.

Overall analyte stability was determined to be high until Day 2, except for the analyte NHPA and several auxin analytes, including the primary auxin IAA and its isotope-labeled IS, d5-IAA. NHPA's chemical instability was expected due to its hydroxylamine group, which is highly reactive and prone to chemical degradation, e.g. by auto-isomerization into oximes [239].

Upon assessing the structures of the affected auxins, it was found that only those with a free carboxylic acid group exhibited decreasing signal intensities. Since these compounds were not known to be chemically unstable, this prompted further investigations. After conducting several additional tests, which are not detailed in this work, it was determined that the chemical surface properties of the column likely changed over time. The column used in both the original and adapted method consists of mildly positively charged particles, referred to as charged surface hybrid (CSH). The FA content of the eluents (0.01% v/v) was found to be outside of the

manufacturer's recommended range (0.1 – 1.0% v/v) for this column type [240]. The observed signal loss in auxins with free carboxylic acid groups suggested the occurrence of electrostatic interactions. Under the low formic acid concentrations used in the original method, the pH likely facilitated an increased partial deprotonation of these acidic analytes, leading to increased retention on the positively charged surface of the CSH stationary phase. The fact that signal intensity drifted without significant peak broadening suggested a loss of analyte mass to the stationary phase rather than a separation issue. The authors of the original method discuss their intentional choice of 0.01% FA addition, which ensured baseline separation of cytokinins [126], but they did not address the resulting instability of the column material.

To summarize, the stability analysis designated the maximum time between analyte solution preparation and injection as 24 h. Furthermore, signal intensity drifts of several auxin metabolites were observed and were concluded to be likely related to the chemistry of the UHPLC column. These effects were assumed to be caused by the eluent chemistry.

4.2.1.2 Eluent Composition

To address the surface-charge-induced instability identified in the previous section, alternative eluent additives were evaluated to optimize analyte response.

FA additions between 0.01% and 0.1% (v/v) were tested, with the latter being at the lower end of the range recommended by the column manufacturer [240]. Similarly, since the manufacturer recommends the combined use of FA and ammonium formate (NH_4FA), this was also assessed. To ensure consistent conditions before each experiment, the column was equilibrated in the same manner with a 1% FA (v/v) solution before transitioning to the standard equilibration at test conditions. The experimental procedures are described in detail in Chapter 3.2.3. Figure 22 shows the changes in signal intensity for all analytes, including the IS, as a heatmap.

First, the FA content of both eluents was varied (Figure 22, left side). Compared to the original FA content of 0.01% (v/v), adding more FA to the eluents resulted in strongly increased auxin signals. For example, the intensity of IAA, the most biologically active auxin, and its isotope-labeled IS increased approximately 10-fold in all experiments that used higher FA ratios. This was assumed to be caused by a lower carboxylic acid deprotonation, thereby reducing electrostatic interactions with the positively charged column surface. Interestingly, the auxin signal intensities did not increase proportionally with the FA content and remained consistent across all tested additions $\geq 0.03\%$.

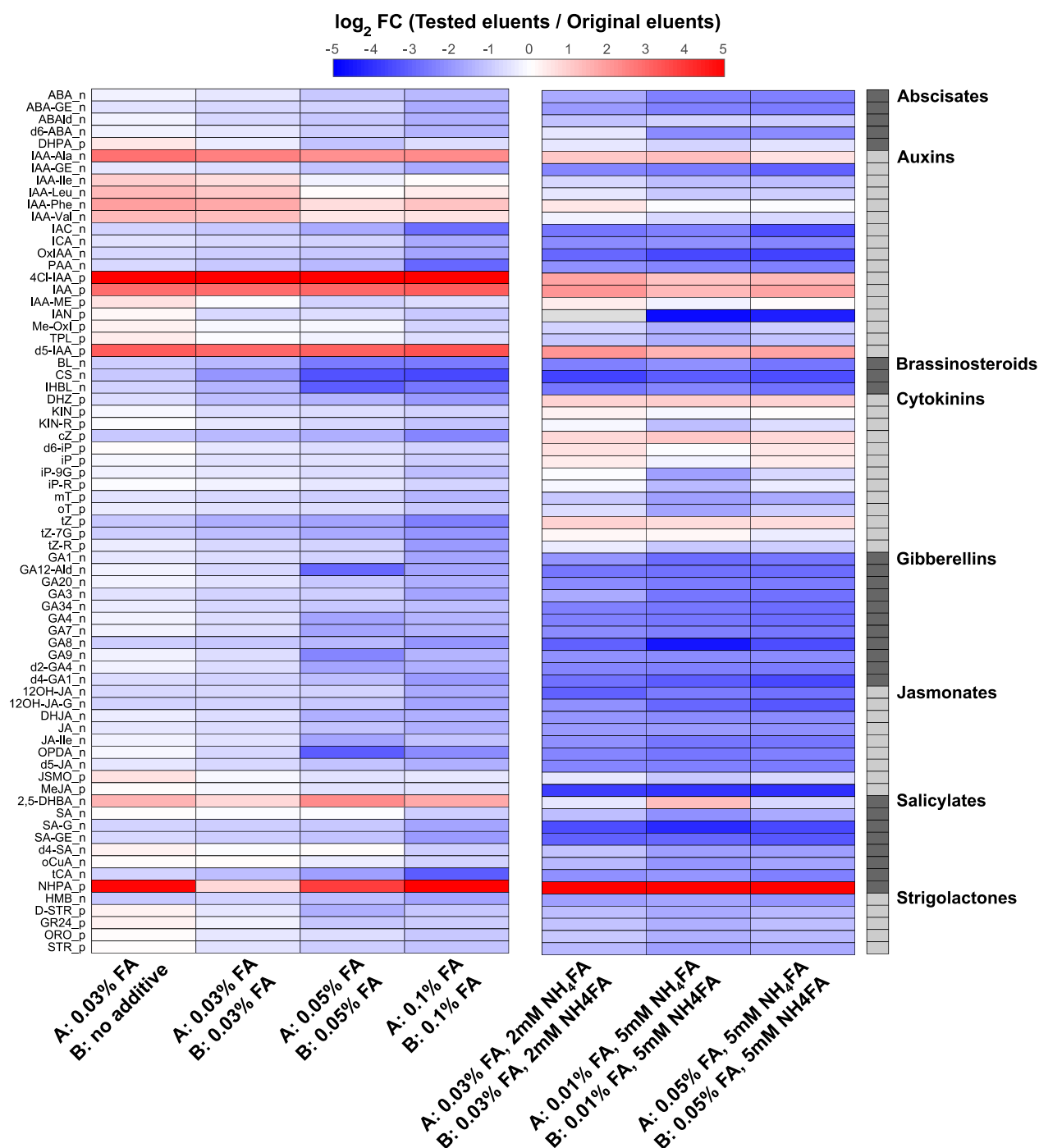


Figure 22: Heatmap visualization of the log₂ FC of the phytohormone analytes (quantifier transitions) measured with different additives to the eluents, sorted by phytohormone class. Analyte transition abbreviations are provided in the Appendix (Table A-19). The original eluent compositions (0.01% FA in both eluents) were used as the reference for FC calculation. Eluents A and B remained the same across experiments (A: Ultrapure water; B: ACN), but 5% water was added to Eluent B when NH₄FA was used. All percentages are volume percentages (v/v). FC, Fold Change; A/B, Eluent A/B; FA, Formic acid; NH₄FA, Ammonium formate.

In contrast to the observations made for the auxin metabolites, increasing the FA content led to lower signal intensities for most other analytes, and their signal intensities decreased further as FA addition increased. The exceptions to this pattern were 2,5-DHBA and NHPA. Since this effect was more pronounced in later-eluting analytes (which reached the MS at a higher Eluent B ratio), adding FA only to Eluent A (at 0.03% v/v) was attempted. Compared to adding 0.03%FA (v/v) to both eluents, this approach retained the higher auxin intensities while leading to lower signal losses for the other analytes, thus performing best out of all the experiments. It was also examined whether changes in FA content led to better auxin signal stability, but this was not the case (Appendix, Figure A-3). However, signal stabilization was observed after > 12 injections, and this was considered in the final data acquisition procedures (see Chapter 3.2.4.1).

Second, NH_4FA was added to both eluents at low concentrations. The best-performing experiments are shown in Figure 22 (right side). Overall, using buffered eluents led to slightly higher signal intensities for auxin analytes, and improved peak shapes and signal intensities for cytokinin analytes. However, a substantial loss in signal intensity was observed for most other analytes. Subsequent experiments with different ratios, including adding NH_4FA to Eluent A only, did not improve this outcome (not presented in this work). Since this trade-off was not considered beneficial overall, NH_4FA addition was ultimately not used.

In summary, the best-performing eluent combination was 0.03%FA in water (Eluent A) and ACN without additives (Eluent B), which replaced the originally published eluents in the adapted method. This combination produced substantially higher signals for several auxins and the metabolites 2,5-DHBA and NHPA. Additionally, further insight was gained into the signal stability of auxins. This insight was used to implement measures into the method to ensure stable signals. Moreover, the equilibration of the UHPLC column with 1% aqueous FA (v/v) used in these experiments was incorporated as standard procedure and performed before each subsequent experiment.

4.2.1.3 UHPLC Gradient Profile

In the phytohormone analysis method adapted in this work [126], each UHPLC run begins with a 5 min isocratic hold, before gradually increasing the organic proportion of the eluent mixture. While adapting the method it was observed that peaks with early RTs, eluting during or shortly after the isocratic segment, exhibited poor peak shapes compared to those with later RTs. The isocratic part of the UHPLC gradient profile was originally used to separate cytokinin and methylthiolated cytokinin metabolites, few of which were included in the adapted method due to unavailability of

respective reference standards (see Chapter 3.2.2). Thus, it was attempted to alter the UHPLC gradient profile to potentially improve analyte peak shapes and shorten analysis times.

Several gradient eluent composition profiles were tested in preliminary experiments and the two best-performing gradient profiles were tested more extensively, as detailed in Chapter 3.2.3. Both of these gradient profiles, Gradient 3 and Gradient 5, decreased the isocratic hold time at the beginning of each analysis to 2 min and 0.5 min, respectively. Starting the gradient earlier than in the original method resulted in a slight but consistent increase in signal intensity for almost all analytes, and no substantial losses in signal intensity. Since this was a consistent trend, the $\log_2$ FC heatmap for these experiments is provided in the Appendix (Figure A-5). Additionally, the alternative gradient profiles improved the peak shapes of analytes with early RTs. This was more pronounced the earlier the compounds eluted, except for very early eluting compounds (NHPA, HMB, cZ, and tZ), which were only minimally affected. Figure 23 demonstrates representative peaks of three analytes measured with the three different gradient profiles.

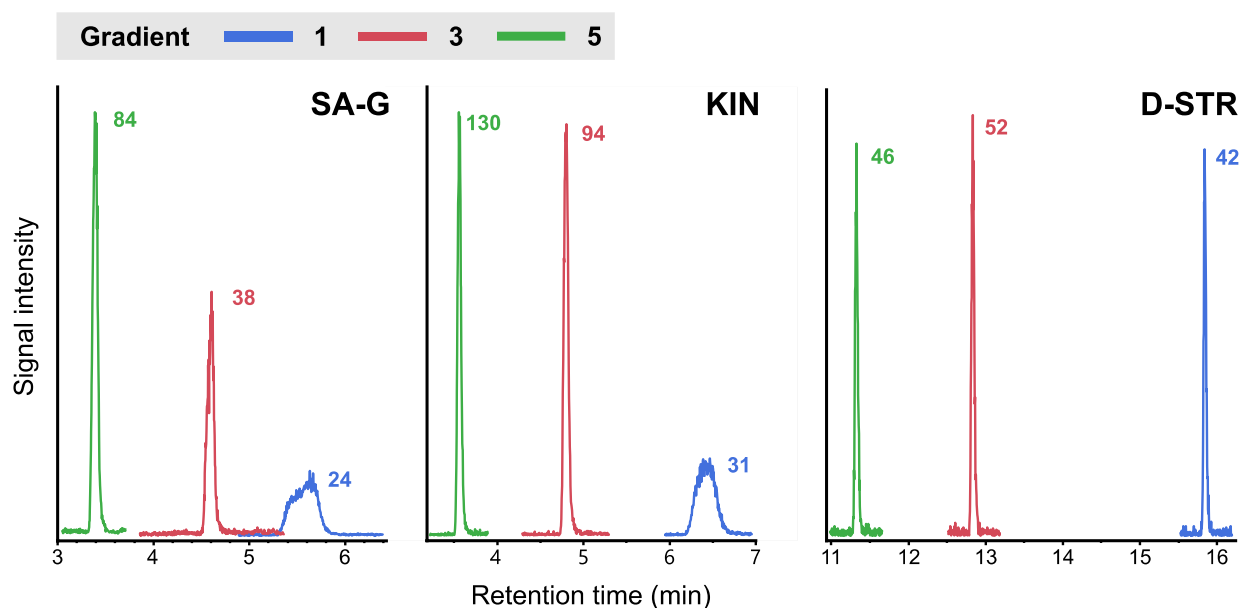


Figure 23: Exemplary peak shapes of two analytes with early retention times (SA-G, KIN) and an analyte with a late retention time (D-STR), measured with three different UHPLC gradient profiles. The numbers by each peak apex provide the peak-to-peak signal-to-noise ratio. D-STR, Deoxystrigol; KIN, Kinetin; SA-G, Salicylic acid glucoside.

Gradient 5, the best-performing gradient profile, was chosen to replace the gradient profile adapted from the original method. Besides improving signal intensities and peak shapes, this shortened analysis times from approximately 22 - 23 min³ to 18.5 min per run. This reduction in analysis run time significantly increased the method's throughput and thus increased its suitability for large-scale studies.

4.2.1.4 Alternative Sample Purification

As detailed in Chapter 1.4, multiple wide-scope analysis methods for phytohormones have been published that differ in their sample purification strategies. A QuEChERS-like approach using graphitized carbon-black (GCB) as an adsorbent in a dispersive SPE [127] was tried out as an alternative to the cartridge-based SPE procedure described by Šimura et al. [126]. While both purification procedures aim to bind and remove chlorophylls from plant material, the QuEChERS-like approach promised quicker and potentially more reproducible extract purification.

Analyte mixtures were prepared using both workflows, and the signal intensities of the prepared and unprepared mixtures were compared. The term "absolute recovery" was used to describe this signal intensity ratio, in accordance with the SANTE 11312/2021 guidelines [174] for method validation. Plant material was not added as this was devised to be an early-stage test. The full sample preparation workflows and the design of the comparative experiment are detailed in Chapter 3.2.4. The absolute recoveries of both experiments are shown as histograms in Figure 24 and the respective CVs as Figure A-4 in the Appendix.

While the precision of some analytes increased with the GCB-based approach, the absolute recoveries decreased substantially across most analytes. This was likely to be caused by strong adsorption to the GCB material, which has a particularly high affinity for aromatic molecules [127]. Because many phytohormones are present at trace levels in plant tissues, high recovery is a prerequisite for reliable detection near the LOD. Thus, it was determined that the signal reductions of the GCB method outweighed potential advantages in preparation speed. No further attempts were made to optimize the GCB-based workflow and apply it to plant materials. The cartridge SPE-based method using HLB adsorbent was used for all further experiments; its final version is evaluated critically in Chapter 4.2.2.1

³ The authors of the original method do not detail the length of their column washing and column reequilibration steps. The lengths of 22 – 23 min were those of the method as it was adapted from the publication. Positive and negative analysis times differed by 1 min.

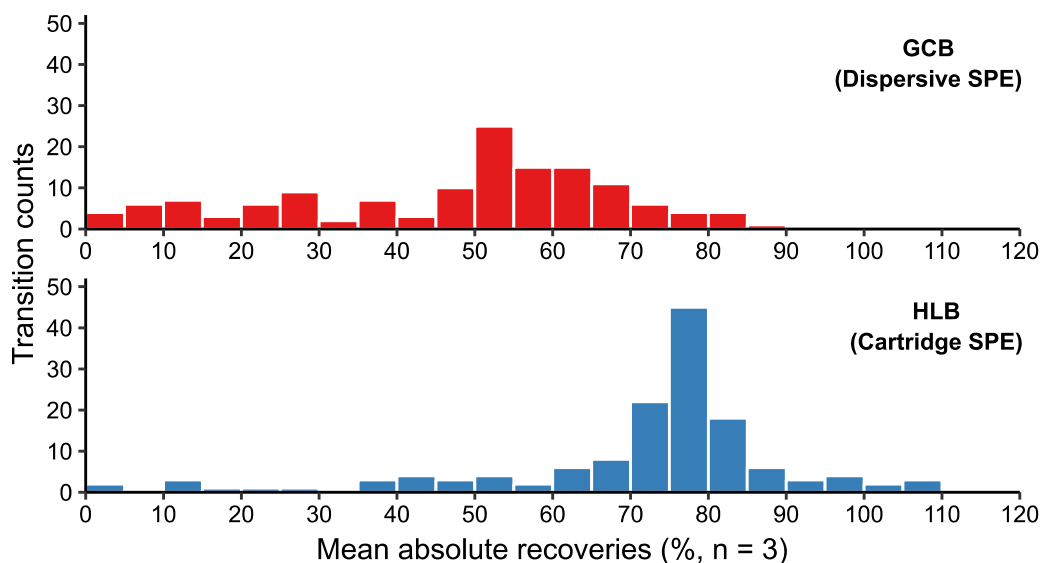


Figure 24: Recovery distributions of analyte mixes prepared with different purification workflows. Absolute recoveries were calculated as the signal intensity ratio between prepared and unprepared samples. GCB, Graphitized carbon black; HLB, Hydrophilic-Lipophilic-Balanced; SPE, Solid phase extraction.

4.2.2 Qualitative Evaluation of the Final Method

Following the optimization of eluent chemistry and gradient profile, which collectively improved signal intensity and throughput, this chapter presents the final UHPLC-MS/MS method. Beyond these chromatographic refinements, the following sections highlight key adaptations made to the "phytohormonomics" method published by Šimura et al. [126]. These include the adaptation of the sample preparation workflow for freeze-dried tissues (Chapter 4.2.2.1), the analyte coverage and chromatographic separation within each phytohormone class (Chapter 4.2.2.2), and the development of a data analysis pipeline in R (Chapter 4.2.2.3). Each aspect is briefly presented and then critically discussed, while details are provided in Chapter 3.2.4.1 (subsection "Data analysis").

4.2.2.1 Sample Preparation

The original sample preparation workflow was intended for use with freshly frozen plant materials. One of the major objectives in adapting the method was to adapt it for freeze-dried, pulverized sample materials (see Chapter 4.2). The finalized sample preparation workflow is shown in Figure 25 and is described in detail in Chapter 3.2.4.1.

4 - Results and Discussion

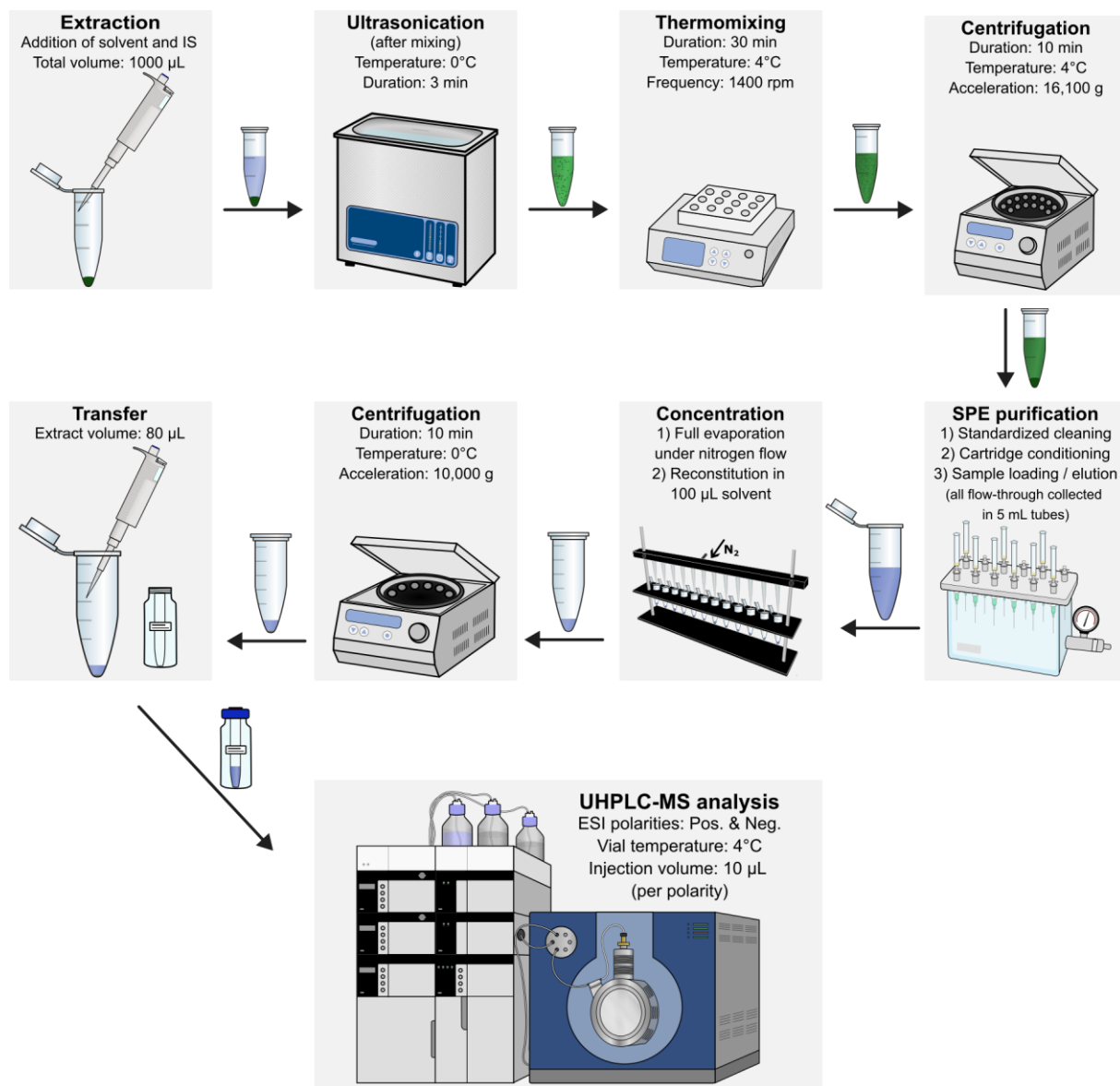


Figure 25: Optimized sample preparation workflow for freeze-dried, pulverized plant materials. Own illustration. IS, Internal standards; Pos./Neg., Positive/Negative; SPE, Solid phase extraction.

The core steps of the original workflow were compatible with freeze-dried plant materials, though several adaptations to laboratory-specific equipment were made. The first step of the adapted workflow was weighing aliquots of the freeze-dried, pulverized samples. A weighing protocol was established to increase reproducibility and minimize analyte losses during the process. The first step of the original method, a simultaneous tissue homogenization and extraction using a vibration mill, was replaced by adding the extraction solvent directly to the sample tubes containing the powdered samples. The subsequent steps, from ultrasonication to SPE, adhered more closely to the original method. The SPE purification process remained largely unchanged, though a standardized pre-cleaning protocol and other measures aimed at improving reproducibility were

implemented. The subsequent evaporation under a gentle nitrogen stream again followed the protocol of the original method. However, major changes were made between the reconstitution step and the sample analysis.

The authors of the original method did not address one aspect of extract concentration, which is the possibility of precipitates forming during evaporation that do not redissolve in the reconstitution solvent. These solids could severely impair the analysis by clogging capillaries or the column of the LC instrument, especially since the authors do not mention using a pre-column. These effects could shorten the longevity of analytical columns and the UHPLC instrument and cause pressure variances and reduced peak resolution, resulting in lower measurement reproducibility. Therefore, the absence of filtration or centrifugation steps in the original method is considered a limitation, particularly given its intended use for analyzing various and complex plant matrices. The adapted method introduces a centrifugation step to prevent solid particles from being transferred into the UHPLC vial. It was expected that this step would greatly improve the robustness and general applicability of the method, especially during longer measurement series. However, this step, together with technical limitations, resulted in a larger reconstitution volume. Ultimately, 100 μL were used, as opposed to the 40 μL used in the original method, resulting in a 2.5-fold lower analyte concentration. At the trace levels at which many phytohormone metabolites are present, this presents a significant drawback.

Ultimately, the transition to a 100 μL reconstitution volume was deemed a necessary trade-off to ensure instrument longevity and analytical robustness. By successfully adapting the core workflow for freeze-dried material and integrating rigorous SPE protocols and a final centrifugation step, the adapted method provides a solid foundation for phytohormone analysis across diverse and complex plant matrices.

4.2.2.2 Analyte Coverage and Performance

The finalized UHPLC-MS/MS method covers 63 phytohormones and related metabolites belonging to eight different classes. Importantly, these classes are used to categorize the analytes by biological pathways, rather than by chemical functionalities (see Chapter 1.4.2). The following subsections cover and critically evaluate each of these classes regarding their coverage of the respective signaling pathway. Additionally, analytical aspects affecting the detection of selected metabolites and options for future expansions of the method are discussed. Since metabolic pathways were described in detail in Chapter 1.4.2, this chapter does not include individual literature references for the roles of metabolites unless they were not addressed previously.

Abscisates

Four abscisates and one IS are included in the method scope. These include the biologically active ABA, its IS d6-ABA, its direct precursor ABAld, its major storage form ABA-GE, and one of its inactivation products, DHPA. Figure 26 shows their chromatographic separation; all analytes had different precursor m/z and did not interfere with each other's analysis.

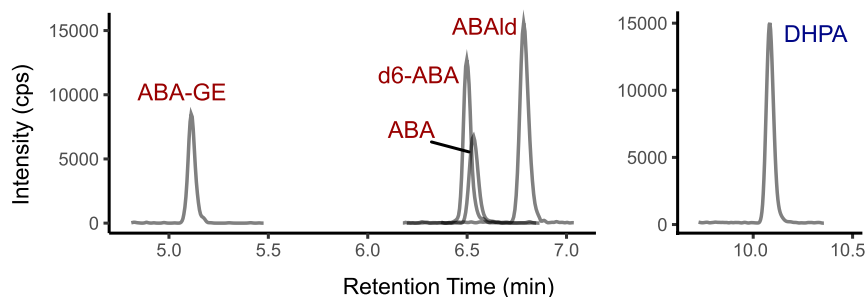


Figure 26: Exemplary chromatographic peaks of abscisates at concentrations close to their respective LLOQ. Analytes labeled in red and blue were measured in positive and negative polarity, respectively. (+)-ABA, (+)-Absciscic acid; ABAld, Absciscic acid aldehyde; ABA-GE; Absciscic acid glucose ester; DHPA, Dihydrophaseic acid.

Together, these metabolites represent key steps of ABA biosynthesis, storage, and degradation. There are no other major storage forms, and further precursors are carotenoids, which are not well soluble in aqueous solvent mixtures and are highly prone to degradation [241]. In future extensions of the method, the inactivation pathway products phaseic acid and DHPA glucoside could be included to further cover ABA degradation.

Auxins

With 16 analytes and one IS (d5-IAA), auxins constitute the largest phytohormone class covered by the targeted method (Figure 27). The method includes IAA, the primary biologically active auxin, as well as the auxins 4Cl-IAA and PAA. Two of the three immediate biosynthetic precursors of IAA are captured through IAC and IAN. However, the pathway through IPY and indole-3-acetaldehyde is only covered by the analyte TPL, which can interconvert with indole-3-acetaldehyde. This was due to the instability of the IPY standard in solution and the unavailability of indole-3-acetaldehyde standards at the time. Reversible IAA conjugates included are IAA-GE, IAA-ME, and five IAA amides with amino acids (IAA-Ala, IAA-Val, IAA-Leu, IAA-Ile, and IAA-Phe). These conjugates are therefore extensively covered. The irreversible conjugate IAA-Asp is also included in the method scope and represents IAA inactivation, along with the primary oxidation product OxIAA, and the reportedly lesser oxidation product ICA.

The most notable analytical effects were the dependency of auxins on column chemistry (see Chapter 4.2.1.1 and 4.2.1.2) and the unexpectedly high LODs and steep response curves of IAA-Asp (see Chapter 4.3.1) and IAA-Glu. The latter metabolite was excluded from the method due to inconsistent detection. Additionally, several auxins exhibited better LODs and LLOQs when measured in negative mode, but this applied only to the quantifier transitions. Consequently, these analytes exhibited a large difference in detection limits for their quantifier and qualifier transitions (see Chapter 4.3.1 or Table A-27). The impact on the identification of these analytes in various plant matrices remains to be determined, as the concentration levels of these compounds have rarely been reported. Despite multiple analytes sharing various fragment ion m/z , all analytes were chromatographically separated well enough to not interfere with each other's detection, except for IAA-Ile and IAA-Leu. These analytes were not baseline separated, so their respective peak heights (rather than their peak areas) were used as the dimension of their signal intensities during method validation.

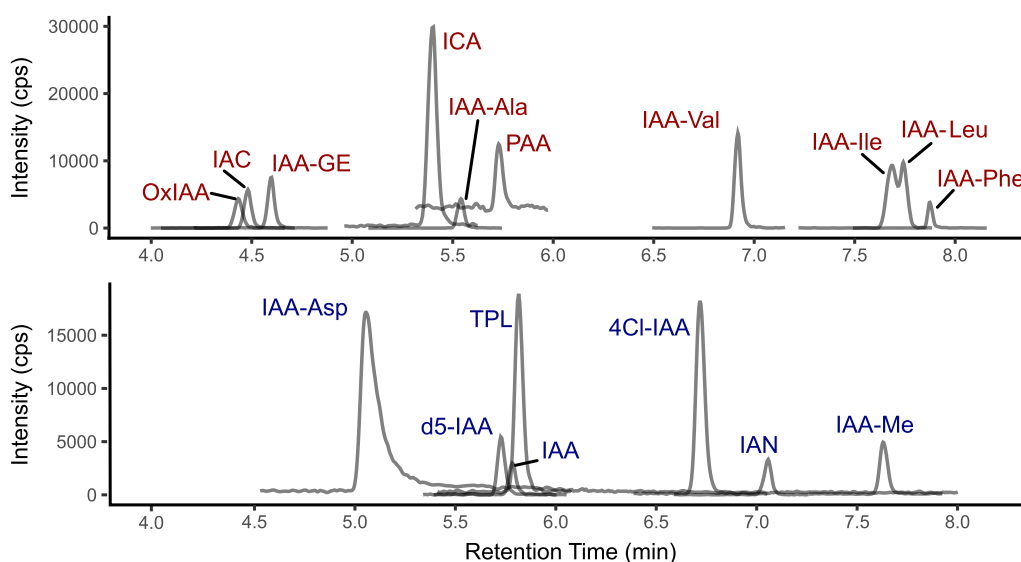


Figure 27: Exemplary chromatographic peaks of auxin analytes at concentrations close to their respective LLOQ. Analytes labeled in red and blue were measured in positive and negative polarity, respectively. 4Cl-IAA, 4-Chloroindole-3-acetic acid; (d5-)IAA, (d5-)Indole-3-acetic acid; IAA-Ala, IAA-alanine; IAA-Asp, IAA-aspartic acid; IAA-GE, Indole-3-acetyl b-D-glucose ester; IAA-Ile, IAA-isoleucine; IAA-Leu, IAA-leucine; IAA-Me, Indole-3-acetate methyl ester; IAA-Phe, IAA-phenylalanine; IAA-Val, IAA-valine; IAC, Indole-3-acetamide; IAN, Indole-3-acetonitrile; ICA, Indole-3-carboxylic acid; OxIAA, 2-Oxindole-3-acetic acid; PAA, Phenylacetic acid; TPL, Tryptophol.

In summary, the established method covers the auxin class well and captures the major aspects of IAA metabolism. If further extensions are attempted in the future, more inactivation products could be incorporated, such as OxIAA-Asp or OxIAA glucose ester, as well as additional reversible conjugates, e.g. IAA-inositol. Furthermore, as part of this work, the minor bioactive auxins IBA and

IAcrA were evaluated and showed acceptable analytical performance. They were removed from the method because they co-eluted with key analytes that were intended to be absolutely quantified. This removal was a preventive measure to ensure a more stable signal for the quantified analytes. However, with more thorough testing and validation, these analytes could be included in the scope of the method again. Lastly, tryptamine could be evaluated for inclusion into the method scope to cover an additional biosynthetic route.

Brassinosteroids

The scope of the method regarding brassinosteroids focuses primarily on the bioactive BL and its immediate precursor, CS (Figure 28). Due to the highly complex biosynthesis and inactivation of brassinosteroids and the limited availability of reference standards for relevant substances, the objective was not to cover the entire BL metabolism. Additionally, brassinosteroid recoveries were the lowest of all analytes in the original method [126]. Consequently, the goal was to evaluate the detection of BL and CS in plant samples before expanding the method to include other brassinosteroids. IHBL was acquired as an IS for BL, since it rarely occurs in plant tissues and differs from BL only by having one methyl group replaced by an ethyl group. This strategy and the analytical performance of BLs are discussed more thoroughly in the validation experiments (see Chapter 4.3.2).

Interestingly, despite their minor structural differences, the analytes BL and IHBL were chromatographically separated. This shows that a chromatographic separation of additional brassinosteroids, which are often closely related structurally, would potentially be possible with this method in the future. However, an exception was observed during the analysis of 24-epi-BL, which was tested during method development and found to coelute completely with BL. Therefore, it was removed from the method. Additionally, the biosynthetic precursor O-CAMP was tested but did not yield any signals in MS detection, so it was not considered further as an analyte.

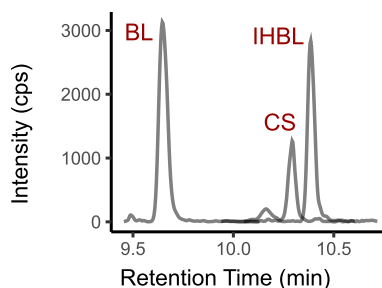


Figure 28: Exemplary chromatographic peaks of brassinosteroid analytes at concentrations close to their respective LLOQ. Analytes were measured in negative polarity. BL, Brassinolide; CS, Castasterone; IHBL, Isohomobrassinolide.

Cytokinins

Of the cytokinin class, twelve analytes and one IS are included in the final method (Figure 29). These analytes cover the most important bioactive compounds, iP and tZ, as well as the related, less bioactive compounds cZ and DHZ. They also include the minor bioactive compounds oT and mT. KIN and KIN-R are additionally included as analytes, even though they were originally intended for use as IS. However, this strategy was later discarded due to their endogenous presence in tomato samples, as described in Chapter 4.3.3. Consequently, d6-iP is the only isotope-labeled standard for the cytokinins in the final method.

Furthermore, the method also covers the tZ and iP ribosides (tZ-R and iP-R), but none of the cytokinin nucleotides because the respective standard substances could not be obtained and were presumed to be unstable in solution. The glucoside conjugates iP-9G and tZ-7G are included, but no others, again due to the limited availability of affordable reference standards. MPO, a product of irreversible iP cleavage, was attempted to be included in the method, but was not detectable during MS optimization. In summary, while the method covers a large number of cytokinins, it does not represent the complete biosynthesis and inactivation mechanisms of this hormone class. Future expansions of the method should aim to include additional conjugates, such as those of cZ and DHZ, as well as more isotope-labeled IS.

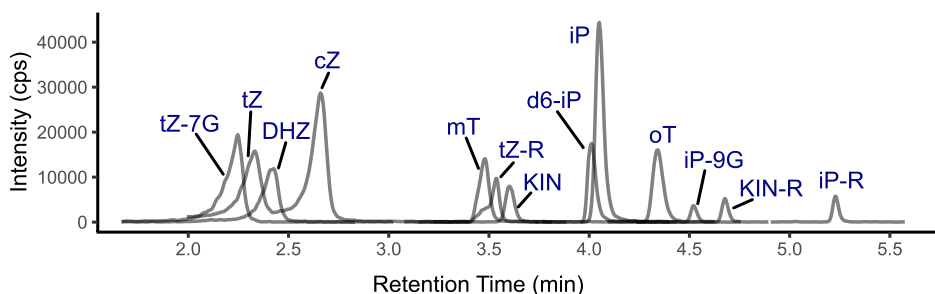


Figure 29: Exemplary chromatographic peaks of cytokinin analytes at concentrations close to their respective LLOQ. Analytes were measured in positive polarity. cZ, *cis*-Zeatin; DHZ, Dehydrozeatin; iP, *N*6-Isopentenyladenine; iP-9G, iP-*N*9-glucoside; iP-R, Isopentenyladenosine (iP riboside); KIN, Kinetin; KIN-R, KIN riboside; mT, *meta*-Topolin; oT, *ortho*-Topolin; tZ, *trans*-Zeatin; tZ-7G, tZ-*N*7-glucoside; tZ-R, tZ riboside.

A discrepancy in peak shapes was observed between the early-eluting and late-eluting cytokinins. As discussed in Chapter 4.2.1.3, this was likely caused by the initial gradient conditions, which could not be improved without compromising the baseline separation of isomers, such as tZ and cZ. This represents a typical trade-off between chromatographic peak shape and the chromatographic resolution required for the separation of structurally similar cytokinin isomers. cZ and tZ performed best with the same precursor-to-fragment *m/z* and MS parameters and were

therefore analyzed with the same MRM transitions, requiring chromatographic separation for reliable differentiation. The peak shapes of these analytes likely affected their LODs and LLOQs, which is discussed in Chapter 4.3.1.

Gibberellins

The final method scope encompasses nine gibberellins and two IS (Figure 30). The bioactive gibberellins GA1 and GA4 are included alongside their IS (d4-GA1 and d2-GA4), as well as GA3 and GA7, which are bioactive in plants but are more commonly associated with fungal biosynthesis. Also included are GA12-Ald, the common precursor to all gibberellins, and GA9 and GA20, the immediate precursors to GA1 and GA4. Similarly, GA8 and GA34 are the major oxidative inactivation products of these primary gibberellins.

The immediate metabolism around GA1 and GA4 is therefore well covered. However, if it is intended to widen the scope of the method, more biosynthetic precursors, and particularly more inactivation products, should be incorporated. Two such metabolites were excluded from the final method due to poor analytical performance. The irreversible conjugate GA4-ME exhibited a very high LOD, while eKA, an early gibberellin precursor, did not produce suitable fragment ions for MRM detection ($m/z > 40$ required).

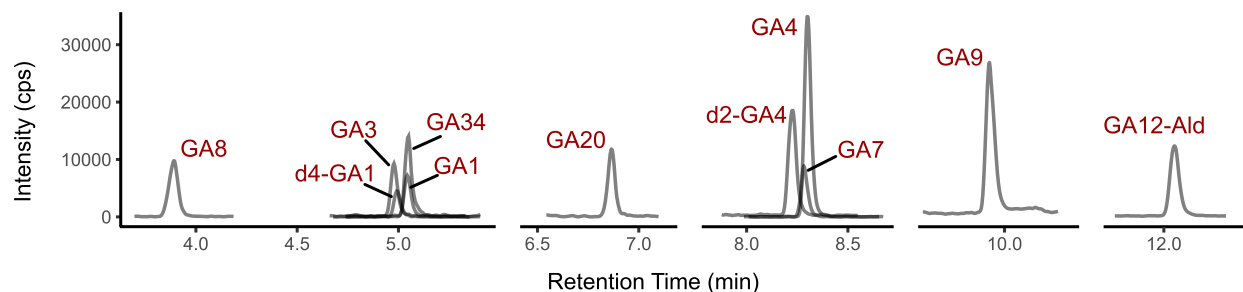


Figure 30: Exemplary chromatographic peaks of gibberellin analytes at concentrations close to their respective LLOQ. Analytes were measured in negative polarity. Gibberellins are referred to by their identifying numbers and use no other names. GA12-Ald, Gibberellin A12 aldehyde.

All gibberellin isomers were fully separated, and the analytes spread over the entire gradient. Their detection limits were favorable as well, which is discussed in Chapter 4.3.1. Thus, it was concluded that the method optimally separates gibberellins and that more such analytes could likely be incorporated into its scope.

Jasmonates

Seven jasmonate metabolites, one IS, and one putative IS were incorporated into the final method (Figure 31). The biologically active conjugate JA-Ile and its central precursor JA were included, as

well as the isotope-labeled d5-JA. Along with the key JA precursor OPDA, which also exhibits biological activity, the analyte DHJA was integrated into the method as a putative OPDA IS. While DHJA has been described as an IS in OPDA quantification [242] and JA quantification [243], little information was found on its natural occurrence. Thus, DHJA was included in the method to evaluate its occurrence in plant materials before applying it as an IS. The inactivation of jasmonates is covered by the reversible conjugate MeJA, as well as the oxidative inactivation products JSMO, 12OH-JA, and 12OH-JA-G.

Overall, jasmonate metabolism is adequately represented for a comprehensive assessment. Standards of other jasmonate metabolites were not commercially available in many cases at the time of development, but this status should be reevaluated regularly. Several JA conjugates are known and could be integrated into the method at a later time. Recently, OPDA conjugates have also been shown to be highly relevant for jasmonate signaling (see [106]) and may therefore deserve attention once the respective standards are more widely available.

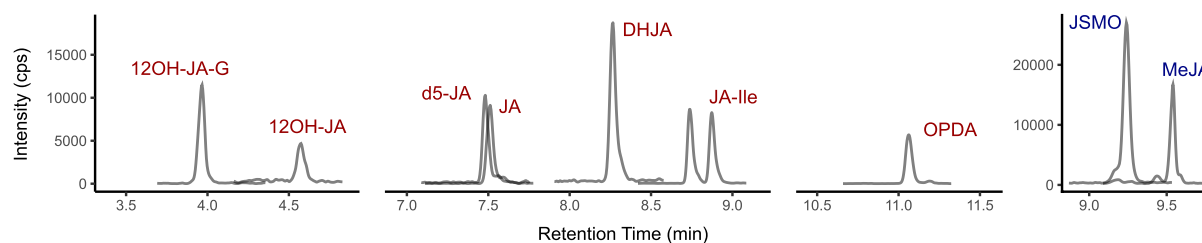


Figure 31: Exemplary chromatographic peaks of jasmonate analytes at concentrations close to their respective LLOQ. Analytes labeled in red and blue were measured in positive and negative polarity, respectively. 12OH-JA(-G), 12-OH-JA (glucoside); DHJA, Dehydrojasmonic acid; JA, Jasmonic acid; JA-Ile, JA-isoleucine; JSMO, *cis*-Jasmone; MeJA, Methyljasmonate (JA methyl ester); OPDA, 12-Oxophytodienoic acid.

Difficulties in jasmonate analysis were mainly caused by the analytes' stereochemistry. JA has two stereocenters, at C3 and C7 (see Chapter 1.4.2.6), leading to four possible stereoisomers, not including double bond isomers. Standard nomenclature is rarely used, and the enantiomers (3R, 7R)-JA and (7R, 7S)-JA are usually referred to as (+)-JA and (-)-JA, respectively. The naturally occurring form is (-)-JA, along with its bioactive C7 epimer, which is referred to as (+)-7-*iso*-JA or (+)-*epi*-JA [104, 244, 245]. All jasmonate standards obtained are (±)-JA derivatives. These enantiomeric forms cannot be separated using non-chiral column materials and therefore yield only one peak per analyte. However, this means that the 7-*iso*-forms are not individually covered, although they likely yield separate chromatographic peaks (see the tomato analysis, Chapter 4.4.3.1). Furthermore, the JA-Ile standard separates into two peaks of approximately equal intensity, with only the latter corresponding to its naturally occurring form. This likely results

from non-stereoselective isoleucine addition during synthesis. Acquiring pure standards containing only one stereoisomer would further improve jasmonate detection with this method, especially with regard to absolute quantification of JA-Ile. Despite these difficulties, the method successfully achieved its purpose of chromatographically separating and selectively detecting jasmonates.

Salicylates

The final method's salicylate scope comprises SA, its IS d4-SA, and six additional analytes (Figure 32). These were the biosynthetic precursors tCA and oCA, the inactivated 2,3-DHBA, SA-G, and SA-GE, and the metabolically distinct signaling molecule NHPA. While these analytes sufficiently capture important aspects of SA inactivation and catabolism, they do not cover the major SA biosynthesis pathway through CHO and isochorismic acid. CHO was evaluated during the initial development of the method but exhibited chemical instability. Additionally, no standard was commercially available for isochorismic acid. Both compounds are known to be highly reactive and unstable in solution [246, 247], which makes their incorporation into the method scope unlikely. The PAL pathway precursors included in this method cover the alternative SA biosynthesis route, but are not specific to SA metabolism. Nevertheless, due to the high endogenous concentrations of SA and the method's ability to cover its inactivation, it was determined that the method was suited for assessing whether SA response systems were triggered.

Several more metabolites were disregarded as potential analytes during the testing and optimization phases. One of these was 2,5-DHBA, which was removed from the method due to irregular detection and a very high LOD. However, since 2,5-DHBA is one of the two primary SA inactivation products, its performance could be systematically reevaluated in the future to potentially reincorporate the compound into the method. Furthermore, BA and MeSA were deemed unsuitable for the method due to interfering signals and a lack of analyte signals in ESI-MS detection, respectively.

Additionally, salicylate analysis presented further challenges for a variety of reasons. Several metabolites have wide peak shapes, likely due to their aromatic carboxyl group interacting with the positively charged column surface. DHBA and SA were affected the most. SA was also detected in almost all solvent samples and, in even higher intensity, in the blank samples that underwent sample preparation. Although these solvent/blank peaks were small, and subtracting them from sample signals was effective, the presence of these peaks in all tested SA transitions clearly indicated that the consumable materials used resulted in minor contaminations. SA

detection was improved by using its third most intense transition as the quantifier and its second most intense transition as the qualifier. These transitions showed lower baselines compared to the most intense transition, improving S/N ratios and leading to more reproducible measurements. Because SA was detected at high intensities in plant samples, this non-conventional transition selection had the additional effect that the detector saturation was only reached at higher concentrations, which effectively widened the dynamic range.

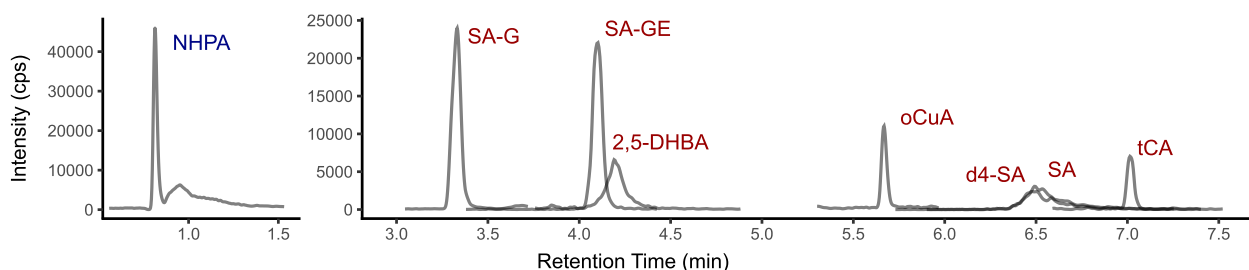


Figure 32: Exemplary chromatographic peaks of salicylate analytes at concentrations close to their respective LLOQ. Analytes labeled in red and blue were measured in positive and negative polarity, respectively. 2,3-DHBA; 2,3-Dihydroxybenzoic acid; NHPA, *N*-Hydroxypipecolic acid; oCuA, *ortho*-Coumaric acid; SA, Salicylic acid; SA-G/-GE, SA glucoside / glucose ester; tCA, *trans*-Cinnamic acid.

NHPA, a small and highly polar compound, is only minimally retained by the column and elutes as the first analyte of the method. Since many other polar compounds elute at the same time, NHPA detection was limited by an unstable baseline, even in matrix-free samples. Additionally, the analyte exhibited a very steep response slope, resulting in a factor of approximately 25-50 between its LLOQ and detector saturation (see Chapter 4.3.2.1). NHPA's immediate precursor, pipecolic acid, which is less polar and should elute later, could potentially be incorporated into the method to ensure NHPA signaling coverage. This metabolite was detected in the potato and tomato metabolome analysis (see Chapters 4.1.3 and 4.5.2), and should therefore be present at detectable levels with this method as well.

Strigolactones

One objective of adapting the method published by Šimura et al. [126] was to incorporate strigolactones, which were not included in the original method. At the time of early development, few strigolactone standards were commercially available. Thus, the final method includes only three bioactive strigolactones (ORO, STR, and D-STR), the strigolactone catabolite HMB, and the synthetic GR24, which is used as an IS (Figure 33). However, these strigolactones are commonly reported to be among the most frequently occurring strigolactones, and HMB is a common catabolite of all strigolactones. Therefore, the method is well suited for assessing its applicability in strigolactone detection in various plant matrices.

It should be noted that strigolactones have often been described as unstable compounds [248, 249]. Although analyte instability was not observed in the stability experiments, the effect of the various stages of sample processing and preparation on the compounds has not been systematically investigated. Their hydrolysis, which could result in the release of HMB, should be studied further.

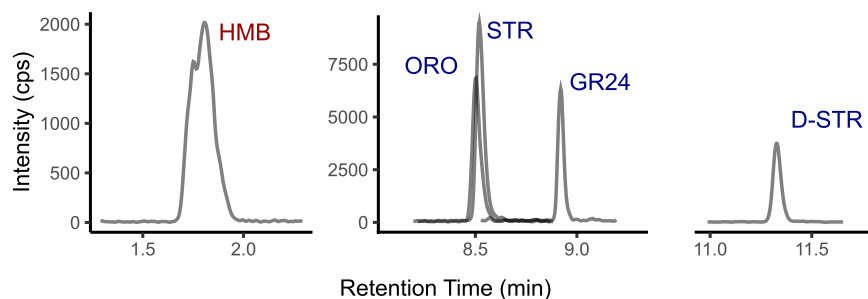


Figure 33: Exemplary chromatographic peaks of strigolactone analytes at concentrations close to their respective LLOQ. Analytes labeled in red and blue were measured in positive and negative polarity, respectively. D-STR, (±)-5-Deoxystrigol; GR-24, (±)-Strigolactone GR24; HMB, 5-Hydroxy-3-methylbutenolide; ORO, (±)-Orobanchol; STR, (±)-Strigol.

Another challenging aspect of strigolactone analysis is their stereochemistry [249, 250]. All three strigolactones were analyzed as racemic mixtures, but since the enantiomers were not chromatographically separated, this did not affect their analysis. However, many more strigolactone compounds are isomers or stereoisomers of each other. For instance, ORO and STR are isomers that differ in their stereochemistry, as well as the position of one hydroxyl group. Although they were not chromatographically separated, MRM transitions were chosen so that they could be selectively detected, even when both analytes are present. Nevertheless, this strategy was only possible because both reference standards were measured together. In contrast, the standard substance for deoxy-ORO was not obtained, so a coelution with D-STR cannot be ruled out. Therefore, these findings demonstrate that detected strigolactones must be thoroughly evaluated to prevent misannotations.

Ethylene Metabolites

In addition to strigolactones, another class of metabolites not covered by the original method [126] was ethylene metabolites. While ethylene itself cannot be analyzed via LC-based methods due to its high volatility, most metabolites involved in ethylene biosynthesis are non-volatile. Therefore, it was aimed to include key ethylene metabolites in the adapted method. However, this attempt was unsuccessful due to the analytical performance of these analytes.

Four ethylene-related standards were acquired and tested: the immediate ethylene precursor ACC, as well as three other metabolites related to ethylene conversion (CN-Ala, MTA, SAM). ACC analysis was hindered by coeluting substances, as was the case for its IS d4-ACC, and also for MTA. The cause for ACC was assumed to be its non-specific formula ($C_4H_7NO_2$) and non-specific fragmentation (decarboxylation and/or deamination), coupled with its low polarity, which resulted in an early RT. Since the analyte eluted with all substances that were not retained by the column material and could not be differentiated by MS detection, interfering isomers were likely present. Furthermore, no substance peaks of the analytes CN-Ala and SAM were detected during UHPLC-MS measurements, despite the absence of detection issues during ESI-MS optimization. Since all candidate transitions were reviewed for these four analytes, including potential adducts as precursor ions, they were removed from the scope of the method.

Consequently, the established phytohormone analysis method does not capture ethylene signaling, which must therefore be assessed indirectly through its crosstalk with other signaling pathways. Alternatively, aspects of ethylene metabolism may be captured through other methods, such as metabolomics, which is addressed in the analysis of tomato plants (see Chapter 4.5.2).

Conclusions

In summary, the finalized method provides targeted coverage of the signaling pathways of eight phytohormone classes. Although the original method [126] included more metabolites (101), it used only one MRM transition per analyte. By establishing two MRM transitions for each of the 63 analytes in this work, the confidence in analyte identification was substantially increased. Almost 20 additional analytes were considered and tested, but ultimately excluded due to analytical constraints, such as standard instability or poor ESI-MS responses (e.g. ethylene precursors and specific brassinosteroids). Nevertheless, the current scope allows for a comprehensive and reliable assessment of phytohormone signaling in plant tissues. Furthermore, identifying potential target analytes for future inclusion laid the groundwork for further method expansions.

4.2.2.3 Data Analysis Pipeline

To streamline data analysis and ensure its reproducibility, a data analysis pipeline was built in R, composed of a series of scripts that automatically fulfill various parts of the analysis process. The pipeline was designed to be modular and easily applicable to future projects. Therefore, it does not use project-specific variables, and all parts of the code structure are extensively commented.

As input, the pipeline requires tables of integrated peaks, as well as several files supplying supplemental information on the samples and analytes. The code uses a master script that serves as the central interface for user-required inputs, and then executes a total of 11 other scripts.

Table 7 presents summaries of all scripts that were created. This chapter will highlight key aspects of the workflow; details are provided in Chapter 3.2.5.

Table 7: Summaries of individual scripts employed in the R data analysis pipeline of the targeted phytohormone method. PCA, Principal component analysis, ANOVA, Analysis of variance, R^2 , Coefficient of determination; RMSE, root mean square error.

Script	Functions	Export (filetype)
1: Masterscript	Loads (and installs, if necessary) project packages. Executes all other scripts Collects most user inputs	
1b: Data structure	Creates folder structure for file export, if not yet existing	
1c: Data import	Imports peak lists and additional files (sample weights, transition info) Creates one data table per matrix Creates table with signal intensities in control samples for signal correction	
2a: Quantification (calibration)	Creates calibration curves for quantified analytes Calculates quality parameters (Residuals, back-calculated concentrations, RMSE, R^2)	Calibration curve graphs (pdf) ¹ Calibration curve data and quality parameters (xlsx) ¹
2b: Quantification (results)	Calculates absolute concentrations of quantified analytes in samples	Absolute concentration tables (xlsx) ¹
3a: All analytes (table generation)	Performs signal corrections for all transitions Applies filters to create lists of reproducibly detected analytes Creates summary tables on all transitions, all detected analytes and the internal standards	Summary tables (xlsx) Summary tables on internal standards, including matrix effects and recoveries (xlsx)
3b: Detected analytes (heatmap generation)	Creates heatmaps of analyte signal intensities per subgroup, using the QC as reference group	Heatmaps on analyte signal intensities (svg, pdf) ¹
3c: Detected analytes (statistics)	Runs statistical tests on analyte signal intensities per subgroup (one- or two-factor ANOVA, Tukey tests)	
4: Detected analytes (identification)	Compares quantifier/qualifier ratios to data of reference standard measurements Allocates identification flags to each analyte ("successfully identified", "not identifiable", "manual check required")	Table with identification information and results (xlsx)
5a: Detected analytes (graphical summaries)	Creates a graphical summary for each analyte, containing signal intensity bar plots, plotted intensities throughout measurement series, statistical results, identification flags	Graphical summary per analyte (pdf)
5b: Internal standards (graphical summaries)	Creates a graphical summary equal to Script 5a, but for IS only	Graphical summary per internal standard (pdf)
6: PCA	Performs a PCA on all samples (using the detected analyte data), including preliminary steps of missing value imputation and data transformation.	PCA 2D score and loading plots (pdf, svg) ¹

¹ Separate files are created for each matrix.

Two crucial parts of the workflow are the absolute quantification of specified analytes and the selection and evaluation of reproducibly⁴ detected analytes. For the absolute quantification of analytes, a workflow was devised that facilitates a fast, comprehensive, and easily adaptable creation of calibration curves. This aspect is discussed in the validation of the quantified analytes (Chapter 4.3.3).

Extensive data tables are generated for all analytes, but subsequent data analysis focuses on analytes that were reproducibly detected in the study samples (present in detected in >66% of samples in at least one subgroup). The quantifier signal intensities of these analytes, referred to as "detected analytes", are visualized in heat maps, bar charts by subgroup, and plots showing their intensities by injection order. The latter serves to highlight QC sample intensities throughout a measurement series, thus providing a monitoring measure. Statistical tests are then performed on the detected analytes, and they are taken through an identification process that allocates identification flags to all analytes. For the identification, observed quantifier-to-qualifier ratios are compared to a "common range" derived from prior measurements of reference standards.

To make data evaluation most accessible, all of these pieces of information (except the heatmaps) are exported together in a pdf report. Each page of the report shows these elements for one analyte. Two example pages are displayed in Figure 34 and demonstrate the report layout for one analyte detected in all samples (ABA-GE) and one analyte detected in only some samples (mT).

Beyond standard processing, the pipeline includes specialized functions to address common challenges in LC-MS/MS data analysis. For instance, the user can globally interchange quantifier and qualifier transitions. This considers matrix-specific interferences and ensures that the analytically better-performing transition is treated as such. Furthermore, analytes can be user-specified to be evaluated by peak height instead of peak area for signal intensity calculations. This is generally used for coeluting analytes (primarily IAA-Ile and IAA-Leu, see Chapter 4.2.2.2), but it can be applied for all analytes when coelutions occur in an analyzed matrix.

In summary, the custom R-based pipeline not only accelerates extensive data analysis, but also greatly improves its reproducibility by eliminating human error in the handling of large datasets. Thus, it transforms a traditionally labor-intensive manual bottleneck into a streamlined, high-throughput process. However, while it was developed with universal applicability in mind, the data analysis pipeline has only been thoroughly tested in the analysis of tomato plants (see Chapter 4.4.3), and future applications will likely reveal the need for further refinements.

⁴ It should be noted that the term „reproducibly“ refers to detection within a measurement series, and therefore differs slightly from its meaning in the context of method validation (see Chapter 4.3).

4 - Results and Discussion

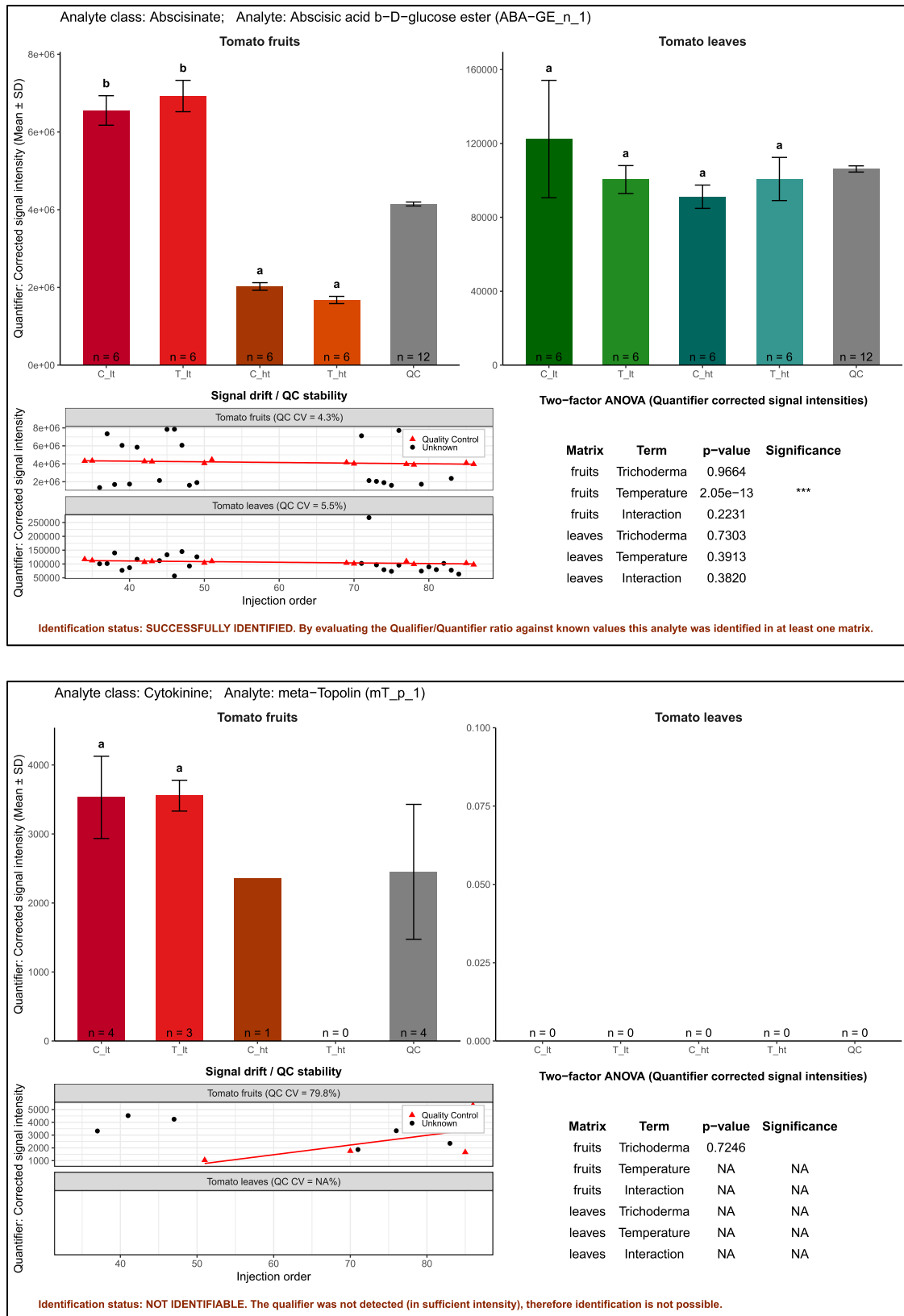


Figure 34: Two exemplary pages of the graphical summary pdf report created automatically by the R data analysis pipeline. Abbreviations are not provided. Original scale of one page is DIN A4.

4.3 Validation Results

The final UHPLC-MS/MS method was validated to ensure its reliability and suitability for the relative and absolute quantification of phytohormones and their metabolites. Analytes that were relatively quantified, i.e. analytes whose signal intensities are intended for intergroup comparisons, are hereafter referred to as semi-quantified analytes.

The SANTE 11312/2021 guidelines for the validation of quantification methods in pesticide residue analyses [174] were adopted to evaluate the method's performance. These guidelines were chosen as the validation framework in this work due to their focus on analytes of low abundance in food and feed. However, these guidelines were originally developed for analytes that have legal reporting limits, to which they often refer. Thus, the validation benchmarks were used as references rather than as strict thresholds. Other validation guidelines, such as the ICH Q2(R2) guidelines [175], were consulted for aspects not extensively covered by the SANTE 11312/2021 guidelines. Eight phytohormones were absolutely quantified; each represents the most important metabolite of its respective class. The targets set by the SANTE 11312/2021 guidelines were applied more rigorously for these analytes, but they were also used as references in the validation of the semi-quantified analytes. This strategy ensured a harmonized assessment of the method's reliability, regardless of the quantification approach used.

The determination of instrument LODs and LLOQs is presented in Chapter 4.3.1. Then, the validation of semi-quantified analytes is described in Chapter 4.3.2. The quantified analytes were validated using a two-phase approach. First, the method performance was assessed using neat reference standards to verify fundamental parameters and the efficiency of sample preparation (Chapter 4.3.3). Second, matrix-specific validation was conducted for tomato leaf and fruit samples (Chapter 4.3.4). This approach is intended to serve as a baseline for future applications. It acknowledges that, while core analytical parameters remain largely consistent, performance must be revalidated for each unique plant species or tissue type to account for varying chemical backgrounds and matrix-induced effects.

4.3.1 Instrumental Detection and Quantification Limits

To establish the functional sensitivity of the finalized method, the LODs and LLOQs of all analytes were determined. For internal consistency, the term LLOQ is applied to both absolutely quantified

and semi-quantified analytes. Since the SANTE 11312/2021 guidelines [174] provide only a broad definition for LLOQ⁵ and none for LOD, other guidelines were consulted for this step.

The ICH Q2(R2) guidelines [175] are aligned with common LC-MS analysis practices [176], and provide an LOD/LLOQ calculation approach based on the S/N ratio between analyte signals and blank (baseline) signals. Following this approach defines the LOD and LLOQ as ratios of 3:1 and 10:1, respectively. Despite concerns about the reproducibility of S/N-based LOD and LLOQ calculations (summarized in [251]), this approach was adopted here because it allowed for a direct, benchmarked comparison with the foundational method [126]. S/N ratios were calculated as peak-to-peak height ratios, which presents a more conservative calculation than using the standard deviation of the noise as reference. Chapter 3.3.2.1 describes the detailed procedures for semi-quantified analytes, and Chapter 3.3.3.1 for the quantified analytes.

The determined LODs and LLOQs are listed in Table A-27 in the Appendix in the preferred dimension of analyte amount per sample, which considers the concentration factor resulting from the reconstitution solvent volume used. To enable comparison with the original method (Figure 35), they were recalculated into instrumental limits (analyte amount per injection). However, interpreting the observed differences between methods was hindered because the original method did not state how S/N ratios were determined. Of the 37 analytes common to both methods, 12 showed comparable LLOQs (LLOQ ratio 0.5 – 2.0) and 8 showed improved LLOQs in this work. LLOQs were lower for OxIAA, two jasmonic acid metabolites (12OH-JA, OPDA) and all five gibberellins (GA1, GA3, GA4, GA8, and GA34). Interestingly, all of these analytes were measured in negative ESI polarity, while most of the analytes with higher LLOQs were measured in positive polarity, with the exception of the brassinosteroids. This finding suggested a higher relative ionization efficiency for this mode on the current instrument platform.

Conversely, several analytes, particularly in the auxin, cytokinin and brassinosteroid classes, exhibited higher LLOQs than previously reported. While the specific sources of these discrepancies remain elusive, they were likely attributable to different ESI source properties and to differences in analyte ionization and particularly desolvation. For the cytokinins, suboptimal peak shapes likely were a contributing factor, since higher LLOQs were only observed for early-eluting analytes (see Chapter 4.2.1.3). The most notable discrepancy was IAA-Asp, whose LLOQ (4,000 fmol) was much higher than reported by Šimura et al. (1 fmol), despite using the same quantifier transition [126]. This analyte displayed an unexpected and anomalous analytical

⁵ SANTE guidelines do not use the term LLOQ but rather the more general LOQ (limit of quantification), which they define as the lowest spike level meeting identification, recovery and precision criteria. Furthermore, LOQ is used synonymously with limit of determination, confusingly abbreviated to LOD.

response, exhibiting a very high slope that resulted in its signal intensity reaching detector saturation at approximately 25- to 50-fold its LLOQ (see next chapter). The reasons for these deviations from the validation results reported by Šimura et al. could not be determined.

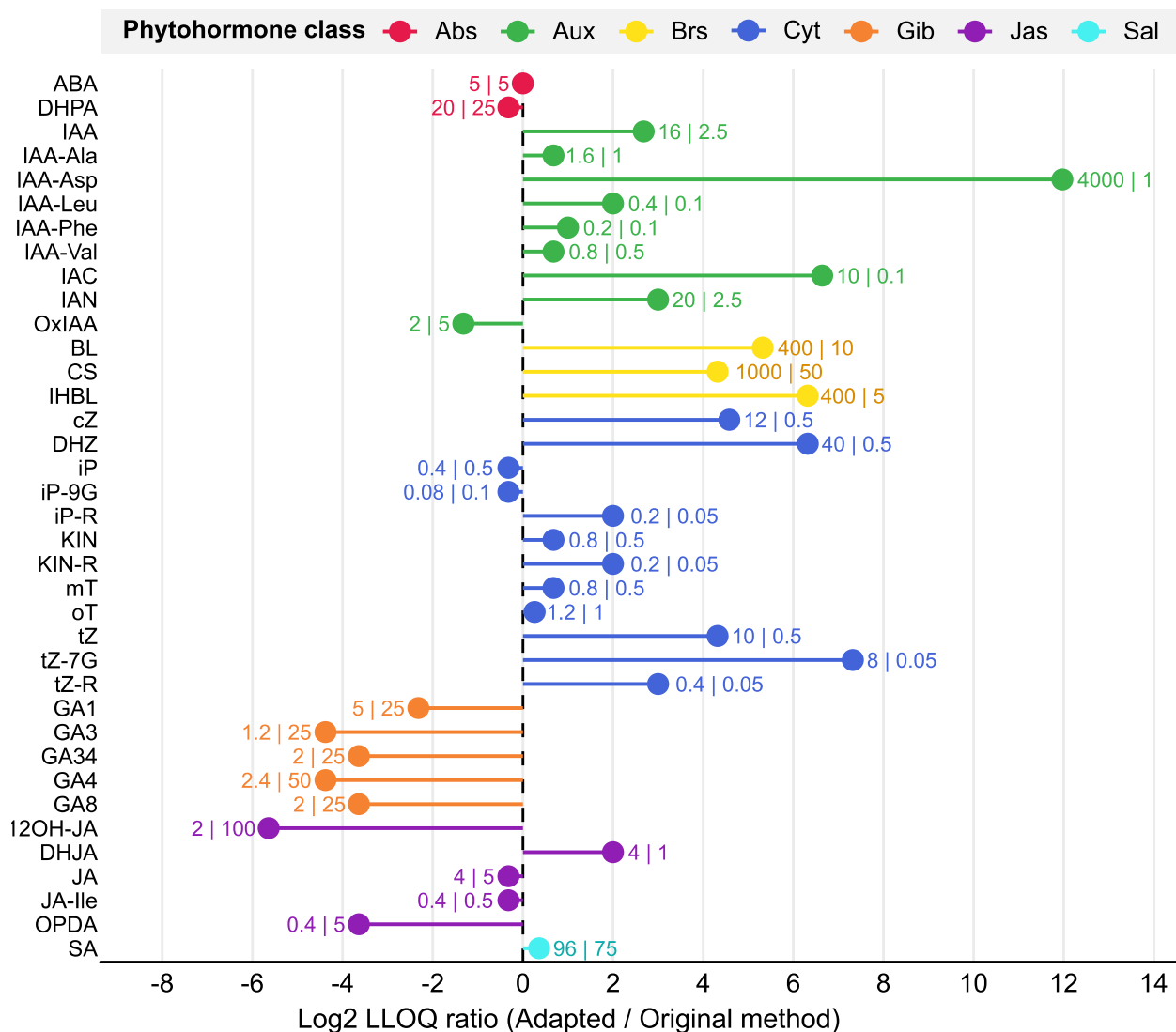


Figure 35: Comparative visualization of LLOQs determined with the adapted method and as published with the original method [126]. Logarithmic ($\log_2$) LLOQ values are used for scaling and actual values are provided next to each analyte point (fmol / injection). Analyte abbreviations are provided in Table A-17. LLOQ, Lower limit of quantification. Abs, Absciscates; Aux, Auxins; Brs, Brassinosteroids; Cyt, Cytokinins; Gib, Gibberellins; Jas, Jasmonates; Sal, Salicylates.

Ultimately, these instrumental limits provided a baseline for the method's application potential. Furthermore, it is important to note that sensitivity is not a static parameter. Professional cleaning of the MS quadrupoles performed shortly before the validation of quantified analytes (see Chapter 3.3.3.1) resulted in a 3- to 10-fold reduction in LLOQs for the quantified analytes. Because the

semi-quantified analytes were not reassessed following this maintenance, their listed LLOQs likely represent a conservative estimate. This underscores that instrumental detection can change over time and necessitates regular reassessment to maintain data integrity. Whether the detection limits determined here are sufficient for the analysis of phytohormones at endogenous levels will be addressed with the method's applications in Chapter 4.4.

4.3.2 Semi-quantified Analytes

The established phytohormone analysis method covered 55 analytes for which no IS were acquired. Although absolute quantification was not attempted for these metabolites, their analytical performance was validated to establish a baseline for relative quantification. The goal was to ensure that intergroup comparisons remained robust and reproducible, even in the absence of IS correction. All validation steps for the semi-quantified analytes aimed to evaluate method and instrument performance for all applications and were thus performed in the absence of matrix. In addition to analyte stability (see Chapter 4.2.1.1) and instrument sensitivity (see the previous chapter), the validated parameters included the instrument's linearity and precision across the dynamic range (Chapter 4.3.2.1), as well as the method's intraday and interday precision and recoveries (Chapter 4.3.2.2).

4.3.2.1 Instrument Precision and Linearity

Based on the determined LLOQs, analyte mixtures were created and used to determine the precision and linearity of signals across a wide dynamic range. To assess each transition individually, mixtures were prepared for both quantifier and qualifier transitions. These experiments included the quantified analytes and IS, but the focus of the interpretation was on the semi-quantified analytes, since the calibration curves for the quantified analytes were assessed more thoroughly in a separate experiment (see Chapter 4.3.3.1).

Measurements were performed in eight replicates per concentration level. The concentration levels included the LLOQ concentration, as well as 5-fold, 10-fold, 25-fold, 50-fold, 100-fold, 500-fold and 1,000-fold LLOQ concentrations. Some analytes' highest two concentration levels were not analyzed to avoid detector saturation (e.g. IAA-Asp, NHPA) or because the concentrations would have been unrealistically high (e.g. brassinosteroids). The experimental procedure is detailed in Chapter 3.3.2.2.

Precision

Precision across each analyte's dynamic range was assessed by evaluating the signal intensities and CVs at each level. All CVs are provided in Table A-27 in the Appendix and are summarized visually in Figure 36. At the lowest level (LLOQ), only one transition exhibited a CV greater than

20% (PAA qualifier transition). Starting at the next-lowest level (5-fold LLOQ), all CVs were below 15% and then remained consistently below 10% at 10-fold LLOQ and higher concentrations. Although no consulted guidelines provide specific benchmarks for instrument precision, these results were considered to satisfy the method's requirements.

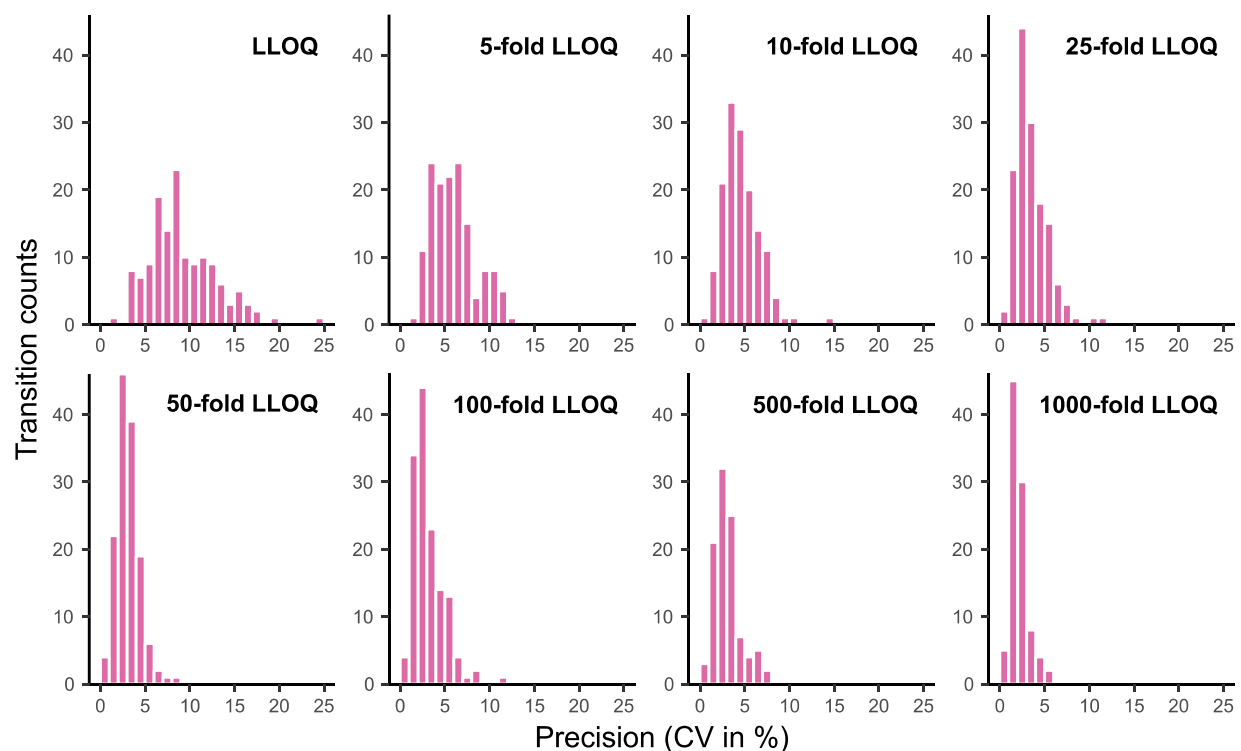


Figure 36: Analyte precision at different concentration levels relative to each analyte's LLOQ. Mixtures were measured in eight replicates per concentration level. CV, Coefficient of variation; LLOQ, Lower limit of quantification.

Linearity

Linearity of analyte responses was assessed by fitting a linear regression to each transition's concentration levels and signal intensities (weighting factor $1/x$). The resulting curves were visually evaluated, and the regression parameters were examined, including the residuals, the RMSE and the R^2 of each curve. Each transition's R^2 value is included in Table A-27 in the Appendix, while the R^2 value distribution of the quantifier transitions is shown in Figure 37. Regression analysis of 63 quantifier transitions yielded an R^2 value > 0.98 , and 52 of those transitions had an R^2 value > 0.99 . The nine analytes with lower values are labeled in Figure 37 and are briefly discussed here. The analytes NHPA and IAA-Asp exhibited very steep slopes in their response curves. Both reached the detector saturation range in less than 50-fold of their LLOQ concentration, resulting in only four data points per curve. Hence, it was concluded that the poor linearity parameters were likely rooted in the high sensitivity and the low number of data points.

The analytes IAN and DHZ exhibited a putative outlier at the 25-fold LLOQ concentration level. Since this pattern was observed for multiple analytes measured in the same batch, it was assumed to be caused by a technical error rather than by underlying chemistry. The same conclusion was drawn for HMB and GA12-Ald, which showed an outlier at the 50-fold LLOQ concentration level.

Consistent nonlinear response curves were only observed for the three brassinosteroids included in the method (BL, CS, and IHBL), which exhibited flattened response curves. As an example, Figure 37 shows the (non-fitted) response curve of the CS quantifier transition. Curiously, several LC-MS studies have reported good linearity for these compounds [82, 126, 252-254], but the injection amounts in this work (0.4 – 40 pmol) were considerably higher than those of the cited studies (see previous chapter). This suggests that the observed nonlinearity was likely a result of ESI source saturation or concentration-dependent ion suppression at these higher levels. Consequently, achieving linear responses over a wide detection range would necessarily require lower detection limits than those obtained in this work. Although the original intention was to absolutely quantify BL (using the naturally much less abundant IHBL as an IS), this approach was abandoned due to these results. While various strategies may be employed for non-linear calibration and quantification [255, 256], it was decided that brassinosteroid detection with the established method needed to be ensured before devising such complex strategies.

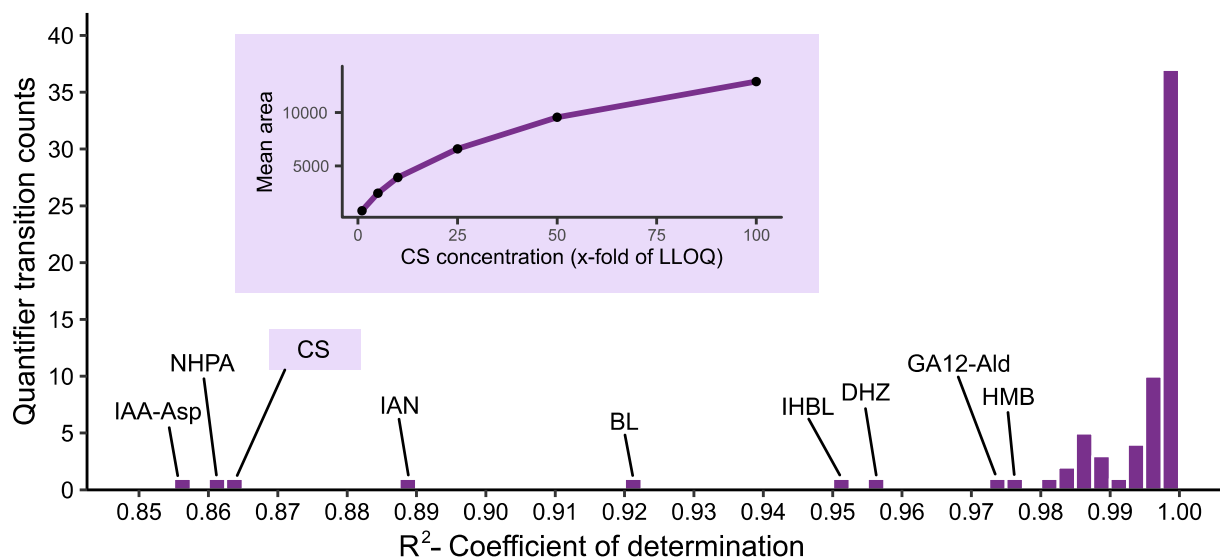


Figure 37: R^2 distribution of the linear regression (weight $1/x$) performed for each analyte quantifier transition over its measured concentration range. Analytes with $R^2 < 0.98$ are labeled with their analyte IDs (see Table A-17), and the response of CS is provided (purple background). CS, Castasterone. Further analyte abbreviations are provided in Table A-17.

Ultimately, the linearity parameters of semi-quantified analytes were evaluated relative to the requirements of comparative analysis, rather than the rigid criteria used for absolute quantification.

While specific analytes displayed sub-optimal linearity or narrow dynamic ranges, they were not excluded from the method to preserve its broad metabolite scope. These documented response profiles were regarded as a critical resource for data interpretation, as they provided the analytical context in which relative biological changes were assessed. Furthermore, these characterizations will serve as a useful resource for future method refinements, particularly for an extension of absolute quantification. For current purposes, the instrumental response was deemed sufficiently robust to support the intended relative quantification goals for all semi-quantified analytes.

4.3.2.2 Method Precision and Absolute Recoveries

In addition to instrument precision, method precision was also determined for all semi-quantified analytes. To characterize the method's performance at the trace levels typical of most phytohormones, intraday and interday precision were evaluated by preparing analytes at 2.5-fold LLOQ concentrations on three separate days. The full experimental procedures are described in Chapter 3.3.2.3. In addition to precision, absolute analyte recoveries were evaluated to compare the final sample preparation workflow with that from an earlier method development stage. As in Chapter 4.2.1.4, absolute recovery refers to the signal intensity ratio between prepared and non-prepared samples, which follows the nomenclature of the SANTE 11312/2021 guidelines [174]. The SANTE guidelines also provide targets for method precision (repeatability $\leq 20\%^6$) and recoveries (70 - 120%), which were used here as a reference and not as target benchmarks, since these limits are intended for determinations in matrix.

Five replicates were analyzed per experiment day and assessed per day (intraday) and collectively (interday). Intra- and interday CVs and absolute recoveries of all semi-quantified analytes are listed in the Appendix (Table A-29). To illustrate differences between preparation days, the respective distributions of the quantifier transitions are shown in Figure 38. Aside from a few exceptions, all intraday CVs were under 20%. Analytes with intraday CVs above 20% were all detected close to their LODs due to analyte losses during preparation. This resulted in higher CVs, especially for analytes that were not detected in all samples (e.g. NHPA and JSMO). Likely for the same reason, the analyte GA12-Ald was not detected at all in the prepared samples. As expected, interday precision was worse than intraday precision. However, 37 out of 55 analyte quantifiers exhibited an interday CV $\leq 20\%$ and 49 an interday CV $\leq 35\%$. Given the low analyte concentrations, these results were deemed to satisfy the method's precision requirements.

⁶ SANTE 11312/2021 guidelines define precision as repeatability relative SDs (RSD_r), specifying that experiments should be repeated "over a short period of time". In this work, interday CVs were considered representative of RSD_r .

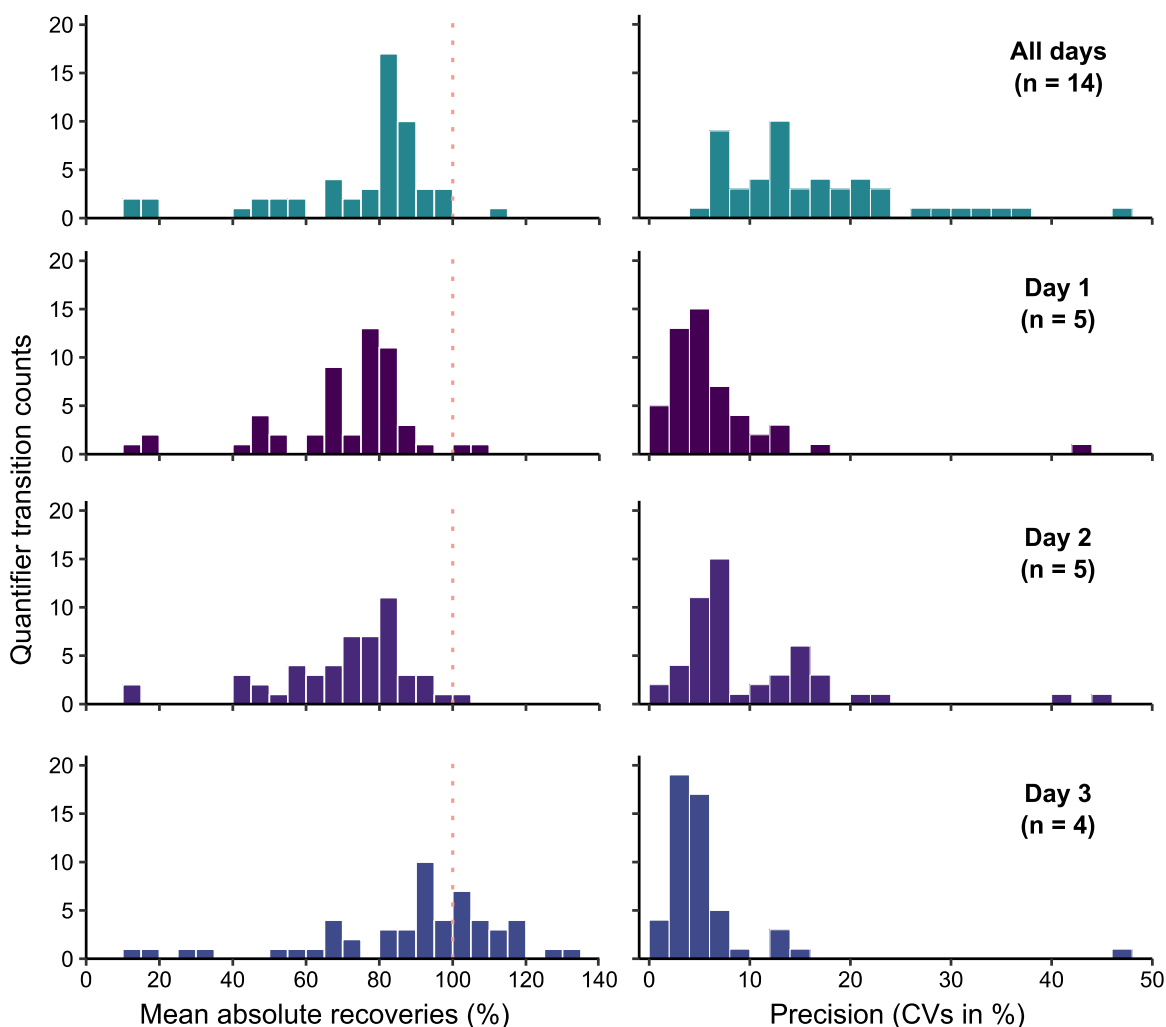


Figure 38: Intra- and interday precision and absolute recoveries of semi-quantified analytes (quantifier transitions) after sample preparation with the established workflow. The number of samples prepared and analyzed to calculate precision and mean recoveries (n) are provided in parentheses. One sample was removed due to sample mishandling; some data points are not displayed due to high values (see Table A-29). CV, Coefficient of variation.

Out of the 55 semi-quantified analytes, absolute recoveries $\geq 80\%$ were obtained for 35 and absolute recoveries $\geq 70\%$ for 40 quantifier transitions. These results were slightly improved when compared with those of earlier applications of the sample preparation workflow (Chapter 4.2.1.4), especially since higher concentrations were used for most analytes in the prior experiment. These results were not only deemed adequately high when comparing them to the SANTE guidelines' reference range, but they also demonstrate the efficacy of the improvements implemented in the sample preparation workflow. However, while most analytes met the target benchmarks, a noticeable increase in recovery was observed on the third experimental day. This shift suggests that the workflow retains a sensitivity to environmental or procedural variables that were not fully

combated by the current harmonization measures. Since such signal variations could be detrimental in longer measurement series, thorough monitoring measures with the option for signal corrections were implemented for the final method application (see Chapter 4.4.3).

In summary, despite the low concentrations tested, over two-thirds of the semi-quantified analytes exhibited CVs and recoveries within the reference thresholds adapted from the SANTE 11312/2021 guidelines. These results demonstrate that the established sample preparation and analysis workflow is capable of providing reproducible data across a broad metabolite scope, despite the lack of IS correction. Furthermore, the identification of inter-batch signal variations led to the development of quality control protocols that were subsequently integrated into the final analytical pipeline.

4.3.3 Quantified Analytes without Matrix

The absolute quantification of selected analytes was first validated using neat reference standard solutions in experiments similar or equal to those used for the semi-quantified analytes. However, the results obtained for the quantified analytes were evaluated more rigorously, using the SANTE 11312/2021 guidelines [174] as the primary reference. Following these guidelines, absolute quantification was strictly limited to compounds for which stable-isotope-labeled IS were acquired, ensuring the highest level of compensation for potential matrix effects.

Absolute quantification was attempted for eight analytes (ABA, IAA, iP, tZ, GA1, GA4, JA and SA), which are presented in Table 4 (Chapter 3.3.3). All are key phytohormones in their respective metabolic pathways and are, in all cases but one, biologically active hormones (see Chapter 1.4). While JA-Ile is the primary bioactive jasmonate, JA was selected for absolute quantification since no isotope-labeled IS could be obtained for JA-Ile. Furthermore, the cytokinin tZ was initially intended to be quantified using KIN as the IS. Although KIN is frequently used in applicatory cytokinin studies, it is mainly produced synthetically and has widely been reported to only occur rarely in plant samples [91, 257]. However, this strategy was revised during the analysis of tomato plants (Chapter 4.4.3), as both KIN and its nucleoside form, KIN-R, were detected in tomato plant samples. Attempts were then made to quantify tZ using the isotope-labeled standard for the cytokinin iP (d6-iP) as a surrogate IS. Both strategies are presented and discussed, together with the calibration curves of all quantified analytes, in Chapter 4.3.3.1. Afterwards, method precision and IS-corrected recoveries are covered in Chapter 4.3.3.2.

4.3.3.1 Evaluation of Calibration Curves

Calibration curves were prepared for all quantified analytes to evaluate their linearity, dynamic range and robustness as well as signal interferences at high concentration levels. Due to the lack

of phytohormone-free plant matrices, external calibrations using neat analyte and IS solutions in solvent were employed throughout this work. All calibration curves were fitted using linear regression with a weight of $1/x$. Further details on the creation of calibration curves and the experiment discussed in this chapter are described in Chapter 3.3.3.2.

Where applicable, the evaluation of calibration curves used criteria set by the SANTE 11312/2021 guidelines [174]. Specifically, these guidelines require at least five calibration levels with deviations of less than 20% (bias < 20%) between each level's back-calculated concentration and its true concentration. An iterative approach to calibration curve creation was developed (see Chapter 3.2.5.4), in which calibration data were processed in R to create graphs and calibration parameter tables. These outputs were designed to enable quick evaluation of all calibration curves, while the code was set up to allow quick removal of data points. In combination, this enabled a fast process of stepwise evaluations until sufficient calibration curves that (ideally) satisfied the desired performance parameters were obtained for all analytes. However, this step was intentionally not automated to remove calibration levels until the calibration parameters satisfied pre-set conditions. This approach was intended to prioritize expert evaluation of linearity and potential interferences over the rigid application of calibration quality thresholds.

To examine both the analyte response curves and the established calibration evaluation workflow, three calibration curves were prepared for each of the eight quantified analytes. The calibration levels for each analyte were its LLOQ and 5-, 10-, 25-, 50-, 100-, 500-, and 1,000-fold LLOQ concentrations. The relevant parameters of the resulting calibration curves are listed in Table A-28 in the Appendix.

All fitted regression curves fulfilled the bias < 20% criterion for back-calculated concentrations, and only one curve exhibited a bias above 10% when examining the concentration levels past the LLOQ. Furthermore, all curves exhibited R^2 values ≥ 0.999 , indicating good linearity. Considering the calibration point distribution, which resulted in lower means for the RMSE calculation, the relative RMSEs were determined to be low overall (all < 10%). However, they were not held to specific benchmarks, as they were intended primarily for comparing calibrations over repeated applications of the method. Evaluating the linear equations produced highly similar curves for the separately prepared batches and calibration curves. Additionally, quantifying tZ with both tested surrogate IS (d6-iP and KIN) yielded quantification curves of high quality. While d6-iP was used in subsequent experiments, these results demonstrate that KIN could alternatively be used as an alternative in future method applications, if it is absent from the sample tissue of interest.

Except for GA4, no more than one level had to be removed for any analytes. This was always the highest level and was mostly caused by the 1,000-fold LLOQ concentrations approaching the detector saturation range in some measurements. For GA4, the signal intensities of the IS (d2-GA4) showed a signal intensity increase at the 500-fold and 1,000-fold concentration levels, which affected the response ratios of GA4 at those levels. This was attributed to heavier isotopes of GA4, which had the same m/z as d2-GA4, bleeding into the IS signal. Since the upper part of the tested range was not expected to be highly relevant for GA4 analysis, its ULOQ was limited to 100-fold or 250-fold LLOQ concentrations in further analyses. No levels were generally excluded for other analytes, and it was concluded that the range between the LLOQ and the 1,000-fold LLOQ covered the entire instrument range well for all analytes. No additional isotope effects on IS signals were observed; however, maximum concentrations were established for multiple IS because analyte peaks were detected at higher concentrations.

In summary, the calibration curves were highly reproducible and linear, consistently fulfilling or exceeding the requirements of the method as well as the criteria of the SANTE 11312/2021 guidelines. Their dynamic range spanned three orders of magnitude and the entire instrument range (from detection to saturation) for all analytes except GA4, for which a separate ULOQ was set. Furthermore, the developed R-based workflow facilitated the creation and comprehensive assessment of calibration curves in less than ten minutes when starting with the peak tables obtained from the integration software. This was expected to effectively streamline data evaluation processes for future applications. To increase calibration confidence in the higher parts of the calibration range, especially when upper levels are removed, an additional 250-fold LLOQ concentration level was introduced for future experiments. The method's robustness was further reinforced by establishing maximum permissible IS levels, effectively preventing false-positive signals arising from isotopic impurities or high-concentration signal interferences.

4.3.3.2 Method Precision and Apparent Recoveries

After ensuring instrumental precision across the dynamic range (see Chapter 4.3.2.1) and establishing a robust calibration strategy, the precision of the method and the IS-corrected recovery of the quantified analytes were assessed. Mirroring the experiments applied to the semi-quantified analytes (see Chapter 4.3.2.2), matrix-free analyte mixtures were prepared and analyzed on separate days. However, to evaluate the method's performance across its dynamic range, three concentration levels (2.5-fold, 75-fold, and 400-fold of the instrument LLOQ) were tested. This experiment was designed to assess repeatability in accordance with the SANTE 11312/2021 guidelines [174]. In line with the guidelines' terminology and in contrast to previous

experiments, the term “apparent recovery” is used here to refer to the ratio between concentrations determined with a calibration curve and the true concentrations.

Five replicates per concentration level and day were used in these experiments. The experiments are detailed in Chapter 3.3.3.3, while the resulting intra- and interday CVs and apparent recoveries are listed in Table 8. As explained in the previous chapter, the ULOQ of GA4 was 100-fold its LLOQ, so the analyte was only determined at the lower two levels. The calibration data is not presented in this work.

Table 8: Intra- and interday precision and apparent recoveries of quantified analytes at low (2,5-fold LLOQ), medium (75-fold LLOQ), and high (400-fold LLOQ) concentration levels. Five replicates were prepared per experiment day; one sample of the low concentration level was removed due to mishandling. Interday values were obtained by combining the datasets of the three individual days. CV, Coefficient of Variation; ID, Abbreviated analyte ID (see Table 4); n.d., not determined.

ID	Level	Method precision (CV, %)				Mean apparent recoveries (%)			
		Day 1	Day 2	Day 3	Interday	Day 1	Day 2	Day 3	Interday
ABA	low	8.3	4.4	2.0	6.6	102.6	94.5	101.7	99.5
	medium	11.9	0.9	1.3	7.3	109.4	102.0	104.4	105.3
	high	1.0	0.7	1.8	3.9	103.9	95.8	102.7	100.8
GA1	low	7.6	7.4	6.0	6.9	118.9	113.1	117.8	116.5
	medium	1.8	5.1	7.1	5.8	111.1	120.0	116.2	115.8
	high	0.9	6.5	2.8	6.0	122.1	109.0	116.1	115.7
GA4	low	11.8	12.8	5.2	10.1	83.6	85.2	84.9	84.5
	medium	2.3	3.7	2.4	3.2	100.8	97.8	102.2	100.3
	high	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
IAA	low	6.1	31.4	6.3	28.2	91.4	103.0	63.6	87.6
	medium	3.0	1.6	3.3	2.8	102.2	101.8	104.2	102.8
	high	1.8	1.6	2.1	2.7	103.2	98.7	102.7	101.5
iP	low	3.4	4.8	4.6	4.7	103.2	104.5	109.1	105.3
	medium	2.5	1.8	1.1	2.6	100.8	99.3	103.9	101.4
	high	2.0	2.1	1.6	3.4	102.3	97.3	103.9	101.2
JA	low	6.0	4.3	2.4	4.6	108.7	111.7	106.9	109.3
	medium	2.4	1.1	1.7	2.1	102.6	100.5	103.2	102.1
	high	1.7	2.3	1.5	2.6	103.2	99.0	102.8	101.7
SA	low	6.1	9.6	3.1	7.1	83.7	81.4	87.9	84.1
	medium	3.6	1.8	1.5	2.8	101.0	100.4	104.0	101.8
	high	3.2	3.5	3.8	3.8	98.3	94.8	99.2	97.4
tZ	low	3.5	8.8	5.5	8.1	108.6	121.5	120.2	116.5
	medium	4.2	10.5	4.5	7.2	103.9	111.1	107.2	107.4
	high	3.8	1.9	2.9	3.2	109.7	106.7	110.9	109.1

The SANTE 11312/2021 guidelines define target criteria of 70 - 120% for recoveries and $\leq 20\%$ for interday CVs⁶ (see Chapter 4.3.2.2) at each spike level, which are intended for in-matrix applications and are primarily used as reference points here. All analytes fulfilled these recovery requirements and all but IAA fulfilled the precision requirements.

The recoveries of IAA exhibited an unexpectedly high CV in the sample with the lowest concentration on one preparation day. Since no such variances were observed for the other analytes in the same samples, technical errors during preparation and analysis were unlikely. However, further explanations could not be determined. Nevertheless, since the mean recoveries were close to the target value and only the lowest concentration, which is prone to higher variances, was affected, this was not considered an indication of poor method performance. At higher analyte levels, IAA and the other quantified analytes exhibited excellent performance criteria, and no further edge cases were observed.

In conclusion, the method precisely and reliably quantified all eight primary phytohormones. Although this experiment was performed in the absence of a matrix, these results demonstrate the efficacy of the core analytical method and provide a solid foundation for matrix-specific validations.

4.3.4 Quantified Analytes with Matrix

In-matrix validations were performed separately for two tomato matrices (fruit and leaves) by spiking analytes into pooled tissue samples at two levels. An approach was devised that was intended to serve as the foundation for future in-matrix validations, including a framework for data analysis in R. As a first step, endogenous analyte concentrations in both sample tissues were analyzed. Next, analyte mixtures were prepared with analyte concentrations adapted to the endogenous concentrations. These mixtures were then used for spiking experiments, and the apparent recovery and precision were evaluated. Furthermore, the analytes were spiked into matrix-containing samples at two different stages of the sample preparation workflow to evaluate matrix effects.

Endogenous Analyte Contents

Pooled samples of freeze-dried tissue powders were created for tomato leaves and fruits. Importantly, these tissue samples were sourced separately from the tomato plants of the final experiment, but were processed in the same manner. The process and subsequent analysis of the pooled samples are detailed in Chapter 3.3.4.1. For each tissue type, two distinct sample masses were weighed in triplicate, prepared, and analyzed. Additional sample masses were considered and in part prepared, but ultimately not measured due to instrument and dynamic range limitations.

The analyte contents of the measured samples are listed in Table A-30 in the Appendix. All eight analytes were detected in the leaf tissue, and all analytes but tZ in the fruit tissue. Their contents were evaluated in two ways, both of which focused on comparing the sample masses of the same

tissue type. First, the concordance of the analysis results was assessed to determine whether strong matrix effects were likely to affect the analysis. This would require an analysis using different techniques or approaches, such as standard addition, to determine endogenous levels, as these baseline values are required for accurately calculating spiking recoveries. However, the analysis of different sample masses (e.g. 3 mg vs. 10 mg) yielded highly similar results for endogenous levels, suggesting that matrix-induced interferences were minimal for these analytes at the chosen tissue amounts. This verification allowed for the use of pooled samples as a reliable baseline for the subsequent spiking experiments. Second, the amounts of analytes per sample were compared with the calibration range to decide which sample masses to use in the spiking experiments. Since multiple analytes were detected near or above the ULOQ in the samples containing higher amounts of tissue material, sample masses were chosen so that spiking was possible without exceeding the ULOQ.

Spiking Experiments

Based on the results of the tomato sample analysis, analyte mixes for the spiking experiments were prepared. The SANTE 11312/2021 validation guidelines [174] recommend spiking at ≥ 3 times the endogenous level when a blank matrix is not used. Here, the tissue samples were spiked at two levels; once at a level close to the endogenous sample content, and once at five times this amount. For analytes with low or no endogenous content, the spiking levels were set to 2.5-fold and 12.5-fold of the LLOQ. For analytes with high endogenous levels, this strategy was not possible without exceeding the ULOQ, so the maximum spiking levels were set to 50-fold and 250-fold of the LLOQ. This was done with the awareness that likely only the higher level could be evaluated for analytes that are naturally very abundant (> 250 -fold LLOQ). Chapter 3.3.4.2 details the preparation of spiking solutions and all subsequent steps of the experiment.

Spiked analytes and their IS were added at two distinct steps, either during the extraction of samples or during the reconstitution of evaporated extracts. For each concentration level, five replicates were spiked at extraction, and four replicates were spiked at reconstitution. Five replicates of non-spiked tissue samples were prepared alongside the spiked samples to serve as a reference for endogenous levels. The results of the analyses are listed in Table A-31 in the Appendix and are visualized in Figure 39 (leaves) and Figure 40 (fruits).

Interestingly, the analytes presumed to be GA1 and GA4 were revealed to be closely eluting isomers of the respective phytohormones. Although their integration and quantification were unaffected by this, the spiked amounts of GA1 were consequently much higher than those of other analytes that were not endogenously present. As in the previous chapter, the SANTE 11312/2021

guidelines [174] target criteria of 70 - 120% for recoveries and $\leq 20\%$ for CVs were used as reference for the evaluation. However, it should be noted that the precision criteria are intended for repeatability CVs, i.e. combined precision data of multiple experiments.

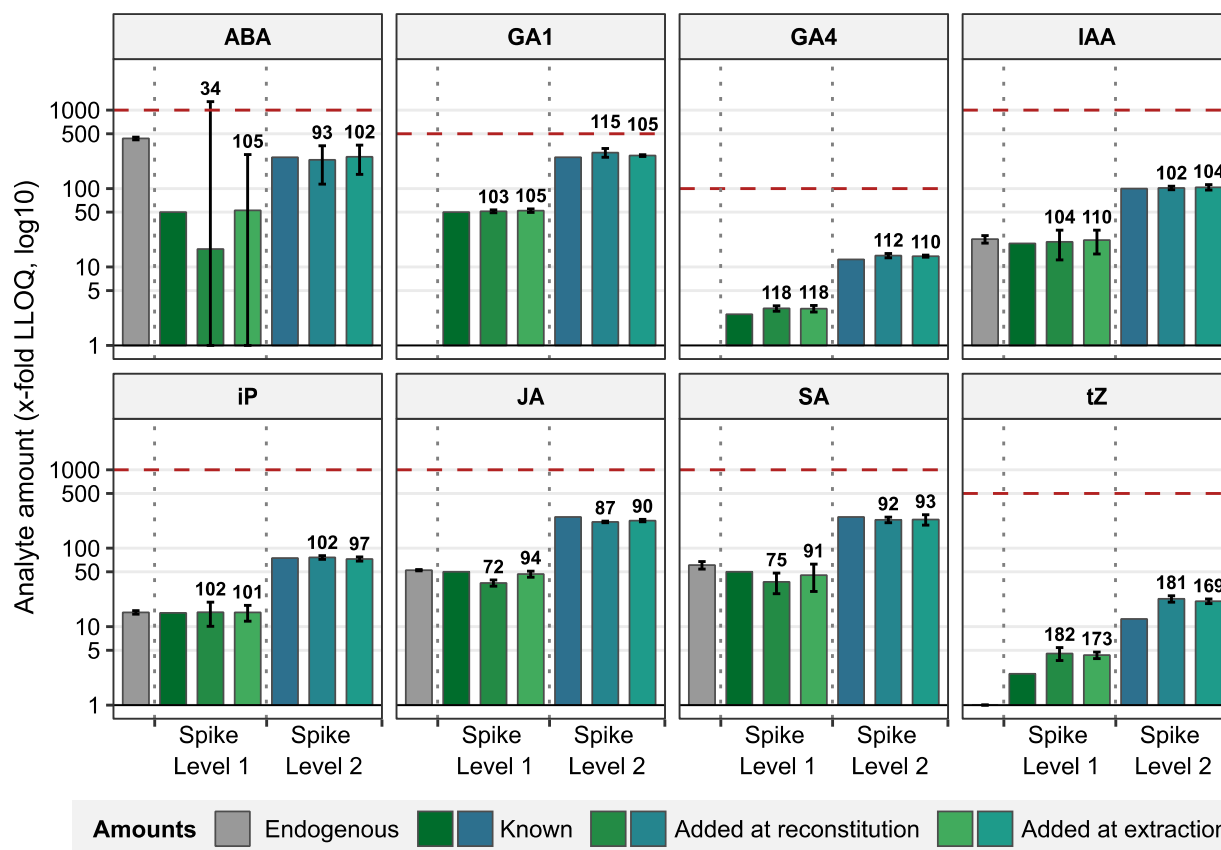


Figure 39: Analyte amounts determined in non-spiked tomato leaf samples (endogenous), in samples spiked at reconstitution and in samples spiked at extraction. Determined amounts are presented on a logarithmic scale with the respective ULOQs marked in red. Apparent recoveries are provided above each spiked sample. See Table 4 for analyte abbreviations. ULOQ/LLOQ: Upper/lower limit of quantification.

As expected, the endogenous levels of ABA in fruit and leaf samples (301- and 435-fold LLOQ) and SA in fruit samples (331-fold LLOQ) were high. This resulted in poor recoveries and very high CVs at the lower spiking level (50-fold LLOQ). Thus, only the higher spiking level (250-fold LLOQ) was evaluated for these analytes, which was still slightly lower than the endogenous concentrations.

Overall, the results were similar between the two tissues, but the leaf samples exhibited slightly better CVs and recoveries. CVs of the determined amounts were below 20% for all analytes in the evaluated leaf sample types. Similarly, recoveries were within the target range of 70 - 120% for all analytes in all evaluated leaf sample types, except for tZ, which was recovered at 173 - 181% in all four spiked sample types. Results for the fruit samples were equally good for the analytes GA1,

GA4, IAA, and iP, as well as for the higher spiking level of ABA. Similarly, tZ quantification in the fruit samples was precise (CVs 4.7 – 7.7%), but the recoveries also exceeded the target range by a considerable margin (142 – 150%). Unlike the leaf samples, the recovery of SA at the higher spiking level exceeded the target range of the SANTE guidelines (136%), and JA determination at the lower level was imprecise, but only for the samples spiked during reconstitution (CV 48.8%).

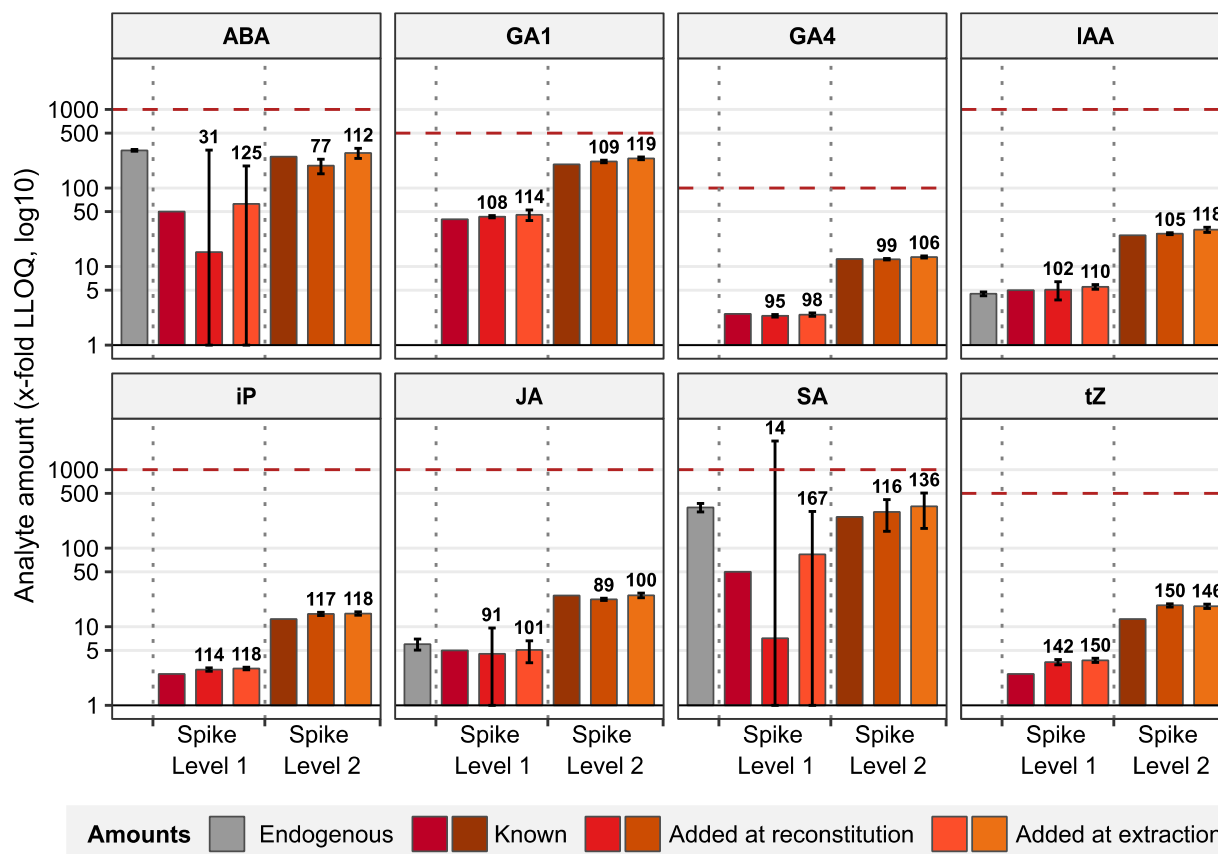


Figure 40: Analyte amounts determined in non-spiked tomato fruit samples (endogenous), in samples spiked at reconstitution and in samples spiked at extraction. Determined amounts are presented on a logarithmic scale with the respective ULOQs marked in red. Apparent recoveries are provided above each spiked sample. See Table 4 for analyte abbreviations. ULOQ/LLOQ: Upper/lower limit of quantification.

In summary, the primary factor leading to decreased precision and recoveries was a high ratio of endogenous to spiked analyte amounts. Given the abundance of some endogenously occurring compounds and the precision of their determinations in non-spiked samples, these results were considered satisfactory for all analytes except tZ.

Because tZ was not quantified with a corresponding isotope-labeled IS, it was assumed that the matrix affected the analyte and the surrogate IS (d6-iP) differently, resulting in precise but inaccurate quantification. Interestingly, when assessing the effects of the matrix on all analytes by comparing the samples spiked at extraction and those spiked at reconstitution, the determined

analyte amounts differed only marginally. This indicated that matrix effects do not occur to a significant degree during sample preparation, but rather during sample analysis, most likely affecting the ESI process.

The molecular structure of tZ differs from that of iP only by an additional hydroxyl group in the isoprene side chain, which could differentially affect analyte ionization in an ESI source, particularly in complex chemical mixtures. These findings underscore the inherent challenges of using surrogate standards in absolute quantification, as even minor variations in functional chemistry can lead to pronounced differences in matrix effects. Nonetheless, quantifying tZ using either KIN or d6-iP remains a viable option for other matrices, provided that matrix effects are thoroughly evaluated. However, the acquisition of an isotope-labeled IS for tZ would remain the gold standard for its absolute quantification.

Conclusions

The established phytohormone analysis method was successfully validated for the absolute quantification of the phytohormones ABA, GA1, GA4, IAA, iP, JA, and SA in tomato fruit and leaf samples. The specific challenges encountered with the quantification of tZ were discussed and the necessity of stable-isotope-labeled standards for absolute quantification in complex biological matrices was highlighted.

While the results directly support the application of this method to tomato plant studies, they also establish a highly reliable and versatile analytical framework. In addition to the absolute quantification of seven key phytohormones, the method enables the simultaneous detection of over 50 supplemental metabolites within the interconnected signaling pathways of plants. This work provides a validated, high-coverage template that significantly lowers the technical barrier for high-sensitivity phytohormone research in diverse and complex plant species.

4.4 Phytohormone Analysis of Plant Materials

The finalized and validated UHPLC-MS/MS method was applied to the analysis of phytohormone metabolites in tomato plants. However, prior to its finalization, the method was applied twice to other plant species while still in development, thus focusing on a narrower scope of analytes. These experiments yielded results of high relevance to this work, and are therefore summarized in the following Chapters 4.4.1 and 4.4.2. The final experiment on tomato plants is then presented and thoroughly discussed in Chapter 4.4.3.

4.4.1 Preliminary Analysis of Potato Plants Expressing Fungal Effector Proteins

One of the main objectives of method adaptation was to align the sample preparation workflow with the in-house metabolomics methods (see Chapter 4.2). Specifically, the goal was to establish a phytohormone analysis method that uses freeze-dried, ball-milled plant material. Since previous applications of freeze-dried, pulverized materials in phytohormone analysis were limited (see Chapter 1.4.3), tests on this type of material were performed at an early stage of the method adaptation process to evaluate the feasibility of this approach. The potato plant samples that were analyzed via metabolomics (see Chapter 4.1) were used for these experiments. At this stage of its development, the targeted method included only the classes identified as potentially relevant to these experiments. Specifically, the scope of the method included the gibberellin, auxin, and strigolactone metabolites. The experimental procedure is outlined in Chapter 3.4.1.1.

Despite multiple attempts at optimization, analyses of potato root and leaf samples did not yield satisfactory results. Out of the 36 analytes included in the method, only the IAA precursor IAN was successfully identified and IAA-Ala, a storage form of IAA, was putatively identified. Neither analyte exhibited significant differences in signal intensity between SP5/7-expressing plant lines and the control lines (Tukey tests, $p > 0.05$). The main reason for the low detection rates was the massive signal suppression exhibited by the sample matrix, which was characterized by examining IS signal intensities (Table 9). Although the components of the matrix causing these suppression effects could not be elucidated, the prior metabolome analyses showed that several metabolites, particularly glycoalkaloids, exhibited very high signal intensities (see Chapter 4.1.3).

To summarize, the analysis of phytohormones in potato leaves and roots did not confirm the viability of analyzing phytohormones in freeze-dried, pulverized plant materials. However, ion suppression during MS analysis was demonstrated to be the main limitation, rather than the type of plant material used. It was therefore concluded that further experiments were needed to test the efficacy of using such materials, or to determine whether more thorough sample purification strategies would be necessary. Additionally, these findings provide insight into the applicability of

the original “phytohormonomics” method [126]. Whereas the authors only validated their method for *A. thaliana* seedlings, the observations of this study highlight the chemical diversity of plant tissues and emphasize the need for matrix-specific validations.

Table 9: Absolute recoveries (mean signal intensities in plant samples / control samples) of IS in potato plant extracts. Plant and control samples both underwent the full sample preparation workflow. IS, internal standard.

Phytohormone class	IS	Mean relative signal intensities (%)
Auxins	d2-IAA	3.2%
Gibberellins	d2-GA4	0.1%
Gibberellins	d4-GA1	16.4%
Strigolactones	GR24	16.0%

4.4.2 Preliminary Analysis of Rice Plants under Short-term Wound Stress

Following the unsuccessful analysis of phytohormones in potato plants, the analysis of other plant species was explored. The primary objective remained to evaluate the feasibility of using freeze-dried, ball-milled samples for phytohormone analysis with the adapted UHPLC-MS method. Rice plants were chosen for analysis because they are fast-growing food crops that are often the focus of phytohormone studies [258-262]. Two experiments were devised to assess the effects of freeze-drying and ball-milling on phytohormone analysis. Both experiments included short-term wounding stress as an additional biological factor. Importantly, the cultivation of rice plants and the phytohormone analysis were performed as part of a Master's thesis conducted within the scope of this project. However, the study was designed and supervised as an integral part of this work to address the methodological questions addressed in the previous chapter. The individual contributions are clearly stated in Chapter 3.4.1.2, which also outlines the experimental designs. The key results of the phytohormone analysis are briefly summarized here, as they are relevant to this work.

At this stage of method development, the phytohormone analysis method included 51 analytes, over 30 of which were detected in the rice plant samples. A total of 22 analytes were successfully identified between the two experiments. The method's ability to capture both large and subtle shifts in phytohormone profiles in response to wounding stress was demonstrated. The influence of ball milling temperature was examined, and it was found that using ice-cooled sample containers instead of room-temperature containers resulted in slightly improved signal intensities for several analytes. This was subsequently adapted as the standard procedure for processing samples in the adapted phytohormone analysis protocol (see Chapter 3.3.4.1).

The analysis of freshly frozen and freeze-dried plant samples produced slightly different phytohormone profiles. However, neither processing method yielded consistently higher signal intensities, and the effects on signal intensities were overall low. Although absolute quantification was not attempted, the two identified analytes that are quantified in this work (ABA and JA) did not show significant changes in signal intensity when comparing freeze-dried and fresh samples (Tukey tests, $p > 0.05$). Combined with the assumption that sample processing affects all samples equally, these results suggest that analyzing freeze-dried materials is a viable alternative to the analysis of freshly frozen samples. Nonetheless, this should be evaluated more extensively across a wider range of plant tissues in future studies.

In conclusion, these results demonstrated that the adapted phytohormone analysis method is suitable for use with either fresh or freeze-dried plant samples. The usefulness of the method in plant research was further evaluated in a direct comparison with an in-house UHPLC-MS metabolomics method, which is presented and discussed in Chapter 4.5.1.

4.4.3 Broad Analysis of Tomato Plants Exposed to Long-term Heat Stress

After being validated for the analysis of freeze-dried tomato leaf and fruit samples, the targeted UHPLC-MS/MS phytohormone analysis method was applied to a study on tomato plants. The experiment employed an experimental design involving long-term heat stress and inoculation with the beneficial fungus *Trichoderma afroharzianum* to evaluate the method's performance in a complex biological context. It should be noted that the primary focus of this experiment was the analytical performance of the method under biologically relevant conditions, rather than the biological characterization of stress responses. The main goal was to demonstrate the method's suitability for analyzing Solanaceae crop species cultivated under varying physiological conditions. The experimental design and procedure are described in Chapter 3.4.2.

As this analysis marks the first full application of the finalized method on plant matrices, analytical aspects were thoroughly evaluated and are presented and discussed in Chapter 4.4.3.1. The findings are then assessed and discussed from a biological viewpoint in the subsequent Chapter 4.4.3.2.

4.4.3.1 Analytical Performance of the Established Method

Of the 63 targeted phytohormones and phytohormone metabolites, 43 were reproducibly detected in the tomato samples. Evaluating quantifier-to-qualifier ion ratios, as recommended by the SANTE 11312/2021 guidelines [174], resulted in the confident identification of 35 analytes, while eight analytes were classified as putatively identified. The eight unsuccessful identifications were mostly due to the qualifier transitions not being detected with sufficient intensity. No anomalous

ratios were observed, which would have indicated misidentification due to coelution. Furthermore, the detected analytes spanned seven of the eight phytohormone classes; only brassinosteroids were not detected, underscoring the broad coverage and sensitivity of the method in plant tissues of distinct compositions.

The quantification results are provided in Table A-32, the signal intensities of all reproducibly detected analytes in Table A-33, and the results of the statistical analysis in Table A-34 (Appendix). The following paragraphs will focus on evaluating the analytical performance of the method in several aspects.

Monitoring Measures

The data acquisition protocol and the data analysis pipeline were both designed to facilitate comprehensive monitoring of analytical performance. As these measures are intended to serve as a template for future method applications, their efficacy is briefly evaluated here.

Measurements were set up in a block design, adapted from in-house metabolomics methods (see Chapter 3.4.2.4). Two QC samples were measured before (and after) each block of six study samples. This enabled monitoring of signal drifts throughout the measurement series and allowed for analyte-specific signal correction in the event of drifts. To easily assess such drifts, the data analysis pipeline included graphs plotting signal intensities against injection order (see Figure 34, Chapter 4.2.2.3).

No notable signal drifts were observed in these measurements, including for the auxin metabolites that had previously exhibited changes in signal intensity over longer measurement series (see Chapter 4.2.1.2). Thus, signal corrections were unnecessary for this dataset. Nevertheless, the implemented graphical evaluation of measurement trends was found to allow for rapid and straightforward assessment of analytical drifts.

Absolute Quantification

Six out of seven quantifiable analytes were successfully detected and identified in both tomato tissues. Only GA4 was not detected in either sample type, likely due to low endogenous levels. To examine the precision of the absolute quantification, analyte CVs were assessed using the QC samples as a reference (Figure 41). While this was done for all analytes, the precision of the absolute quantification was investigated in greater detail, since the IS-correction minimizes analytical variance and allows for an assessment of biological variance.

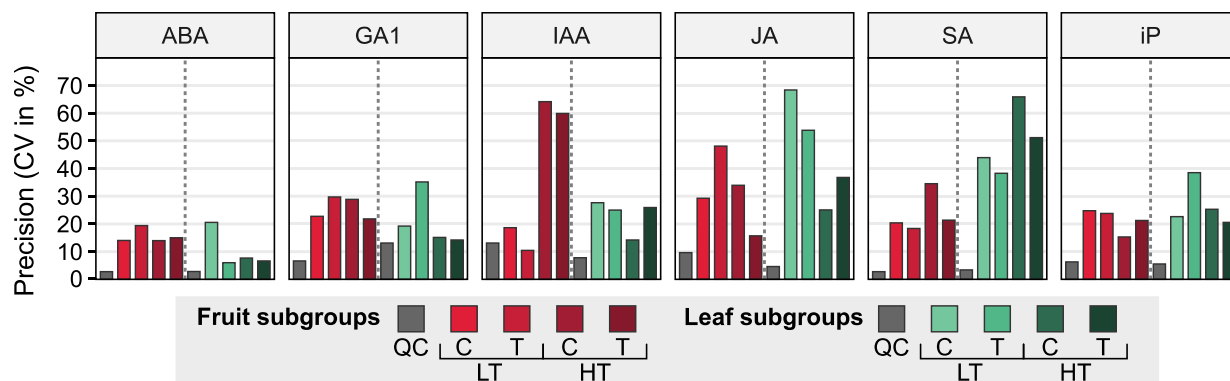


Figure 41: Precision of phytohormone quantification in tomato fruit and leaf treatment subgroups and in QC samples. All analytes were detected in all six samples of each subgroup and in all twelve QC samples. CV, Coefficient of variation; C/T, *Trichoderma* control/treatment; LT/HT, Low/High temperature cultivation; QC, quality control. See Table 4 for analyte abbreviations.

The CVs of the analytes in the QC samples ranged from 2.8 to 13.1% in both tissue types. In contrast, the CVs of analytes in the samples were much higher, and reached up to 68.4% in some subgroups. No difference was observed between tissue types, and the high CVs were inconsistent between subgroups. Given the high precision observed in the QC measurements, the large variance is very likely driven by the biological variability between samples. In this experiment, several leaves and fruits from each plant were pooled together as one biological replicate. Consequently, it was not possible to attribute these high biological variances to the status of the plants themselves or to individual fruits or leaves being heavily affected (e.g. due to an unnoticed infection).

These findings have strong implications for the sampling process in future applications of the method. Robust sampling strategies are a key part of any metabolome analysis [131, 263]. However, since phytohormones respond strongly to external factors, sampling strategies for their analysis must be even more carefully designed. Future experiments could incorporate more quantitative measurements of plant health and physiological status, which could then serve as the basis for the developing more rigorous sample harvesting protocols.

Matrix Effects

Understanding the impact of matrix chemistry on analysis is crucial for interpreting MS data and should be an essential part of every method application [49, 53, 67]. Due to the strong signal suppression caused by the matrix during the analysis of potato plants (see Chapter 4.4.1), the data analysis workflow was designed to automatically calculate matrix effects (see Chapter 3.2.5.6). The impact of matrix effects on signal intensities was assessed using IS, which covered

all phytohormone classes except brassinosteroids (Figure 42). The CVs of all the IS were below 20% for each subgroup, and no signal drifts were observed over the course of the measurements.

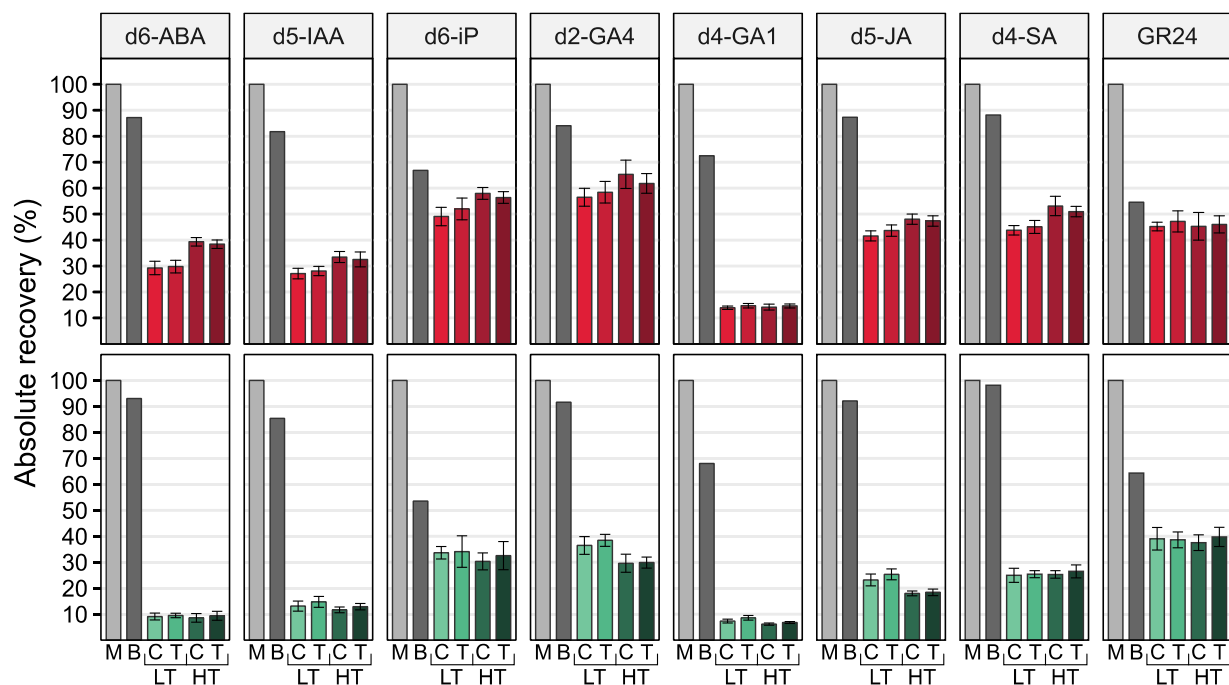


Figure 42: Signal intensities of internal standards in unprepared mixes (M), in prepared but matrix-free blanks (B), and tomato fruit (top) and leaf (bottom) samples; study samples are separated by treatment subgroups. M measurements were used as reference for recovery calculations. M and B measurements were performed with two replicates each, and sample measurements with six replicates per subgroup. C/T, *Trichoderma* control/treatment; LT/HT, Low/High temperature cultivation. Analyte abbreviations are provided in Table 4 (for the non-labeled analytes).

Overall, the effects of the matrix on signal intensity differed between tissues and were consistently stronger for leaf samples. For the fruit samples, the observed signal intensities were similar to those of the blank samples for d6-iP, d2-GA4, and GR24. Strong signal suppression was only observed for d4-GA1, which exhibited a fivefold reduction in signal intensity in the fruit samples compared to the blank samples. In the leaf samples, only the signal intensities of d6-iP and GR24 exceeded 50% of the blank sample signal intensities, and several IS (d6-ABA, d5-IAA, and d4-GA1) were only recovered at approximately 10% of the signal intensity observed in the IS mix.

Interestingly, differences in matrix effects were observed not only when comparing tissues, but also when comparing subgroups within a tissue. These effects were smaller than the differences between tissues but were still substantial for some IS, particularly when comparing samples from plants cultivated at higher and lower temperatures. The most pronounced differences in matrix suppression between subgroups were observed for d6-ABA in the fruit samples as well as for

d2-GA4 and d5-JA in the leaf samples. For each of these analytes, relative differences between plants cultivated at higher and lower temperatures were as high as 30%.

During the in-matrix validations, it was determined that the matrices' major effects likely resulted from interactions during the ESI process (see Chapter 4.3.4). Since no other sources of analytical interference were identified here (e.g. baseline intensity levels or coeluting matrix compounds), this was assumed to be the cause of the observed differences as well. These findings underscore that signal intensities are affected not only by analyte abundance, but also by the chemical environment, and that matrix effects can occur even between subgroups of the same tissue type.

While these findings do not affect the absolute quantification due to IS-correction, they have strong implications for the interpretation of data from semi-quantified analytes. Primarily, it was demonstrated that relative quantification data should only be compared between similar tissues and must be supported by thorough monitoring measures. Furthermore, data interpretation should focus on broad trends rather than individual analytes, particularly when individual FCs are small. These considerations were applied during the biological data evaluation in the following chapter.

Discussion of Notable Analytes

Several unusual and unexpected compounds were detected in this study, though they were often only putatively identified. Additionally, no metabolites were detected or identified for some analyte classes. These findings warrant further discussion as they highlight both the method's current reach and potential avenues for future investigations.

As discussed during the method validation (see Chapter 4.3.3), both KIN and KIN-R were detected in the tomato plant samples, but only KIN was identified successfully. KIN differs from tZ and iP in that its side chain is cyclized into a furfuryl structure. Interestingly, KIN was the first cytokine discovered, when it was isolated from autoclaved DNA, but is rarely detected endogenously in plant tissues [91, 257]. However, other authors have argued that KIN can be formed through DNA oxidation and can therefore be found in all DNA-carrying organisms, if the detection method is sensitive enough [264]. Although KIN is often applied exogenously to tomato plants to induce biological effects (see e.g. [265-267]), its endogenous occurrence in tomato plants has not yet been reported. Further investigation of the presence of KIN and KIN-R in tomato plants is necessary, particularly to ensure that they are not formed as byproducts of sample processing, e.g. during lyophilization.

The reduced form of JA, DHJA, was detected in tomato leaves and fruit, but was only putatively identified. This analyte was originally incorporated into the method to specifically assess its occurrence in plants (see Chapter 4.2.2.2). This was done because DHJA was considered to be

used as an IS, as it has been in other methods [242, 243], including applications on tomato plants. Although DHJA is not commonly mentioned as a JA metabolite, it has been putatively identified in tomato fruits and leaves before [140]. Therefore, its presence in these tissues should be investigated further. If the natural occurrence of DHJA in plant tissues is confirmed, it could provide novel insights into jasmonate metabolism.

Several other jasmonates, including JA-Ile, 12OH-JA, 12OH-JA-G, and OPDA, were detected in a double peak. These observations align with the known stereochemistry of jasmonates, since two epimers that are both bioactive occur naturally (see Chapter 4.2.2.2). These epimers can easily be interconverted through tautomerization at the C7 position, which is next to a carbonyl function at C6 (see Chapter 1.4.2.6). Here, these peaks were co-integrated and not viewed as separate analytes, since it has not been examined whether the epimers are stable or tautomerize during the sample preparation process. Additional information on the natural distribution of jasmonate stereoisomers might be gained if such investigations were carried out.

The analyte GA3 was both detected and successfully identified in both tomato leaves and fruits. This finding was unexpected since GA3 has been reported to occur almost exclusively in monocotyledonous plants [98], which tomatoes are not. Therefore, it must be considered whether the GA3 detected in the samples originated in the rhizosphere microbes. While this study's data did not allow for the determination of the biological source of GA3 in the samples, future studies could focus specifically on its endogenous occurrence in tomato plants and potentially provide new insights into gibberellin metabolism.

The strigolactone analytes ORO and STR were detected, but not identified successfully due to only one transition being detected. Since these analytes were not part of the original method [126], it still cannot be concluded whether strigolactones are detectable in plant matrices using this method. Nevertheless, these results are promising for further method applications and more targeted strigolactone analysis methods.

Of the analytes covered by the established analysis method, only the brassinosteroids were not detected. As discussed during the method validation, the brassinosteroid analytes performed poorly in analytical aspects (see Chapter 4.3.1 and 4.3.2.2), and this method's capability to detect them in plant tissues remains to be proven. However, it should be noted that micro-tomato varieties, like the one used in this study, are brassinosteroid-deficient mutants and therefore exhibit lower levels of these phytohormones [268]. The detection of brassinosteroids with this method should be examined further, as capturing CS and BL signaling would be of high value to this method.

Identification of Putative Isomers

Additional substance peaks were detected in the detection windows of multiple analytes. While these isomeric (or isobaric) compounds could be unrelated matrix components that coelute by chance, they could also constitute biologically relevant derivatives of the analytes. Some of these cases are summarized here, as identifying them could be a goal of future studies that aim to expand the scope of the method.

The MRM chromatograms of several analytes with known isomers exhibited peaks in close proximity to the analytes. This was especially pronounced for cytokinin glycosides such as tZ-7G and iP-9G, as well as tZ-R and iP-R. Regarding the latter compounds, it is unclear what other deoxyhexoside types could be present in plant matrices if these substances are indeed tZ-R and iP-R derivatives. 2,5-DHBA was not detected in any samples, but an intense substance peak next to its expected RT was recorded, and it was assumed to potentially be 2,3-DHBA, a primary SA inactivation product. The irreversible IAA conjugate IAA-Asp was detected at low intensities. However, shortly after the RT of the analyte itself, a highly intense peak was detected that reached detector saturation. Due to the intensity of the nearly co-eluting substance, investigating its identity further would be worthwhile. Lastly, a medium-intensity peak eluting shortly before ABA could belong to an isomer of the primary phytohormone.

In some cases, peaks of unknown analytes exhibited a clear biological response, strongly suggesting their relevance to the plant stress response. For example, GA4 was not detected, but a low-intensity peak was present exclusively in the leaf samples of heat-stressed plants shortly before its expected RT. Similarly, a peak eluting shortly before the expected RT for IAA-ME appeared only in the leaf samples of tomato plants inoculated with *Trichoderma* and exposed to heat stress (subgroup HT + T).

Systematic investigation of such occurrences could lead to the identification of biologically relevant metabolites that have already been shown to be present at detectable levels in plant materials. As a first step, metabolites detected in this manner should be monitored strategically in future applications of the method. The identities of the unknown substances could then be confirmed by measuring them with the same UHPLC method on a high-resolution MS and performing systematic fragmentation experiments. Next, if available, reference standards could be acquired to confirm the identified structures. Although not yet widely used, specific projects have created databases for identifying unknown phytohormone signals that could greatly facilitate such identifications [269]. Metabolome analysis data could also aid in elucidating unknown, potentially relevant metabolites by cross-referencing with phytohormone analysis data, or independently.

This will be further discussed in Chapter 4.5.2 regarding the tomato samples analyzed via metabolomics.

Conclusions and Outlook

In its application to tomato leaves and fruits, over two-thirds of the analytes covered by the targeted phytohormone method were reproducibly detected, and over half were successfully identified. Six out of seven key analytes were absolutely quantified with high precision, as demonstrated by the QC samples. These results clearly demonstrate the applicability of the method to the analysis of freeze-dried plant materials.

Moreover, the QC-based measures integrated into the workflow proved to be robust tools for performance monitoring. Examining biological variance in isolation, using the IS-corrected absolute quantification results, provided insights into sampling strategies for future applications. Furthermore, the assessment of IS signal intensities in various sample types allowed for the systematic characterization of matrix-induced ion suppression effects, which were discussed in the context of further data interpretation. Since the analyzed matrices presented a high level of chemical complexity, as characterized by the high abundance of secondary metabolites observed (see Chapter 4.5.2), these findings demonstrate the method's versatility and its suitability for application to a wide range of plant matrices.

Regarding future research possibilities, noteworthy analytes were identified that warrant further attention and investigation. Similarly, the evaluation of non-specific MRM transitions led to the detection of multiple putative phytohormone metabolites not yet included in the method. Strategies to monitor and identify such substances were discussed. Employing these strategies in further applications of the method could provide an approach for the continuous expansion of the method's scope and potentially lead to the discovery of novel phytohormone metabolites.

4.4.3.2 Biological Interpretation of Results

To gain insight into the effects of treatment factors (heat stress and *Trichoderma* inoculation) on phytohormone signaling pathways, the plants and the samples harvested from them were assessed in multiple aspects. The following subsections examine the plants' morphology, the exploratory data analysis, and then the signal intensity changes under each treatment factor. Lastly, conclusions are summarized and future outlooks are discussed.

Plant Morphology and Tomato Yields

First, the plants themselves were assessed, focusing on external phenotype and fruit yields. Figure 43 shows exemplary images of representative plants from each subgroup, together with

the results of the tomato fruit harvest. Although plant growth and appearance were not quantitatively evaluated in this study, the impact of the stress treatment on the plants was apparent nonetheless. Plants grown under optimal temperatures (LT plants) exhibited much darker leaf colors than plants grown under high temperatures (HT plants). As described in Chapter 3.4.2.2, fruits from LT plants ripened earlier, and their first tomatoes were ready to harvest one week before those of the HT plants. Additionally, the total mass of harvested tomatoes, which were collected until no new fruits formed, was substantially and significantly lower for the HT plants. No notable differences were observed between the *Trichoderma*-inoculated plants and the non-inoculated control plants for any of these factors.

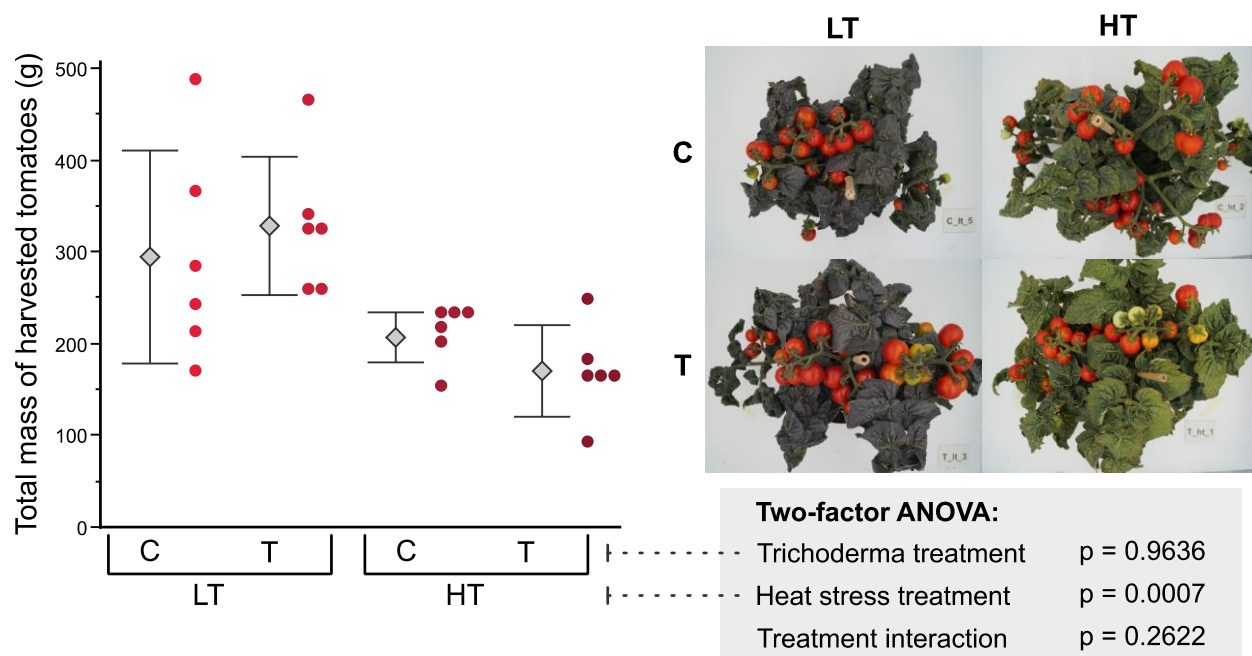


Figure 43: Tomato fruit yields per treatment subgroup ($n = 6$), including statistical analysis and exemplary pictures taken at harvest. ANOVA, Analysis of variance; C/T, *Trichoderma* control/treatment; LT/HT, Low/High temperature cultivation.

Lower tomato yields of plants exposed to heat stress were previously found by many studies (summarized in [180]). However, comparing further developmental and morphological changes of the plants with these previous studies is difficult, because the results were strongly affected by experimental design and plant genotype. Additionally, several studies combined the application of heat stress and drought stress, which was not the case in this study, as all plants were sufficiently watered. One study performed on a micro-tomato variety, similar to the one used in this study, exposed plants to the same temperature (32°C) heat stress [270]. The authors also reported reduced yield, an earlier onset of fruit ripening, and changes to leaf pigments for the heat-stressed

plants. Although the authors did not provide pictures of their plants for visual comparison of morphologies, these results align well with the findings of this experiment.

Exploratory Data Analysis

Following the sample analysis and peak integration, the results were first assessed in a PCA to gain an overview of the samples. Figure A-6 (Appendix) shows the 2D score and loading plots for the tomato fruits and leaves. The PCA calculation is detailed in Chapter 3.2.5.11. No unusual clustering was observed for either tissue and no samples were identified as outliers.

Additionally, it was evaluated which factors had the greatest impact on the variance between all samples. As in the previous section, the cultivation temperature was identified as the largest effect on analyte variances. HT and LT plant samples were separated along PC1, which accounted for 31.4% (fruits) and 37.5% (leaves) of the total variance. In comparison, PC2 explained 11.5% and 13.4%, respectively, but could not be associated with any known experimental factors. Higher PCs were evaluated (data not shown) to look specifically for an effect of the *Trichoderma* inoculation. For the leaf samples, no such effect was observed in the first five PCs. For the fruit samples, C and T samples were separated along PC4, which accounted for 8.1% of the total variance, but only the LT plants were fully separated. Thus, it was concluded that several unknown biological or environmental factors impacted the tomato plants more than their inoculation with *Trichoderma afroharzianum*.

Signal Intensity Changes

Individual analytes were evaluated by examining their FCs between the plants of a treatment group and the respective control group. Figure 44 presents the $\log_2$ FCs, together with the results of the two-factor ANOVA. These statistical tests consider analyte signal intensity variance, which was previously found to be substantial, even for IS-corrected analytes (see previous chapter). Here, the FCs of some analytes were dominated by single samples showing unexpectedly high or low intensities. For example, tZ-G was detected at 23-fold higher intensity in one sample of the heat-stressed, non-inoculated tomato fruits (subgroup HT + T) compared to the mean intensity of the other samples in the subgroup. No other technical or biological aspects clearly indicating the sample as an outlier were identified, so the data point was not removed. Consequently, tZ-7G exhibited a high FC, but due to the resulting large variance, it was not statistically significant.

The overall trends seen in the heat maps were consistent with previous results and showed greater shifts between heat stress treatment groups than between *Trichoderma* treatment groups. The following two subsections individually address the shifts in phytohormones and phytohormone metabolites affected by the two biological factors.

Effects of Heat Stress

Significant and large FCs were observed for the phytohormones of every identified class under heat stress. This strongly suggests that the plant signaling pathways underwent major, long-lasting alterations following their exposure to heat stress. Interestingly, the primary effect of heat stress was an increase in analyte signal intensities, indicating an increase in metabolic regulation. In this subsection, the phytohormone metabolites are first evaluated separately and then in conjunction.

The phytohormone class exhibiting the most substantial adaptations between LT and HT plants was the jasmonates, though intensity shifts were much more consistent in leaves than in fruits. JA and JA-Ile were both present at significantly higher intensities in both tissue types, but their FCs were higher in the fruit samples. Several other jasmonate metabolites were also significantly increased in the stressed leaf samples ($p < 0.01$). These metabolites included OPDA, a JA precursor and minor bioactive jasmonate, as well as the oxidation products 12OH-JA and JSMO, and the reversibly formed MeJA (FCs 1.5 - 3.1). In contrast, OPDA was the only jasmonate other than JA and JA-Ile that was significantly increased ($p < 0.05$) in the fruits (FC C | T: 1.1 | 1.8), while the inactivated form 12OH-JA-G was significantly reduced (FC C | T: 0.69 | 0.62). Together, these changes indicate a highly increased activation of jasmonate signaling under heat stress.

ABA and its storage form ABA-GE were detected, but only exhibited significant changes in the fruit samples, where they were both significantly reduced in the HT plants ($p < 0.01$). This reduction was much stronger for ABA-GE (FC C | T: 0.31 | 0.24) than for ABA itself (FC C | T: 0.91 | 0.70), which hints at a depletion of stored ABA but could also result from less glucose being available for conjugation. In the tomato leaves, changes in signal intensity of ABA and ABA-GE were small and not significant ($p > 0.05$).

Changes in auxin signaling were similarly different between tissues. In the fruits, IAA levels were slightly increased (FC C | T: 1.3 | 3.0), though there was inconsistency between subgroups which resulted in limited statistical significance ($p < 0.05$). IAA-GE levels were also increased (FC C | T: 2.1 | 2.9), but IAC and TPL levels decreased (FCs 0.34 – 0.66). Curiously, the contrast between a decrease in biosynthetic precursors and an increase in the IAA storage hints at an increased deactivation of IAA under heat stress, despite the higher levels of IAA. This could potentially indicate a recent inactivation. In the tomato leaves, more compounds exhibited significantly altered FCs. IAA levels were slightly but consistently increased (FC C | T: 1.7 | 1.7) in the HT samples, while TPL levels were elevated more strongly (FC C | T: 3.1 | 2.8). In contrast, the levels of IAN, the immediate IAA precursor, were significantly decreased (FC C | T: 0.66 | 0.65). The intensities of the oxidation products ICA and OxIAA were also significantly increased, and particularly the

intensity shifts of ICA (FC C | T: 6.1 | 8.6) were among the highest FCs in this study. Contrary to the results for fruits, the observed accumulation of inactivated IAA suggests higher IAA activity under heat stress, though the time-dependence of these shifts were not assessed in this study.

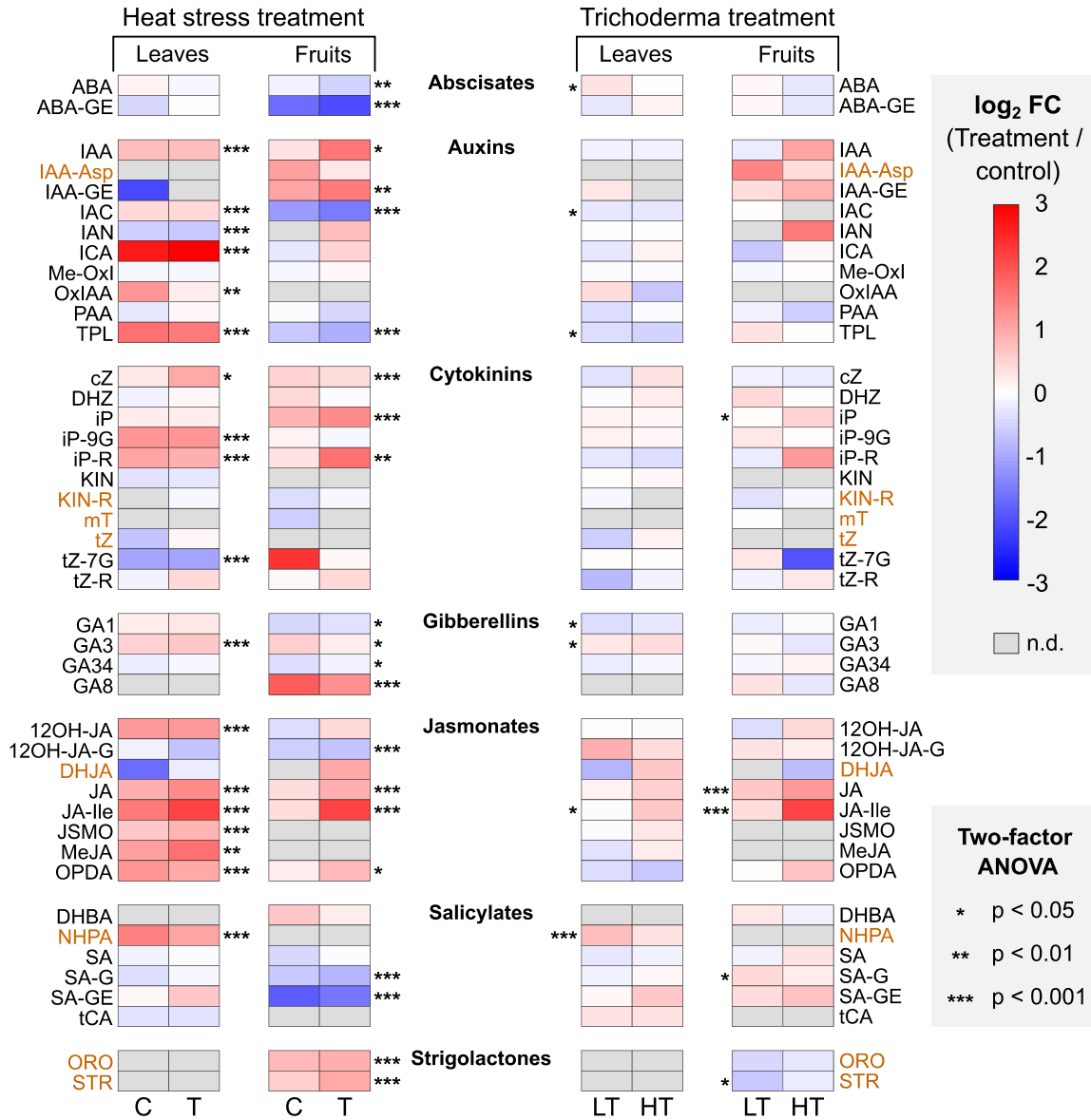


Figure 44: Log₂ FC heatmaps of phytohormone analytes in tomato fruit and leaf samples. FC calculations were performed between means of treatment and control groups (T / C and HT / LT), which was done separately for the groups of the non-observed factor, resulting in two columns per plant tissue. Significance of the treatment effect (two-factor ANOVA) is shown as star codes for each analyte in each tissue. Interaction terms are provided in Table A-34 and analyte abbreviations in Table A-17 (Appendix). FC, Fold change, C/T, *Trichoderma* control/treatment; LT/HT, Low/High temperature cultivation; n.d., not determined (in at least one group of the comparison).

The identified cytokinins exhibited less significant changes. In both tissues, cZ (FC C | T: 1.2 | 2.0) and iP-R (FC C | T: 2.0 | 1.9) exhibited slight but significant increases in the HT samples. Meanwhile, iP contents were significantly elevated in the fruits of stressed plants (FC C | T: 1.9 | 2.5). Interestingly, the two other metabolites that changed significantly in the leaf samples were the iP 9G and tZ 7G glucosides, which exhibited opposite metabolic responses to heat stress. While iP-9G levels were higher in the leaf samples of HT plants (FC C | T: 2.4 | 2.3), tZ-7G levels were lower (FC C | T: 0.48 | 0.47). The reasons for this differential glucosylation trend could not be elucidated.

Four gibberellins were identified, and all four were significantly altered in the fruit samples, but only GA3 in the leaf samples. GA3 levels were slightly increased in both tissues (FCs 1.2 – 1.5). GA1 levels were slightly decreased in the HT fruit samples (FCs 0.73 – 0.81), while its 2-hydroxylated form GA8 was strongly increased (FC C | T: 3.7 | 2.5), suggesting an increase in gibberellin signaling in the fruits. In contrast, the equivalent GA4 inactivation product, GA34, was slightly reduced in the fruits (FC C | T: 0.76 | 0.90).

Several salicylate metabolites were identified, but none exhibited significantly changed FCs in the leaves of the stressed plants, while SA-G and SA-GE levels were reduced in the fruits with high significance ($p < 0.001$). As with ABA-GE, these reductions in glucose conjugate levels could be caused by changes in phytohormone signaling, but could also be indirect effects due to reduced glucose levels. The SA-related signaling metabolite NHPA was detected in the leaves, where its signal intensity was elevated under heat stress (FC C | T: 2.8 | 2.1) with highest significance ($p < 0.001$). However, it was only putatively identified, as its qualifier transition was not detected. This makes biological interpretation unreliable, especially since NHPA was the only salicylate metabolite exhibiting significant changes in the leaves.

ORO and STR were the only strigolactones that were detected, and both were only putatively identified. Therefore, their biological responses should not be overinterpreted, particularly since ORO and STR are coeluting isomers (see Chapter 4.2.2.2). Both were only detected in the fruit samples, where they were increased in the heat stressed samples (FCs 1.5 – 2.0) with highest significance ($p < 0.001$).

In summary, heat stress induced tissue-specific intensity shifts across multiple metabolite classes. Assessing the tomato fruits suggests an increase in jasmonate, auxin, cytokinin, and gibberellin signaling activities. These classes are all involved in fruit growth and ripening [271, 272], with both auxins and jasmonates being the classes most closely linked to the metabolism of ethylene. This increase in signaling is likely related to the faster development and earlier induction of fruit ripening

in the HT plants. These results align with known plant processes, as plants shift towards reproduction when their environment does not allow for sustainable growth [273]. Such an early-flowering process has been described in tomatoes as a response to multiple abiotic stress factors, including heat stress [270, 274], salt stress [275] and mechanical stress [276]. Higher levels of IAA inactivation products, combined with lower levels of IAA biosynthetic precursors, could potentially be explained by the recent inactivation of auxin signaling, which occurs during the late stage of fruit ripening [271]. Another observed trend in the tomato fruits was that five out of seven of the metabolites that were present in significantly changed intensities in the tomato fruits were glucoside conjugates. These findings are discussed with the metabolome analysis of the tomato fruits (see Chapter 4.5.2), which covered sugar levels in the fruits.

Major changes in signaling processes of tomato leaves under heat stress included a substantial increase in all branches of JA metabolism, as well as significant alterations in auxin and cytokinin signaling. In contrast, SA, gibberellin, and ABA signaling metabolite signal intensities were only marginally altered under heat stress. This is particularly notable for ABA, which is the major mediator of water regulation in plants [277]. However, ABA levels primarily increase during water-deficient conditions to induce leaf stomata closure, limiting water loss. As previously mentioned, the plants in this study were provided with sufficient water and did not experience drought stress. In such cases, limiting ABA accumulation and maintaining a steady transpiration rate presents a cooling mechanism for the plant [278-280]. Additionally, the magnitude of the observed changes in other phytohormones indicates that heat-stressed plants may actively work to maintain ABA homeostasis and stabilize water transport and transpiration through leaf stomata. However, neither the ABA precursor ABAld nor the ABA inactivation product DHPA was identified, which hinders further evaluation.

Effects of *Trichoderma* Application

The *Trichoderma* treatment induced notably less change in both tomato tissue types, as evidenced by the number of analytes with significant changes (two-factor ANOVA, $p < 0.05$) and the FCs of these analytes (see Figure 44). Only three analytes were significantly changed at the highest level of significance used here ($p < 0.001$). These were JA (FC LT | HT: 1.5 | 2.3) and JA-Ile (FC LT | HT: 1.3 | 4.6) in the fruit samples, and the putatively identified NHPA in the leaf samples (FC LT | HT: 1.7 | 1.3). Of the other analytes that exhibited significant changes ($p < 0.05$), none were accompanied by strong FCs (0.65 - 1.34). Overall, these results indicate that interactions between tomato plants and *Trichoderma afroharzianum* in the soil alter signaling processes in the plant leaves and fruits. However, the magnitude of the observable effects was overall small, except for an apparent induction of jasmonate signaling.

These changes in jasmonate signaling were particularly noteworthy when examining the interaction of the treatment factors. JA and JA-Ile, together with iP, were the only major phytohormone metabolites that exhibited a significant interaction term (see Appendix, Table A-34), and were the only metabolites that were significant in both factors as well as the interaction term ($p < 0.05$). This was mainly caused by high intensities in the samples of tomato plants inoculated with *Trichoderma* and exposed to heat stress (subgroup HT + T). For iP, however, this only applied to the fruit samples. These findings indicate that *T. afroharzianum* differentially affects plants growing under optimal versus suboptimal conditions. However, these effects were not reflected in plant morphology or yields, and could therefore not be categorized as beneficial in this experiment.

In summary, while *T. afroharzianum* did not substantially alter the physical phenotype or yield of the tomato plants, it did induce subtle but measurable shifts in hormonal signaling, most notably in the jasmonate pathway under heat stress. These findings underscore the fact that plant-microbe interactions in the rhizosphere can exert modulating effects on the plant's physiological state, even in the absence of obvious morphological changes. Future studies are necessary to investigate this effect further, ideally by including more rigorous assessments of plant morphology and health parameters. Integrating multiple sampling timepoints would also be particularly valuable to better capture the dynamics of these biological processes.

Conclusions and Outlook

Targeted analysis of phytohormone metabolites in tomato leaves and fruits provided comprehensive insight into how the plants adapt to external influences. The signaling pathways of seven phytohormone classes were represented by the detected analytes, demonstrating the method's broad capacity for characterizing hormonal crosstalk. The established method was shown to capture not only large-scale signaling pathway restructuring under heat stress, but also more subtle alterations induced by inoculation of the soil with *Trichoderma afroharzianum*.

The biological changes induced by both factors were put into context with established plant physiological processes, which further validated the method's suitability to accurately capture complex biological processes. Therefore, this work establishes a versatile framework that can be adapted to further studies on tomato plants, or to other high-complexity crop species. Finally, as this method was designed for integration into a multi-omics framework, its synergy with metabolome analysis is further explored in Chapter 4.5.2.

4.5 Comparative Application of LC-MS-Metabolomics

The main objective of this work was to develop an integrated framework that combines untargeted UHPLC-MS metabolome analysis with targeted UHPLC-MS phytohormone analysis. To assess this framework in its entirety, metabolome analysis was performed on rice and tomato plant samples that had previously been analyzed with the targeted method. The following chapter outlines the results and highlights how the two methods complement each other, ultimately offering a more holistic view of plant adaptation.

4.5.1 Metabolome Analysis of Wounded Rice Plants

The metabolome of the rice plants exposed to wounding stress (see Chapter 4.4.2) was analyzed. The primary objective of this analysis was to compare both methods' performance in capturing biological changes in response to short-term stress. The metabolomics method and the general data processing were the same as, or highly similar to, those used for the analysis of potato plants (see Chapter 4.1), and are detailed in Chapter 3.5.1. Unlike the phytohormone analysis, evaluation of the rice plant metabolomics data was performed independently as part of this work.

Of the two rice plant experiments, only the one that assessed short-term wounding stress and the effect of ball-milling temperatures was analyzed via UHPLC-MS metabolomics. The evaluation presented here focuses solely on the biological stress factor. Prior to data processing, the standards of all analytes included in the phytohormone analysis method were measured using the metabolomics method and incorporated into the in-house database.

Two approaches were taken to evaluate the metabolomics method. First, the validity of features annotated as phytohormone metabolites was examined. While eight features were putatively annotated as phytohormone metabolites, only three metabolites were identified. The other matches lacked sufficient-quality MS/MS spectra, likely due to their low abundance. The three identified metabolites were ABA-GE, SA (with two features), and SA-G. Notably, only ABA-GE was also identified using the targeted phytohormone method. However, none of the metabolites showed a stress-relevant response in the metabolome dataset. Comparing the signal intensities in stressed plants with those in control plants revealed only marginal changes for ABA-GE and SA (FC 0.99 – 1.07). Meanwhile SA-G showed contrary FCs in samples milled at different temperatures (FC -0.78 at 0°C and 0.78 at room temperature). This matched the result of the phytohormone analysis for ABA-GE, where its FCs were also negligible.

The second aspect evaluated was to assess all significantly changed metabolites in the metabolome data to identify all metabolites with matches in the in-house database. This strategy aligned with the potato plant analysis (see Chapter 4.1.2), but used less stringent filtering criteria.

Rigorous filtering of metabolites was unnecessary due to the low number of significantly changed metabolites. Out of approximately 13,000 features, fewer than 300 changed significantly between wounded plants and control plants (two-factor ANOVA, $p < 0.01$)⁷.

Using this approach, three metabolites and one metabolite group were identified and are listed in Table 10 alongside their respective FCs. The biological relevance of nicotinic acid, a metabolite of the cofactor NAD(P), and *trans*-ferulic acid, a key metabolite of the phenylpropanoid metabolism, were previously discussed in Chapter 4.1. Hypoxanthine is a purine base resulting from the deamination of adenine nucleotides and nucleosides, which is a process that occurs more frequently during the breakdown of cells or cellular components [281, 282]. The signal intensities of sucrose isomers can only be evaluated as a sum parameter (see Chapter 4.1.3). Their signal intensities, as well as those of hypoxanthine and nicotinic acid, were increased in the samples of stressed plants. In contrast, the signal intensity of ferulic acid was slightly decreased. Together, these observations suggest an increase in oxidative or antioxidant enzyme activities, as well as damage to nucleotides or nucleosides. The decrease in ferulic acid and increase in sucrose-like disaccharides also hints at cell-wall modifications, though the level of evidence for this hypothesis is low. Since the wounded tissues were harvested along with the rest of the seedlings, these metabolic shifts likely reflect processes occurring in tissues directly affected by wounding.

Table 10: Fold changes (FCs) of metabolites significantly altered in response to wounding stress in rice plant seedlings. FCs were calculated as the ratio of mean signal intensities between wounded and non-wounded plant samples.

Method	Metabolite (group)	Role in plant metabolism	FC
Metabolomics	Sucrose-like disaccharides	Unclear; often cell structure formation	1.4
	Hypoxanthine	Purine base salvage pathway	1.5
	Nicotinic acid	NAD(H) cofactor metabolism	1.6
	<i>trans</i> -Ferulic acid	Phenylpropanoid metabolism	0.86
Targeted	Jasmonic acid-isoleucine (JA-Ile)	Jasmonate signaling (active form)	41
	Jasmonic acid (JA)	Jasmonate signaling (JA-Ile precursor)	97
	12-OH-JA	Jasmonate signaling (deactivated JA form)	2.9
	Absciscic acid (ABA)	Abscisate signaling (active form)	1.9
	Salicylic acid glucose ester (SA-GE)	Salicylate signaling (deactivated SA form)	1.8
	Indole-3-acetic acid (IAA)	Auxin signaling (primary active form)	0.88
	IAA-aspartic acid (IAA-Asp)	Auxin signaling (IAA intermediate)	0.84
	Gibberellin A9 (GA9)	Gibberellin signaling (GA4 precursor)	0.63

⁷ Two factors were included, stress treatment and ball milling temperature. Only the stress treatment was considered here. Additionally, the p value threshold was conservatively chosen since no FDR-correction (see Chapter 3.1.4.6) was calculated for the over 10,000 calculated ANOVAs.

The magnitude of the metabolic shifts exhibited by the compounds identified using the metabolomics method was low overall, and the insight gained was constrained by the limited number of identified metabolites. To illustrate the contrast between the two methods, Table 10 includes the FCs of metabolites identified as significantly changed in the targeted phytohormone analysis (two-factor ANOVA, $p < 0.05$). The intensity shifts of key phytohormones were much higher, particularly those of JA and JA-Ile. However, the FCs of 12OH-JA, ABA, SA-G, IAA, IAA-Asp, and GA9 were also more pronounced than those of metabolites identified as significant with the metabolomics method. This corresponds well with the known signaling processes that occur upon wounding. Jasmonate signaling is often one of the first phytohormone pathways activated and mediates other pathways (see Chapter 1.5 and [262, 283, 284]).

In conclusion, the targeted method revealed a high state of activation in the plants' stress-defense pathways, likely capturing the initial signaling phase of the wounding response. This provided much more insight than the changes captured by the metabolomics method, which primarily reflected broad metabolic shifts that offered limited insight into the specific stress status of the plants. These findings strongly emphasize the value of employing a targeted phytohormone analysis in conjunction with the metabolome analysis of plant materials.

4.5.2 Metabolome Analysis of Heat-stressed Tomato Plants

To also compare the metabolomics method with the finalized and validated version of the targeted phytohormone analysis method, the former was applied to study the metabolome of tomato leaves and fruits. Unlike the previous metabolome analysis of rice plants, this experiment involved two biological factors: long-term heat stress and inoculation with *T. afroharzianum* (see Chapter 4.4.3). The in-house database was used to identify metabolites, resulting in a focus on high-confidence identification, as described in Chapter 3.5.2. This chapter presents and discusses the results from both biological and analytical perspectives, focusing on the capabilities of both methods to analyze phytohormones and their metabolites.

Metabolic Trends in Tomato Leaves and Fruits

A total of 91 metabolites were identified with high confidence in the tomato samples, spanning multiple substance classes. Many of these compounds had high signal intensities, including various structurally diverse secondary metabolites. This could potentially explain the matrix effects observed in Chapter 4.4.3.1. The FC heatmaps of all identified metabolites are presented in Figure A-7 and Figure A-8 in the Appendix.

Corroborating the findings of the phytohormone analysis (see Chapter 4.4.3), the observed metabolic shifts were considerably more pronounced between heat-stressed plants (HT plants) and control plants (LT plants) than between *Trichoderma*-inoculated plants and their control plants. Similarly, the specific effects were also starkly different for the two analyzed tissues.

Under heat stress, leaf tissues exhibited a considerable decrease in the signal intensities of all identified flavonoids, dopamine, and caffeic and chlorogenic acids. Meanwhile, the signal intensities of *N*-malonyltryptophan, feruloyltyramine, and cumaroyltyramine increased considerably. Notably, these metabolic shifts mirror those observed in potato plants expressing fungal effector proteins (see Chapter 4.1.4), suggesting that adaptations in the phenylpropanoid metabolism are a general, stress-related mechanism in *Solanum* plants. *N*-malonyltryptophan, which cannot be distinguished between L-tryptophan and D-tryptophan forms, has previously been described as a compound accumulating strongly in tomato leaves under heat stress [285]. It has further been proposed as a potential precursor to IPyA, the primary precursor of IAA [286].

In the tomato fruits, the signal intensities of most flavonoids were increased in the HT plant samples. The differential flavonoid responses in leaves and fruits suggest that flavonoids are transported from leaves to the fruit, likely to protect them from oxidative damage under stress conditions. An increase in flavonoid levels to protect reproductive tissues commonly occurs in tomatoes under heat stress [287, 288], though the effect and the resulting total flavonoid content of the fruit depend on the tomato genotype [289]. Other significant shifts in tomato fruits under heat stress include increased amino acid levels, with the notable exception of L-proline, and decreased levels of histamine, feruloyl- and cumaroyltyramine, and sugars. All of these metabolites were previously discussed as being highly relevant to plant metabolism (see Chapters 4.1.2 and 4.1.3). This study could not evaluate glucose-like hexoses, but the lower levels of all other sugars indicate that sugars may be needed to supply energy for adaptive stress mechanisms. Alternatively, they might also be caused by the early ripening of the tomatoes.

In contrast to the substantial shifts observed under heat stress, inoculation of the soil with *T. afroharzianum* did not lead to any major changes; all FC values were between 0.5 and 2.0. Due to the wide coverage of some substance classes, minor but consistent trends could nonetheless be observed. The signal intensity levels of chlorogenic acid, ferulic acid 4-*O*-glucoside, and several flavonoids were increased in the fruits of *Trichoderma*-treated plants. Meanwhile, the levels of flavonoids and most amino acids were slightly increased in the leaves. Conversely, the signal intensities of amino acids and fatty acids were lower in the fruit samples of inoculated plants.

In addition to these general trends observed in all plants inoculated with *Trichoderma* spores, HT and LT plant samples exhibited differential changes for some biologically relevant metabolites. Most notably, the signal intensities of L-pipecolic acid (the precursor to NHPA) and MTA (an ethylene metabolite) were increased in the HT plants, but decreased in the LT plants. The same shift is shown by L-proline, which is relevant to osmotic stress adaptation (see Chapter 4.1.3). Additionally, several flavonoid aglycones, feruloyl- and cumaroyltyramine, and histamine also show altered FCs when comparing LT and HT plants. These observations support the findings of the phytohormone analysis (see Chapter 4.4.3.2) that *Trichoderma* modulates how tomato plants adapt to stress, albeit moderately.

In summary, the metabolome analysis yielded high-confidence annotations of endpoints that are highly relevant to plant metabolism, including proteinogenic and specialized amino acids, enzyme cofactors, and many metabolites of the phenylpropanoid pathway. Additionally, many of the identified metabolites are relevant to evaluating the quality of tomato fruit, including organic acids, amino acids, sugars, and flavonoids. These results demonstrate the value of metabolomics in assessing tomato plants and their primary product. Expanding the identification process to include additional spectral databases beyond the in-house database would undoubtedly reveal more relevant metabolites, as demonstrated in Chapter 4.1.2, if the goal is to achieve maximum metabolome coverage.

Phytohormone Coverage

Along with the extensive coverage of various metabolite classes, several phytohormone metabolites were detected and identified using the in-house database, which included all analytes of the targeted method. Specifically, six of the targeted method's analytes were identified and are illustrated in Figure 45. All of these were also detected with the targeted method, where they were among the metabolites with the highest signal intensities. Their detection in the untargeted scan serves as a cross-method validation, since the biological responses align well. Capturing these metabolites with the metabolomics method may also be valuable when highly concentrated phytohormones exceed the ULOQ of the targeted method.

Except for ABA and SA, all analytes were detected with at least two different adducts, which increases confidence in their biological interpretations. Nevertheless, illuminating the complex phytohormone signaling pathways based on these six analytes would be challenging. Unlike the more than 40 metabolites of seven phytohormone classes identified with the targeted method, these analytes belong to only three classes. While they all exhibit biologically relevant stress responses and are highly informative in an independent metabolome analysis, the additional

targeted analysis captured a much higher proportion of the hormonal crosstalk occurring in the tomato plants. Ultimately, these results demonstrate that while broad metabolome analysis provides the range of physiological outcomes, the targeted analysis is indispensable for resolving the low-abundance phytohormone metabolites that orchestrate the stress response. Without the supplemental targeted analysis, the metabolome analysis would not have covered most signaling pathways of the plants.

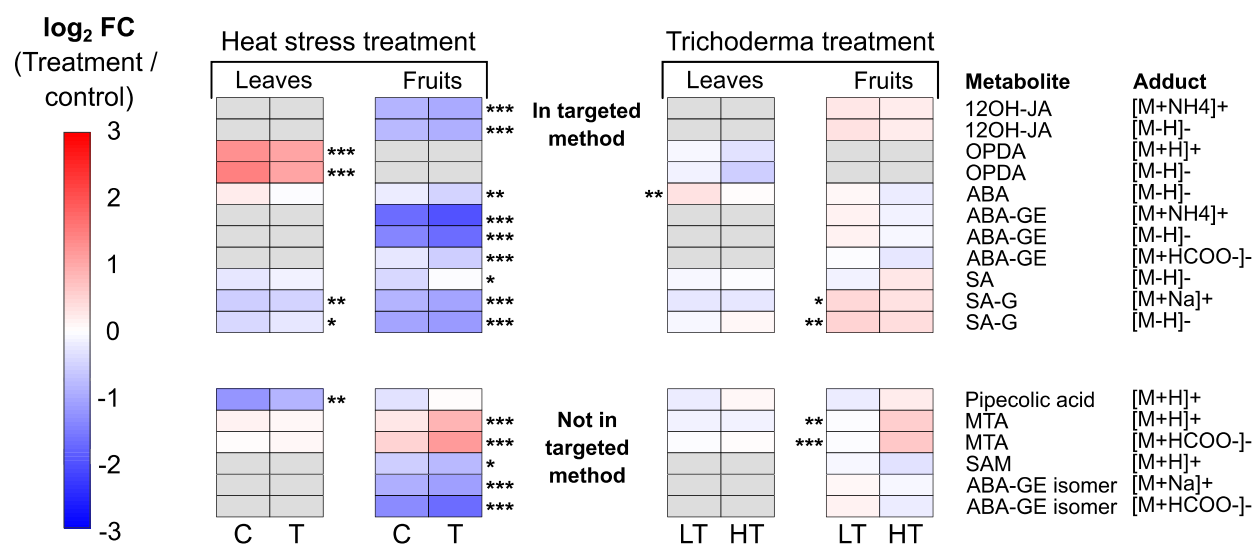


Figure 45: Log₂ FC heatmaps of phytohormone metabolites identified with the in-house metabolomics database in tomato fruit and leaf samples. FC calculations were performed between means of treatment and control groups (T / C and HT / LT), which was done separately for the groups of the non-observed factor, resulting in two columns per plant tissue. Significance of the treatment effect (two-factor ANOVA) is shown as star codes for each analyte in each tissue (see Figure 44); interaction terms are not provided. Analyte abbreviations are provided in Table A-17. FC, Fold change, C/T, *Trichoderma* control/treatment; LT/HT, Low/High temperature cultivation; n.d., not determined (in at least one group of the comparison).

The glucose conjugates identified here exhibit decreased intensities in the fruits of HT plants, similar to many glucose conjugates identified with the targeted method (see Chapter 4.4.3.2). However, the metabolome analysis of the tomato fruits also demonstrated lower sugar levels for the covered sum parameters. Thus, these changes in phytohormone glucose conjugates cannot be confidently attributed to adaptations in signaling pathways because they could also be caused by lower sugar availability. This finding underscores the fact that phytohormone metabolites are not decoupled from broader plant metabolism and highlights the efficacy of the integrated application of these methods.

Furthermore, the metabolome analysis identified several metabolites closely connected to phytohormone signaling that are not included in the targeted method. One such metabolite is pipecolic acid, the direct precursor to NHPA. Its signal intensities were substantially and

significantly decreased ($p < 0.01$) in the leaves of HT plants. This could represent a link to the putatively identified NHPA, whose signal intensities were increased, suggesting elevated conversion of pipecolic acid. Since pipecolic acid was also identified in the analysis of potato plants (Chapter 4.1.3) and has therefore been demonstrated to be present at detectable levels in plant materials, it could be considered for inclusion into the targeted method. This would enhance the interpretation of NHPA by providing biological context to a metabolite currently characterized in isolation. Furthermore, an ABA-GE isomer was identified based on its high similarity to ABA-GE in RT and MS/MS spectra. Since its biological response to heat stress indicates a connection to ABA metabolism, further investigations into its structure are warranted, as outlined in Chapter 4.4.3.1 for unidentified phytohormone isomers.

Lastly, MTA and SAM were identified, though SAM was found in fruit samples only. The detection of MTA and SAM is a particularly high-value outcome of the untargeted analysis, as it provides a window into ethylene metabolism, which is not covered by the targeted method. Specifically, MTA and SAM were removed from the scope of the method due to analytical performance issues (see Chapter 4.2.2.2). As explained in Chapter 1.4.2.9, SAM is converted into MTA and ACC, the precursor to ethylene. The increased MTA intensity in leaves of HT plants, coupled with a reduction in SAM intensity, suggests that SAM is increasingly converted into MTA under heat stress. This further indicates an increase in ethylene production, providing a more direct link to the observed differences in fruit ripening. Additionally, MTA appears relevant to *Trichoderma*'s effect on heat-stressed plants because its levels are much higher in the leaves of HT plants inoculated with *Trichoderma* than in the leaves of inoculated LT plants, when compared with their respective controls.

Conclusions and Future Prospects

The analysis of tomato plants has demonstrated the targeted phytohormone method's capability to elucidate complex signaling processes. However, its combined application with metabolome analyses led to a complementary gain of information. By linking broad metabolic shifts with specific signaling data, this integrated approach provided a level of insight into plant adaptation that would not have been possible using either method in isolation.

While the long-term stress study in tomato revealed broad metabolic restructuring, the results from the wounded rice plants emphasize the specific value of phytohormone profiling during short-term adaptation. Therefore, applying this dual framework of methods to studies designed to monitor stress adaptation at multiple sampling points would be highly valuable. Such experiments could

also facilitate the identification of additional stress-relevant metabolites, as several high-priority candidates for future expansion of the targeted method were already identified in this work.

Moreover, the ability of this framework to resolve subtle hormonal modulations, as demonstrated for the *T. afroharzianum* inoculation of tomato plants, makes it particularly well suited for investigating plant-microbe interactions. In these contexts, interactions in the rhizosphere may exert biologically relevant effects that are not necessarily reflected in large changes in plant morphology. Further studies on these interactions could contribute to the elucidation of beneficial effects of these microbes, and therefore aid in making plants more resilient to stressors.

Given the limited availability of high-coverage phytohormone methods validated for a wide variety of matrices, applying this framework to a wider range of plant tissues remains a logical next step. The use of freeze-dried materials, an approach demonstrated to be feasible in this work, should ideally be systematically investigated for these different tissues. Ultimately, the future integration of this dual-platform approach into even broader multi-omics frameworks, together with methods such as transcriptomics, will be instrumental in achieving a more complete understanding of how plants manage environmental stress.

5 Conclusion

Plants adapt to environmental changes through chemical signaling networks. These networks are regulated by specialized metabolites known as phytohormones, which coordinate the metabolic adjustments required for responses to stress and mediate critical interactions with the microbial environment. Understanding these signaling mechanisms and how they change under external influences is essential for improving crop resilience and developing sustainable agricultural strategies. Such efforts are crucial for ensuring global food production amid increasing environmental pressures, particularly those resulting from climate change.

Untargeted metabolomics is a powerful tool for monitoring a plant's physiological state and assessing the quality of agricultural products. However, the broad analytical scope of these methods often fails to capture the low-abundance phytohormones that orchestrate these metabolic endpoints. To address this limitation, this work developed an analytical framework combining untargeted metabolomics with a highly sensitive, targeted phytohormone analysis. This framework was applied to study plant stress adaptations and plant-microbe interactions, using Solanaceae crops as model plants. The high abundance of secondary metabolites in these tissues provided significant chemical complexity, demonstrating the method's potential for wider applications across diverse and challenging plant matrices.

Initially, an in-house UHPLC-MS metabolomics method was applied to analyze transgenic potato plants that express effector proteins of the arbuscular mycorrhizal fungus *Rhizophagus irregularis*. Mycorrhizal symbiosis is reported to result in multifaceted benefits to host plants, making the elucidation of its underlying mechanisms an important research target. Metabolic profiling across leaves, stems, and roots revealed significant shifts in the phenylpropanoid pathway, characterized by a redirection of metabolic flux from lignin formation to flavonoid biosynthesis. Furthermore, multiple metabolic links to stress response mechanisms were identified, but the analytical coverage of key phytohormones was insufficient to fully elucidate these adaptive mechanisms. This limitation confirmed the necessity for a dedicated, targeted phytohormone analysis to complement the untargeted profiling in subsequent studies.

To address these gaps, a targeted phytohormone analysis method was established, covering 63 metabolites including bioactive forms, precursors, and inactivation products across eight major signaling pathways. A state-of-the-art UHPLC-MS/MS phytohormone analysis method was selected for adaptation, and optimized in several crucial aspects to ensure applicability across diverse plant matrices. Specifically, eluent compositions were refined to maintain column pH stability and resolve previous issues in the analysis of auxin metabolites. Further chromatographic

adjustments improved peak resolution while simultaneously shortening analysis times by several minutes, resulting in runtimes below 20 minutes. To modernize the workflow, a custom data analysis pipeline was developed in R, ensuring reproducible results and high-throughput reporting. Additionally, the sample preparation was successfully adapted for freeze-dried materials, a strategy previously underutilized in phytohormone analysis. The feasibility of this approach was evaluated in studies on potato and rice plants, ultimately confirming that freeze-drying provides a valid, highly reproducible alternative to processing fresh material.

The finalized method covered 63 phytohormone metabolites across eight major classes: abscisates, auxins, brassinosteroids, cytokinins, gibberellins, jasmonates, salicylates, and strigolactones. To balance high coverage with the accessibility of isotope-labeled internal standards, the scope included 55 analytes for relative quantification and eight key phytohormones for absolute quantification. The method was fully validated using the SANTE 11312/2021 guidelines as a reference framework, and met or exceeded all relevant performance criteria. Comprehensive in-matrix validation was performed on tomato leaf and fruit samples, while also establishing a standardized framework for the analysis of future plant tissues.

The established method was applied to study tomato plants cultivated in a complex biological context, analyzing both leaf and fruit tissues. In this study, plants were exposed to long-term heat stress and inoculated with the beneficial fungus *Trichoderma afroharzianum*. The framework successfully captured both biological factors, despite the significant differences in the magnitude of their effects on plant metabolism. Specifically, while heat stress induced large-scale restructuring of signaling pathways, the method also identified the more subtle hormonal modulations associated with fungal inoculation. Of the 55 targeted analytes, over 40 were detected, and 35 were identified with high confidence. The detected analytes spanned seven phytohormone-related metabolic pathways, demonstrating the method's capability to capture hormonal signaling crosstalk effectively. Furthermore, high precision of the absolute quantification was shown for quality control samples, whereas the large biological variance observed within subgroups provided critical insight for the optimization of future sampling strategies. The assessment of matrix effects also highlighted the inherent limitations of relative quantification in complex tissues, reinforcing the value of the absolute quantification approach established in this work. Additionally, the results confirmed the efficacy of the data analysis workflow and identified several candidate analytes for future method expansions.

The tomato plant samples were further analyzed using the in-house UHPLC-MS metabolomics method, and the observed metabolic changes were integrated with the phytohormone data. Evaluating this dual framework confirmed substantial synergistic information gain, as the two

methods complemented each other's analytical strengths and limitations. While the targeted phytohormone analysis characterized the signaling crosstalk of low-abundance hormones, the untargeted metabolomics provided the broader metabolic context and downstream metabolic responses. Notably, the untargeted method also captured ethylene-related metabolites, a signaling pathway not included in the targeted method.

The importance of this dual approach was further underscored by the metabolome analysis of rice plants harvested shortly after wounding. While long-term heat stress in tomato plants led to large-scale shifts across both platforms, the short-term response in rice plants was primarily reflected in the phytohormone analysis results. This emphasizes the necessity of targeted hormone profiling to capture early signaling events before significant metabolic restructuring occurs.

Taken together, this work establishes an integrated analytical framework that combines untargeted metabolomics with high-sensitivity phytohormone analysis, enabling comprehensive characterization of plant stress responses and plant–microbe interactions across diverse plant tissues. Future applications may include time-resolved plant stress experiments to further elucidate the metabolic adaptation mechanisms of plants and to identify more metabolites that are relevant to these processes. Additionally, future studies integrating this analytical framework with other omics methods, such as transcriptomics, would constitute another step toward holistic assessments of plant physiology.

6 Literature

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7 Chemicals and Instruments

Chemicals

Table A-1: Authentic reference standards of phytohormone metabolites used in this work. Impurities and isotopic purities were calculated into purities when specified by the manufacturer. Relational operators (<, >) have been removed from purity specifications to improve readability. n.a., not available.

Substance	CAS	Formula	Manufacturer	Purity
(+)-Absciscic acid	21293-29-8	C ₁₅ H ₂₀ O ₄	Merck, Darmstadt, DE	98%
(+)-Absciscic acid b-D-glucose ester	21414-42-6	C ₂₁ H ₃₀ O ₉	TRC, Vaughan, CA	99.64%
(+)-Absciscic aldehyde	41944-86-9	C ₁₅ H ₂₀ O ₃	TRC, Vaughan, CA	95.35%
Dihydrophaseic acid	41756-77-8	C ₁₅ H ₂₂ O ₅	Biorbyt, Cambridge, UK	98%
2-Oxindole-3-acetic acid	2971-31-5	C ₁₀ H ₉ NO ₃	Biosynth, Compton, UK	98%
3-Methyloxindole	1504-06-9	C ₉ H ₉ NO	TargetMol, Boston, USA	99.34%
4-Chloroindole-3-acetic acid	2519-61-1	C ₁₀ H ₈ ClNO ₂	ChemScene, New Jersey, USA	95.04%
Indole-3-acetamide	879-37-8	C ₁₀ H ₁₀ N ₂ O	Merck, Darmstadt, DE	99%
Indole-3-acetate methyl ester	1912-33-0	C ₁₁ H ₁₁ NO ₂	MedChemExpress, New Jersey, USA	99.12%
Indole-3-acetic acid	87-51-4	C ₁₀ H ₉ NO ₂	Merck, Darmstadt, DE	98%
Indole-3-acetonitrile	771-51-7	C ₁₀ H ₈ N ₂	Merck, Darmstadt, DE	98%
Indole-3-acetyl b-D-glucose ester	19817-95-9	C ₁₆ H ₁₉ NO ₇	TRC, Vaughan, CA	96.34%
Indole-3-acetyl-L-alanine	57105-39-2	C ₁₃ H ₁₄ N ₂ O ₃	Merck, Darmstadt, DE	98%
Indole-3-acetyl-L-aspartic acid	2456-73-7	C ₁₄ H ₁₄ N ₂ O ₅	Biosynth, Compton, UK	95%
Indole-3-acetyl-L-glutamic acid	57105-48-3	C ₁₅ H ₁₆ N ₂ O ₅	Biosynth, Compton, UK	99%
Indole-3-acetyl-L-isoleucine	57105-45-0	C ₁₆ H ₂₀ N ₂ O ₃	Biosynth, Compton, UK	100%
Indole-3-acetyl-L-leucine	36838-63-8	C ₁₆ H ₂₀ N ₂ O ₃	Biosynth, Compton, UK	90%
Indole-3-acetyl-L-phenylalanine	57105-50-7	C ₁₉ H ₁₈ N ₂ O ₃	MedChemExpress, New Jersey, USA	97.14%
Indole-3-acetyl-L-valine	57105-42-7	C ₁₅ H ₁₈ N ₂ O ₃	Biosynth, Compton, UK	95%
Indole-3-acrylic acid	29953-71-7	C ₁₁ H ₉ NO ₂	ABCR, Karlsruhe, DE	98%
Indole-3-butyric acid	133-32-4	C ₁₂ H ₁₃ NO ₂	Santa Cruz Biotechnology, Dallas, USA	n.a.
Indole-3-carbinol	700-06-1	C ₉ H ₉ NO	Merck, Darmstadt, DE	97%
Indole-3-carboxylic acid	771-50-6	C ₉ H ₇ NO ₂	Merck, Darmstadt, DE	99%
Indole-3-pyruvate	392-12-1	C ₁₁ H ₉ NO ₃	Merck, Darmstadt, DE	98.90%
Phenylacetic acid	103-82-2	C ₈ H ₈ O ₂	Merck, Darmstadt, DE	99%
Tryptophol	526-55-6	C ₁₀ H ₁₁ NO	TargetMol, Boston, USA	99.18%
24-Epibrassinolide	78821-43-9	C ₂₈ H ₄₈ O ₆	Biosynth, Compton, UK	90%
6-Oxocampestanol	168113-32-4	C ₂₈ H ₄₈ O ₂	OIChemIm, Olomouc, CZ	95%
Brassinolide	72962-43-7	C ₂₈ H ₄₈ O ₆	Biosynth, Compton, UK	95%
Castasterone	80736-41-0	C ₂₈ H ₄₈ O ₅	OIChemIm, Olomouc, CZ	95%
Isohomobrassinolide	80483-89-2	C ₂₉ H ₅₀ O ₆	MedChemExpress, New Jersey, USA	98%
4-Methylpent-3-en-2-one	141-79-7	C ₆ H ₁₀ O	Merck, Darmstadt, DE	n.a.
cis-Zeatin	32771-64-5	C ₁₀ H ₁₃ N ₅ O	Biosynth, Compton, UK	99%
Dihydrozeatin	23599-75-9	C ₁₀ H ₁₅ N ₅ O	Biosynth, Compton, UK	99%
Kinetin	525-79-1	C ₁₀ H ₉ N ₅ O	MP Biomedicals, Irvine, USA	98%
Kinetin riboside	4338-47-0	C ₁₅ H ₁₇ N ₅ O ₅	MedChemExpress, New Jersey, USA	99.28%
meta-Topolin	75737-38-1	C ₁₂ H ₁₁ N ₅ O	Biosynth, Compton, UK	98%
N6-Isopentenyladenine	2365-40-4	C ₁₀ H ₁₃ N ₅	ABCR, Karlsruhe, DE	97%
N6-Isopentenyladenine-N9-glucoside	83087-94-9	C ₁₆ H ₂₃ N ₅ O ₅	Biosynth, Compton, UK	95%
N6-Isopentenyladenosine	7724-76-7	C ₁₅ H ₂₁ N ₅ O ₄	Biosynth, Compton, UK	99%
ortho-Topolin	20366-83-0	C ₁₂ H ₁₁ N ₅ O	Biosynth, Compton, UK	99%
trans-Zeatin	1637-39-4	C ₁₀ H ₁₃ N ₅ O	Biosynth, Compton, UK	99%

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Substance	CAS	Formula	Manufacturer	Purity
<i>trans</i> -Zeatin riboside	6025-53-2	C ₁₅ H ₂₁ N ₅ O ₅	Biosynth, Compton, UK	97%
<i>trans</i> -Zeatin-N7-glucoside	38165-56-9	C ₁₆ H ₂₃ N ₅ O ₆	Biosynth, Compton, UK	95%
1-Aminocyclopropane-1-carboxylic acid	22059-21-8	C ₄ H ₇ NO ₂	Biosynth, Compton, UK	98%
5'-Methylthioadenosine	2457-80-9	C ₁₁ H ₁₅ N ₅ O ₃ S	MedChemExpress, New Jersey, USA	97%
b-Cyano-L-alanine	6232-19-5	C ₄ H ₆ N ₂ O ₂	TRC, Vaughan, CA	99.85%
S-Adenosylmethionine chloride	24346-00-7	C ₁₅ H ₂₃ N ₆ O ₅ SCl	Chromadex, Los Angeles, USA	n.a.
ent-Kaurenoic acid	6730-83-2	C ₂₀ H ₃₀ O ₂	MedChemExpress, New Jersey, USA	99.56%
Gibberellin A1	545-97-1	C ₁₉ H ₂₄ O ₆	MedChemExpress, New Jersey, USA	95.31%
Gibberellin A12 aldehyde	19436-07-8	C ₂₀ H ₂₈ O ₃	OlChemIm, Olomouc, CZ	97.7%
Gibberellin A20	19143-87-4	C ₁₉ H ₂₄ O ₅	OlChemIm, Olomouc, CZ	97.8%
Gibberellin A3	77-06-5	C ₁₉ H ₂₂ O ₆	Merck, Darmstadt, DE	90%
Gibberellin A34	32630-92-5	C ₁₉ H ₂₄ O ₆	TRC, Vaughan, CA	93.48%
Gibberellin A4	468-44-0	C ₁₉ H ₂₄ O ₅	Biosynth, Compton, UK	90%
Gibberellin A4 methyl ester	19124-90-4	C ₂₀ H ₂₆ O ₅	OlChemIm, Olomouc, CZ	97.7%
Gibberellin A7	510-75-8	C ₁₉ H ₂₂ O ₅	TargetMol, Boston, USA	98%
Gibberellin A8	7044-72-6	C ₁₉ H ₂₄ O ₇	TRC, Vaughan, CA	97.82%
Gibberellin A9	427-77-0	C ₁₉ H ₂₄ O ₄	TRC, Vaughan, CA	99.60%
(±)12-Hydroxyjasmonic acid	140631-27-2	C ₁₂ H ₁₈ O ₄	TRC, Vaughan, CA	98.51%
(±)12-Hydroxyjasmonic acid glucoside	124649-25-8	C ₁₈ H ₂₈ O ₉	Ambinter, Orléans, FR	90%
(±)Jasmonic acid	77026-92-7	C ₁₂ H ₁₈ O ₃	Merck, Darmstadt, DE	95%
(±)Jasmonic acid isoleucine	120330-93-0	C ₁₈ H ₂₉ NO ₄	Cayman Chemical, Ann Arbor, UK	98%
12-Oxo-Phytodienoic acid	85551-10-6	C ₁₈ H ₂₈ O ₃	Cayman Chemical, Ann Arbor, UK	95%
<i>Cis</i> -3-hexenal	6789-80-6	C ₆ H ₁₀ O	Fisher Scientific, Schwerte, DE	n.a.
<i>cis</i> -Jasmone	488-10-8	C ₁₁ H ₁₆ O	MedChemExpress, New Jersey, USA	95%
Dihydrojasmonic acid	3572-64-3	C ₁₂ H ₂₀ O ₃	MedChemExpress, New Jersey, USA	99.26%
Methyl jasmonate	39924-52-2	C ₁₃ H ₂₀ O ₃	Merck, Darmstadt, DE	95%
<i>Trans</i> -2-hexenal	6728-26-3	C ₆ H ₁₀ O	Merck, Darmstadt, DE	98%
(-)-Chorismic acid	617-12-9	C ₁₀ H ₁₀ O ₆	Merck, Darmstadt, DE	96.10%
2,3-Dihydroxybenzoic acid	303-38-8	C ₇ H ₆ O ₄	Merck, Darmstadt, DE	99%
2,5-Dihydroxybenzoic acid	490-79-9	C ₇ H ₆ O ₄	Merck, Darmstadt, DE	98%
Benzoic acid	65-85-0	C ₇ H ₆ O ₂	Merck, Darmstadt, DE	99%
Methyl salicylate	119-36-8	C ₈ H ₈ O ₃	Fisher Scientific, Schwerte, DE	n.a.
<i>N</i> -Hydroxyphenylpicolic acid	115819-92-6	C ₆ H ₁₁ NO ₃	MedChemExpress, New Jersey, USA	98.00%
<i>ortho</i> -Coumaric acid	614-60-8	C ₉ H ₈ O ₃	Merck, Darmstadt, DE	97%
Salicylic acid	69-72-7	C ₇ H ₆ O ₃	Merck, Darmstadt, DE	99%
Salicylic acid 2-O-b-D-glucoside	10366-91-3	C ₁₃ H ₁₆ O ₈	TRC, Vaughan, CA	98.88%
Salicylic acid b-D-glucose ester	60517-74-0	C ₁₃ H ₁₆ O ₈	TRC, Vaughan, CA	94%
<i>trans</i> -Cinnamic acid	140-10-3	C ₉ H ₈ O ₂	Fisher Scientific, Schwerte, DE	99%
(±)5-Deoxystrigol	139540-45-7	C ₁₉ H ₂₂ O ₅	Chiralix, Nijmegen, NL	99%
(±)-Orobanchol	220493-64-1	C ₁₉ H ₂₂ O ₆	OlChemIm, Olomouc, CZ	94.4%
(±)Strigol	11017-56-4	C ₁₉ H ₂₂ O ₆	Chiralix, Nijmegen, NL	99.20%
5-Hydroxy-3-methylbutenolide	931-23-7	C ₅ H ₆ O ₃	ChemScene, New Jersey, USA	98.91%
(±)-Strigolactone GR24	188062-52-4	C ₁₇ H ₁₄ O ₅	Chiralix, Nijmegen, NL	99.20%
d2-Gibberellin A4	84693-21-0	C ₁₉ H ₂₂ D ₂ O ₅	OlChemIm, Olomouc, CZ	90.73%
d2-Indole-3-acetic acid	24420-86-8	C ₁₀ H ₇ D ₂ NO ₂	TRC, Vaughan, CA	94.55%
d4-Aminocyclopropane-carboxylic acid	84392-07-4	C ₄ H ₃ D ₄ NO ₂	TRC, Vaughan, CA	95%
d4-Gibberellin A1	unknown	C ₁₉ H ₂₀ D ₄ O ₆	OlChemIm, Olomouc, CZ	98%
d4-Salicylic acid	78646-17-0	C ₇ H ₂ D ₄ O ₃	TRC, Vaughan, CA	94.78%
d5-(±)-Jasmonic acid	2750534-78-0	C ₁₂ H ₁₃ D ₅ O ₃	TRC, Vaughan, CA	98.03%
d5-Indole-3-acetic acid	76937-78-5	C ₁₀ H ₄ D ₅ NO ₂	MedChemExpress, New Jersey, USA	97.94%
d6-(+)-Absciscic acid	35671-08-0	C ₁₅ H ₁₄ D ₆ O ₄	OlChemIm, Olomouc, CZ	96.81%
d6-N6-Isopentenyladenine	175733-28-5	C ₁₀ H ₇ D ₆ N ₅	OlChemIm, Olomouc, CZ	98%

Table A-2: Solvents used for the untargeted metabolome and targeted phytohormone analysis.

Analysis	Solvent	Specification	Hersteller
Metabolomics	Acetonitrile	LiChroSolv®	Merck, Darmstadt, DE
	Methanol	LiChroSolv®	Merck, Darmstadt, DE
	Formic acid (FA)	LiChropur®	Merck, Darmstadt, DE
	Isopropanol	LiChroSolv®	Merck, Darmstadt, DE
	NH ₄ FA	Supelco EMSURE®	Merck, Darmstadt, DE
	Water	LiChroSolv®	Merck, Darmstadt, DE
Phytohormones	Acetonitrile	HiPerSolv Chromanorm®	VWR International, Darmstadt, DE
	Formic acid	MS-grade, Art. 94318	Honeywell Fluka, Morristown, USA
	Isopropanol	HiPerSolv Chromanorm®	VWR International, Darmstadt, DE
		LiChrosolv®	Merck, Darmstadt, DE
	Methanol	HiPerSolv Chromanorm®	VWR International, Darmstadt, DE
	Ultrapure water	In-house purification apparatus	see Table A-7

Table A-3: Chemicals used to prepare nutrient solution for the tomato plant cultivation.

Substance	Formula	Nutrient	CAS	Manufacturer
Calcium nitrate tetrahydrate	Ca(NO ₃) ₂ x 4H ₂ O	Ca,N	13477-34-4	Roth, Karlsruhe, DE
Iron EDTA complex	FeNaEDTA x 3H ₂ O	Fe	15708-41-5	Roth, Karlsruhe, DE
Boric acid	H ₃ BO ₃	B	10043-35-3	Merck, Darmstadt, DE
Manganese sulfate heptahydrate	MnSO ₄ x H ₂ O	Mn	10034-96-5	Merck, Darmstadt, DE
Zink sulfate heptahydrate	ZnSO ₄ x 7H ₂ O	Zn	7446-20-0	Merck, Darmstadt, DE
Copper sulfate pentahydrate	CuSO ₄ x 5H ₂ O	Cu	7758-99-8	Merck, Darmstadt, DE
Sodium molybdate dihydrate	NaMoO ₄ x 2H ₂ O	Mo	10102-40-6	Roth, Karlsruhe, DE
Ammonium dihydrogen phosphate	NH ₄ H ₂ PO ₄	N,P	7722-76-1	Roth, Karlsruhe, DE
Potassium dihydrogen phosphate	KH ₂ PO ₄	K, P	7778-77-0	Merck, Darmstadt, DE
Potassium Sulfate	K ₂ SO ₄	K,S	7778-80-5	Roth, Karlsruhe, DE
Potassium nitrate	KNO ₃	K,N	7757-79-1	Merck, Darmstadt, DE
Magnesium sulfate heptahydrate	MgSO ₄ x 7 H ₂ O	Mg,S	10034-99-8	Merck, Darmstadt, DE
Potassium nitrate	KNO ₃	K,N	7757-79-1	Merck, Darmstadt, DE

Plant Cultivation Equipment

Table A-4: Equipment used for the cultivation of tomato plants.

System/Product	Model/Name	Manufacturer
Sodium vapor lamps	Son-T PIA Green Power	Philips (Eindhoven, NL)
LED lighting system	Apollo Vertical	Future LED (Berlin, DE)
Plant chamber control module	Tecon 2300	Tecon AG (Oberuzwil, CH)
Plant chamber control software	JC ESC300	Johnson Controls (Milwaukee, US)
Humidity control system	EDB20	WLG GmbH (Frankfurt a.M., DE)
Substrate	Gramoflor „Topf/Pikier Substrat“	Gramoflor GmbH (Vechta, DE)

Analytical Instruments and Appliances

Table A-5: UHPLC-MS instrumental setup used in metabolome analysis.

Part	Model	Manufacturer
UHPLC	1290 Infinity UPLC	Agilent, Santa Clara, USA
MS	TripleTOF 5600+	AB Sciex, Darmstadt, DE
Column	Acquity UPLC HSS T3 Premier; 2,1 × 150 mm; 1,8 µm	Waters, Milford, USA
Pre-Column	Acquity HSS T3 Premier; 2,1 × 5 mm; 1,8 µm	Waters, Milford, USA
Operational Software	Analyst (Version 1.6.2)	AB Sciex, Darmstadt, DE

Table A-6: UHPLC-MS instrumental setup and software used in phytohormone analysis.

Part	Model	Manufacturer
UHPLC	Nexera LC-System Control module: CBM-20A Degasser: DGU-20A5 Pumps: LC-30AD Autosampler: SIL-30AC Column oven: CTO-20AC	Shimadzu, Duisburg, DE
MS (ESI Triple Quad)	5500 QTrap	AB Sciex, Darmstadt, DE
Column	Acquity UPLC Peptide CSH C18 Column (150 mm x 2,1 mm; 1,7 µm, 130 Å)	Waters, Milford, USA
Pre-Column	Acquity UPLC CSH C18 VanGuard Pre-column (5 mm x 2,1 mm; 1,7 µm, 130 Å)	Waters, Milford, USA
Operational Software	Analyst (Version 1.6.2)	AB Sciex, Darmstadt, DE
HPLC Software Plugin	Shimadzu HPLC Control (Version 6.5.0.13)	Shimadzu, Duisburg, DE
Analysis Software	PeakView (Version 2.2) Sciex OS-Q (Version 1.6.1.29803)	AB Sciex, Darmstadt, DE
Integration Software	MultiQuant (Version 2.1.1)	AB Sciex, Darmstadt, DE

Table A-7: Laboratory appliances used in this work.

Appliance	Model	Manufacturer
Analytical balance (0.1 mg)	analytic BP221S	Sartorius, Göttingen, DE
Analytical balance (0.01 mg)	analytic CPA225D	Sartorius, Göttingen, DE
Universal balance (10 mg)	universal U6100D	Sartorius, Göttingen, DE
Pipettes	Research (Plus) series	Eppendorf, Hamburg, DE
Freeze drying	Alpha 2-4 LSC	Christ, Osterode, DE
	Alpha 2-4 LSC basic	Christ, Osterode, DE
	VaCo 5-II	Zirbus, Bad-Grund, DE
Ball milling	MM 400	Retsch, Haan, DE
	MM 500 Control	Retsch, Haan, DE
Thermomixer	Comfort 5355	Eppendorf, Hamburg, DE
Ultrasonic bath	Sonorex digitec	BANDELIN, Berlin, DE
	Sonorex Super RK255H	BANDELIN, Berlin, DE
Vortex	Vortex Genie 2	Scientific Industries, Bohemia, USA
Centrifuges	Centrifuge 5415R	Eppendorf, Hamburg, DE
	Multifuge X Pro series	Fisher Scientific, Schwerte, DE
SPE chamber (incl. Accessories)	Chromabond series	Macherey-Nagel, Düren, DE
Evaporation racks	In-house custom built	
Ultrapure water apparatus	LaboStar, 0,2 µm filter	Siemens, Günzburg, Deutschland

8 Appendix

8.1 Materials and Methods

Potato Metabolome Analysis

Table A-8: Preparation of 1 L of the Murashige & Skoog medium used for growing potato plants in an in-vitro gel culture. The medium was prepared and used at the KIT Molecular Phytopathology Department, the origins and purity of the individual chemicals are not known.

Substance class	Substance	Stock conc (g/L)	Added vol. (mL)	Final conc.
Macronutrients	CaCl ₂ x 2 H ₂ O	4.5	100	0.45 g/L
	KH ₂ PO ₄	1.7	100	0.17 g/L
	KNO ₃	19	100	1.9 g/L
	MgSO ₄ x 7 H ₂ O	3.7	100	0.37 g/L
	NH ₄ NO ₃	16.5	100	1.65 g/L
Micronutrients	H ₃ BO ₃	6.2	1	6.2 mg/L
	KI	0.83	1	0.83 mg/L
	ZnSO ₄ x 7 H ₂ O	8.6	1	8.6 mg/L
	MnSO ₄ x H ₂ O	16.9	1	16.9 mg/L
	CuSO ₄ x 5 H ₂ O	0.03	1	0.03 mg/L
	CoCl ₂ x 6 H ₂ O	0.03	1	0.03 mg/L
	Na ₂ MoO ₄ x 2 H ₂ O	0.25	1	0.25 mg/L
	Na-FeEDTA	3.6	10	36 mg/L
	Myo-Inositol	10	10	100 mg/L
Vitamins	Nicotinic acid	0.1	10	1 mg/L
	Pyridoxine HCl	0.1	10	1 mg/L
	Thiamine HCl	1	10	10 mg/L
Sugar	Sucrose	Direct addition		30 g/L
Gelling agent	Phytigel	Direct addition		3 g/L

Table A-9: Samples prepared and measured on each study day of the metabolome analyses of potato plants. QC, Quality control.

Day	Polarity	Study samples	QC samples	Controls	Total samples	Date
A1	Positive	19	6	5	30	30.01.2023
A2	Positive	34	3	0	37	31.01.2023
A3	Positive	32	3	0	35	01.02.2023
A4	Positive	33	3	0	36	02.02.2023
A5	Negative	19	6	5	30	08.02.2023
A6	Negative	34	3	0	37	09.02.2023
A7	Negative	32	3	0	35	10.02.2023
A8	Negative	33	3	0	36	11.02.2023

Table A-10: Preparation of 20 mL IS-Mix 1 for metabolome analysis. Resulting sample concentrations were 50-fold lower than the IS mix concentrations. Conc, Concentration; IS, Internal standard(s).

Substance	Stock conc. (mM)	Solvent	Added volume (μ L)	IS-Mix conc. (μ M)
D-[$^{13}\text{C}_6$] Mannitol	5.0	H ₂ O	500.0	125
[$^{13}\text{C}_6$] Saccharose	5.0	H ₂ O	500.0	125
[$^{13}\text{C}_5$, ^{15}N] Glucose	10.0	0.5 mM HCl in H ₂ O	400.0	200
[$^{13}\text{C}_5$, ^{15}N] Valine	10.0	0.5 mM HCl in H ₂ O	400.0	200
[$^{13}\text{C}_9$, ^{15}N] Phenylalanine	10.0	0.5 mM HCl in H ₂ O	400.0	200
Histamine- <i>d</i> ₄	10.0	H ₂ O	250.0	125
Methyl-D-Glucose	50.0	H ₂ O	100.0	250
D-Pinitol	100.0	H ₂ O	50.0	250
Methyl-2-desoxy-D-ribose	100.0	H ₂ O	50.0	250
Phenyl-D-glucose	100.0	H ₂ O	50.0	250
Ultrapure water	/	/	17300	/

Table A-11: Preparation of 20 mL IS-Mix 2 for metabolome analysis. Resulting sample concentrations were 50-fold lower than the IS mix concentrations. Conc, Concentration; IS, Internal standard(s).

Substance	Stock conc. (mM)	Solvent	Added vol. (μ L)	IS-Mix conc. (μ M)
2-(4-Chlorophenyl)ethylamine	100.0	Methanol	50.0	250
Ferulic acid- <i>d</i> ₃	10.0	Methanol	200.0	100
5-Bromine-2-Hydroxyhippuric acid	10.0	Methanol	500.0	250
[$^{13}\text{C}_6$] <i>trans</i> -Resveratrol	10.0	DMSO	200.0	100
2-Amino-6-chlorine purine	2.5	Methanol	1,000.0	125
3,4-(Methylenedioxy)cinnamic acid	2.5	Methanol	1,000.0	125
Hexylamine	100.0	Methanol	50.0	250
Benzoic acid- <i>d</i> ₅	10.0	Ethanol	500.0	250
Methanol	/	/	16,500.0	/

Table A-12: Settings, components and solutions used in metabolomics analyses

Component / Parameters	Used setting / material
Oven temperature	40 °C
Flow	0,4 mL/min
Eluent A	2 mM Ammonium formate + 0,05% Formic acid (v/v) in H ₂ O (pH 3,1)
Eluent B	Acetonitrile
Injection volume	2 μ L
Needle level	-1 mm
Needle flush solution	H ₂ O /Acetonitrile 50:50 (v/v)
Piston backflush solution	H ₂ O /Isopropanol 90:10 (v/v)

Table A-13: UHPLC-Gradient used for metabolomics analyses

Time (min)	% Eluent B
0	3
1	3
14	99
20	99
20,5	3
25,5	3

Table A-14: MS-parameter settings during metabolomics analyses

Component	Parameter	Setting
Ion source (ESI)	Capillary position	5 (vertical), 5 (horizontal)
	Temperature	550°C
	Ion spray voltage floating	+5500 V (ESI+), -5500 V (ESI-)
	Curtain gas pressure (CUR)	45 psi
	Ion source gas 1 pressure (GS1)	60 psi
	Ion source gas 2 pressure (GS2)	60 psi
	Declustering potential (DP)	70 V
Scan-Mode	Product Ion Scan	High Sensitivity
Quadrupole (Q1)	Mass range precursor ions	<i>m/z</i> 80-1500
	Cycle time	100 ms
Collision chamber (Q2)	CAD-Gas	high
	Collision energy (CE)	35 V
	Collision energy spread (CES)	± 15 V
TOF	Mass range of fragments	<i>m/z</i> 50-1500
	Accumulation time	25 ms
Detector	Detector voltage	2150 V (Q2 and ToF)
		2200 V (Q1)

Table A-15: Parameters of the independent data acquisition performed during metabolomics analyses

Parameter	Setting
Candidates	3
Charge State	No limitation
Threshold	> 450 cps
Exclude isotopes	1 Da
Mass tolerance	25 ppm
Exclude target ion	after: 2 occurrences for: 3 s

Phytohormone Analysis Method Development

Table A-16: Phytohormone metabolites considered for inclusion into the targeted analysis method. Grouped metabolite categories are marked in bold. “Catabolite” refers to any irreversibly inactivated form in this table.

Phytohormone	PH class	Metabolite type	Prioritization	Acquisition
Abscisic acid	Abscisates	Bioactive form	0	Available
8'-Hydroxyabscisic acid	Abscisates	Catabolite	2	Not acquired
Dihydrophaseic acid	Abscisates	Catabolite	1	Acquired
Dihydrophaseic acid glucoside	Abscisates	Catabolite	2	Not acquired
Phaseic acid	Abscisates	Catabolite	2	Not acquired
Abscisic acid glucose ester	Abscisates	Intermediate form	1	Acquired
9- <i>cis</i> -Neoxanthin	Abscisates	Precursor	3	Not acquired
Abscisic aldehyde	Abscisates	Precursor	1	Acquired
Xanthoxin	Abscisates	Precursor	3	Not acquired
4-Chloroindole-3-acetic acid	Auxins	Bioactive form	2	Acquired
Indole-3-acetic acid	Auxins	Bioactive form	0	Available
Indole-3-acrylic acid	Auxins	Bioactive form	0	Available
Indole-3-butyric acid	Auxins	Bioactive form	0	Available
Phenylacetic acid	Auxins	Bioactive form	0	Available
2-Oxoindole-3-acetic acid	Auxins	Catabolite	1	Acquired
3-Methylen-2-oxoindole	Auxins	Catabolite	2	Not acquired
3-Methyloxoindole	Auxins	Catabolite	1	Acquired
Glucose 2-oxoindole-3-acetate	Auxins	Catabolite	2	Not acquired
Indole-3-acetyl glucose ester	Auxins	Catabolite	1	Acquired
Indole-3-acetyl-L-aspartic acid	Auxins	Catabolite	1	Acquired
Indole-3-carboxylic acid	Auxins	Catabolite	0	Available
Indole-3-methanol	Auxins	Catabolite	2	Not acquired
Indolenin epoxide	Auxins	Catabolite	3	Not acquired
Indole-3-acetate methyl ester	Auxins	Intermediate form	2	Acquired
Inositol indole-3-acetate	Auxins	Intermediate form	2	Not acquired
Other Indole-3-acetic acid amino acid conjugates	Auxins	Intermediate form	2	6 acquired
Indole	Auxins	Precursor	2	Not acquired
Indole-3-acetaldoxim	Auxins	Precursor	2	Not acquired
Indole-3-acetonitrile	Auxins	Precursor	0	Available
Indole-3-pyruvate	Auxins	Precursor	0	Acquired
L-Tryptophane	Auxins	Precursor	0	Available
Indole-3-acetamide	Auxins	Precursor	2	Acquired
24-Epibrassinolide	Brassinosteroids	Bioactive form	0	Available
Brassinolide	Brassinosteroids	Bioactive form	0	Available
26-Hydroxybrassinolide	Brassinosteroids	Catabolite	1	Not acquired
Brassinolide-23-O-glucoside	Brassinosteroids	Catabolite	1	Not acquired
C2/C3-Hydroxy epimers of brassinolide or castasterone	Brassinosteroids	Catabolite	3	Not acquired
Castasterone glucosides	Brassinosteroids	Catabolite	2	Not acquired
Hydroxycastasterone	Brassinosteroids	Catabolite	2	Not acquired
Other brassinosteroid catabolites	Brassinosteroids	Catabolite	2	Not acquired
Other brassinosteroid glucosides	Brassinosteroids	Catabolite	2	Not acquired
Other Hydroxybrassinolides	Brassinosteroids	Catabolite	2	1 acquired
6-Oxocampestanol	Brassinosteroids	Precursor	1	Acquired
Cathasterone	Brassinosteroids	Precursor	1	Not acquired
Castasterone	Brassinosteroids	Precursor / bioactive form	1	Acquired
<i>meta</i> -Topolin	Cytokinins	Bioactive form	0	Available
<i>N</i> 6-Isopentenyladenine (iP)	Cytokinins	Bioactive form	0	Available
<i>ortho</i> -Topolin	Cytokinins	Bioactive form	2	Acquired
<i>trans</i> -Zeatin (tZ)	Cytokinins	Bioactive form	0	Available

Phytohormone	PH class	Metabolite type	Prioritization	Acquisition
(E)-5-hydroxy-4-methylpent-3-en-2-one	Cytokinins	Catabolite	1	Not acquired
4-Methylpent-3-en-2-one	Cytokinins	Catabolite	0	Available
Dihydrozeatin	Cytokinins	Catabolite	1	Acquired
Imines from oxidative cytokinin degradation	Cytokinins	Catabolite	3	Not acquired
iP-N7-glucoside	Cytokinins	Catabolite	1	Not acquired
iP-N9-glucoside	Cytokinins	Catabolite	2	Acquired
tZ-N7-glucoside	Cytokinins	Catabolite	1	Acquired
tZ-N9-glucoside	Cytokinins	Catabolite	2	Not acquired
Other iP/tZ glycosides and conjugates	Cytokinins	Catabolite / intermediate form	2	Not acquired
tZ-O-glucoside	Cytokinins	Catabolite / intermediate form	2	Not acquired
<i>cis</i> -Zeatin	Cytokinins	Precursor / bioactive form	1	Acquired
iP riboside	Cytokinins	Precursor / intermediate form	1	Acquired
other iP/tZ nucleotides	Cytokinins	Precursor / intermediate form	2	Not acquired
tZ riboside	Cytokinins	Precursor / intermediate form	0	Available
Ethylene	Ethylene	Bioactive form	3	Not acquired
Malonyl-ACC	Ethylene	Catabolite-like	1	Not acquired
<i>N</i> -Glutamyl-ACC	Ethylene	Catabolite-like	2	Not acquired
1-Aminocyclopropane-1-carboxylic acid	Ethylene	Precursor	0	Available
S-Adenosylmethionine	Ethylene	Precursor	0	Available
Cyanoformic acid	Ethylene	Precursor-related	3	Not acquired
Methylthioadenosin	Ethylene	Precursor-related	2	Acquired
β-Cyanoalanine	Ethylene	Precursor-related	2	Acquired
Dehydro-Gibberellin A12	Gibberellins	Bioactive form	2	Not acquired
Gibberellic acid	Gibberellins	Bioactive form	0	Available
Gibberellin A1	Gibberellins	Bioactive form	1	Acquired
Gibberellin A4	Gibberellins	Bioactive form	0	Available
Gibberellin A7	Gibberellins	Bioactive form	2	Acquired
16,17-Dihydroxy-Gibberellin A4	Gibberellins	Catabolite	2	Not acquired
Gibberellin A34	Gibberellins	Catabolite	1	Acquired
Gibberellin A4 methyl ester	Gibberellins	Catabolite	2	Acquired
Gibberellin A8	Gibberellins	Catabolite	1	Acquired
Other gibberellin catabolites	Gibberellins	Catabolite	2	Not acquired
ent-Kaurenoic acid	Gibberellins	Precursor	2	Acquired
Gibberellin A12	Gibberellins	Precursor	1	Not acquired
Gibberellin A15	Gibberellins	Precursor	2	Not acquired
Gibberellin A19	Gibberellins	Precursor	2	Not acquired
Gibberellin A20	Gibberellins	Precursor	1	Acquired
Gibberellin A24	Gibberellins	Precursor	2	Not acquired
Gibberellin A44	Gibberellins	Precursor	2	Not acquired
Gibberellin A53	Gibberellins	Precursor	1	Not acquired
Gibberellin A9	Gibberellins	Precursor	1	Acquired
Gibberellin A12 aldehyde	Gibberellins	Precursor	2	Acquired
(±) Jasmonic acid isoleucine	Jasmonates	Bioactive form	0	Available
<i>Cis</i> -3-hexenal	Jasmonates	Bioactive form	0	Available
<i>Trans</i> -2-hexenal	Jasmonates	Bioactive form	0	Available
(±)12-COOH-JA-Ile	Jasmonates	Catabolite	2	Not acquired
(±)12-OH-JA-Ile	Jasmonates	Catabolite	1	Not acquired
Other jasmonate catabolites	Jasmonates	Catabolite	2	Not acquired
(±)12-Hydroxyjasmonate	Jasmonates	Catabolite /	1	Acquired

Phytohormone	PH class	Metabolite type	Prioritization	Acquisition
		bioactive form		
(±)12-Hydroxyjasmonate glucoside	Jasmonates	Catabolite / bioactive form	1	Acquired
<i>cis</i> -Jasmone	Jasmonates	Catabolite / bioactive form	2	Acquired
Other Jasmonate conjugates	Jasmonates	Catabolite / intermediate / bioactive forms	2	Not acquired
(±) Jasmonic acid	Jasmonates	Precursor	0	Available
12-Oxo-Phytodienoic acid	Jasmonates	Precursor	0	Available
Stereochemically pure jasmonates	Jasmonates	Precursor	2	Not acquired
α-Linolenic acid	Jasmonates	Precursor	2	Not acquired
Methyl jasmonate	Jasmonates	Transport form	0	Available
Salicylic acid	Salicylates	Bioactive form	0	Available
2,3-Dihydroxybenzoic acid	Salicylates	Catabolite	1	Acquired
2,5-Dihydroxybenzoic acid	Salicylates	Catabolite	1	Acquired
Sulfonated salicylate catabolites	Salicylates	Catabolite	2	Not acquired
Salicylate aspartate	Salicylates	Catabolite / intermediate form	2	Not acquired
Glucosyl salicylate	Salicylates	Intermediate form	1	Acquired
Methyl salicylate	Salicylates	Intermediate form	2	Acquired
Salicylate-2- <i>O</i> -glucoside	Salicylates	Intermediate form	1	Acquired
<i>N</i> -Hydroxypipicolinic acid	Salicylates	Intermediate form / related metabolite	2	Acquired
(-)-Chorismic acid	Salicylates	Precursor	1	Acquired
Benzoic acid	Salicylates	Precursor	0	Available
Isochorismate-9-glutamate	Salicylates	Precursor	3	Not acquired
Isochorismic acid	Salicylates	Precursor	1	Not acquired
<i>ortho</i> -Coumaric acid	Salicylates	Precursor	0	Available
<i>trans</i> -Cinnamic acid	Salicylates	Precursor	0	Available
5-Deoxystrigol	Strigolactones	Bioactive form	1	Acquired
7-Oxo-orobanchyl acetate	Strigolactones	Bioactive form	2	Not acquired
Non-canonical strigolactones	Strigolactones	Bioactive form	2	Not acquired
Orobanchol	Strigolactones	Bioactive form	1	Acquired
Orobanchyl acetate	Strigolactones	Bioactive form	1	Not acquired
Solanacol	Strigolactones	Bioactive form	2	Not acquired
Strigol	Strigolactones	Bioactive form	1	Acquired
5-Hydroxy-3-methylbutenolide	Strigolactones	Catabolite	1	Acquired
Carlactone	Strigolactones	Precursor	1	Not acquired
Carlactonic acid	Strigolactones	Precursor	1	Not acquired
Methyl carlactonoate	Strigolactones	Precursor	1	Not acquired

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Table A-17: Candidate analytes tested for inclusion in the method, their stock solution compositions and, where applicable, reasons for non-inclusions in the final MRM method (highlighted in orange). ID, Abbreviated analyte ID; LOD, Limit of detection; RT, Retention time.

Substance	ID	PH class	Solvent	Stock (mM)	Stock availability	In final method	Reason for exclusion
(+)-Absciscic acid	ABA	Abscisates	Heptane	10	available	Yes	
(+)-Absciscic acid b-D-glucose ester	ABA-GE	Abscisates	Methanol	10	prepared	Yes	
(+)-Absciscic aldehyde	ABAlD	Abscisates	Methanol	10	prepared	Yes	
Dihydrophaseic acid	DHPA	Abscisates	Methanol	10	prepared	Yes	
2-Oxindole-3-acetic acid	OxIAA	Auxins	Methanol	10	prepared	Yes	
3-Methyloxindole	Me-OxI	Auxins	Methanol	10	prepared	Yes	
4-Chloroindole-3-acetic acid	4Cl-IAA	Auxins	Methanol	10	prepared	Yes	
Indole-3-acetamide	IAC	Auxins	Methanol	10	prepared	Yes	
Indole-3-acetate methyl ester	IAA-ME	Auxins	Methanol	10	prepared	Yes	
Indole-3-acetic acid	IAA	Auxins	Methanol	10	available	Yes	
Indole-3-acetonitrile	IAN	Auxins	Methanol	10	prepared	Yes	
Indole-3-acetyl b-D-glucose ester	IAA-GE	Auxins	Methanol	10	prepared	Yes	
Indole-3-acetyl-L-alanine	IAA-Ala	Auxins	Methanol	10	prepared	Yes	
Indole-3-acetyl-L-aspartic acid	IAA-Asp	Auxins	DMSO	10	prepared	Yes	
Indole-3-acetyl-L-glutamic acid	IAA-Glu	Auxins	DMSO	10	prepared	No	Very high LOD (UHPLC-MS)
Indole-3-acetyl-L-isoleucine	IAA-Ile	Auxins	Methanol	10	prepared	Yes	
Indole-3-acetyl-L-leucine	IAA-Leu	Auxins	Methanol	10	prepared	Yes	
Indole-3-acetyl-L-phenylalanine	IAA-Phe	Auxins	Methanol	10	prepared	Yes	
Indole-3-acetyl-L-valine	IAA-Val	Auxins	Methanol	10	prepared	Yes	
Indole-3-acrylic acid	IACrA	Auxins	Ethanol	10	available	No	RT overlap with important analytes
Indole-3-butyric acid	IBA	Auxins	Methanol	10	prepared	No	RT overlap with important analytes
Indole-3-carbinol	IC	Auxins	Methanol	10	available	No	No analyte signal (UHPLC-MS)
Indole-3-carboxylic acid	ICA	Auxins	Ethanol	10	available	Yes	
Indole-3-pyruvate	IPY	Auxins	Methanol	10	prepared	No	No analyte signal (UHPLC-MS)
Phenylacetic acid	PAA	Auxins	Ethanol	8	available	Yes	
Tryptophol	TPL	Auxins	Methanol	10	prepared	Yes	
24-Epi brassinolide	epiBL	Brassinosteroids	Ethanol	10	available	No	Coelution with BL (UHPLC-MS)
6-Oxocampestanol	O-CAMP	Brassinosteroids	DMSO	1	prepared	No	No analyte signal (MS only)
Brassinolide	BL	Brassinosteroids	DMSO	10	prepared	Yes	
Castasterone	CS	Brassinosteroids	Methanol	10	prepared	Yes	
Isohomobrassinolide	IHBL	Brassinosteroids	Acetonitrile	10	prepared	Yes	
4-Methylpent-3-en-2-one	MPO	Cytokinins	Methanol	10	prepared	No	No analyte signal (MS only)
cis-Zeatin	cZ	Cytokinins	Methanol	10	prepared	Yes	
Dihydrozeatin	DHZ	Cytokinins	Methanol	10	prepared	Yes	
Kinetin	KIN	Cytokinins	Methanol	10	prepared	Yes	
Kinetin riboside	KIN-R	Cytokinins	Methanol	10	prepared	Yes	

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Substance	ID	PH class	Solvent	Stock (mM)	Stock availability	In final method	Reason for exclusion
<i>meta</i> -Topolin	mT	Cytokinins	Methanol	10	prepared	Yes	
N6-Isopentenyladenine	iP	Cytokinins	Ethanol	10	available	Yes	
N6-Isopentenyladenine-N9-glucoside	iP-9G	Cytokinins	Methanol	10	prepared	Yes	
N6-Isopentenyladenosine	iP-R	Cytokinins	Methanol	10	prepared	Yes	
<i>ortho</i> -Topolin	oT	Cytokinins	DMSO	10	prepared	Yes	
<i>trans</i> -Zeatin	tZ	Cytokinins	Ethanol	10	available	Yes	
<i>trans</i> -Zeatin riboside	tZ-R	Cytokinins	Ethanol	10	available	Yes	
<i>trans</i> -Zeatin-N7-glucoside	tZ-7G	Cytokinins	DMSO	10	prepared	Yes	
1-Aminocyclopropane-1-carboxylic acid	ACC	Ethylene metabolites	Methanol	10	prepared	No	Interfering signals (UHPLC-MS + Sample prep.)
5'-Methylthioadenosine	MTA	Ethylene metabolites	Methanol	10	prepared	No	Interfering signals (UHPLC-MS + Sample prep.)
b-Cyano-L-alanine	CN-Ala	Ethylene metabolites	DMSO	5	prepared	No	No analyte signal (UHPLC-MS)
S-Adenosylmethionine chloride	SAM	Ethylene metabolites	Wasser	10	prepared	No	No analyte signal (UHPLC-MS)
ent-Kaurenoic acid	eKA	Gibberellins	Methanol	10	prepared	No	No fragmentation (MS only)
Gibberellin A1	GA1	Gibberellins	Methanol	10	prepared	Yes	
Gibberellin A12 aldehyde	GA12-Ald	Gibberellins	Methanol	10	prepared	Yes	
Gibberellin A20	GA20	Gibberellins	Methanol	10	prepared	Yes	
Gibberellin A3	GA3	Gibberellins	Ethanol	10	available	Yes	
Gibberellin A34	GA34	Gibberellins	Methanol	5	prepared	Yes	
Gibberellin A4	GA4	Gibberellins	Ethanol	10	available	Yes	
Gibberellin A4 methyl ester	GA4-ME	Gibberellins	Methanol	10	prepared	No	Very high LOD (UHPLC-MS)
Gibberellin A7	GA7	Gibberellins	Methanol	10	prepared	Yes	
Gibberellin A8	GA8	Gibberellins	Methanol	10	prepared	Yes	
Gibberellin A9	GA9	Gibberellins	Methanol	10	prepared	Yes	
(±)12-Hydroxyjasmonic acid	12OH-JA	Jasmonates	Methanol	50	prepared	Yes	
(±)12-Hydroxyjasmonic acid glucoside	12OH-JA-G	Jasmonates	Methanol	10	prepared	Yes	
(±)Jasmonic acid	JA	Jasmonates	Ethanol	10	available	Yes	
(±)Jasmonic acid isoleucine	JA-Ile	Jasmonates	Ethanol	5	available	Yes	
12-Oxo-Phytodienoic acid	OPDA	Jasmonates	Methanol	0.1	prepared	Yes	
<i>Cis</i> -3-hexenal	C3H	Jasmonates	Ethanol	10	available	No	No analyte signal (MS only)
<i>cis</i> -Jasmone	JSMO	Jasmonates	Methanol	10	prepared	Yes	
Dihydrojasmonic acid	DHJA	Jasmonates	Methanol	10	prepared	Yes	
Methyl jasmonate	MeJA	Jasmonates	Methanol	10	prepared	Yes	
<i>Trans</i> -2-hexenal	T2H	Jasmonates	Methanol	10	prepared	No	No analyte signal (MS only)
(-)-Chorismic acid	CHO	Salicylates	Methanol	10	prepared	No	Interfering signals (UHPLC-MS)
2,3-Dihydroxybenzoic acid	2,3-DHBA	Salicylates	Methanol	100	prepared	Yes	
2,5-Dihydroxybenzoic acid	2,5-DHBA	Salicylates	Ethanol	8	available	No	Very high LOD (UHPLC-MS)

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Substance	ID	PH class	Solvent	Stock (mM)	Stock availability	In final method	Reason for exclusion
Benzoic acid	BA	Salicylates	Ethanol	8	available	No	No analyte signal (MS only)
Methyl salicylate	MeSA	Salicylates	Methanol	10	prepared	No	No analyte signal (MS only)
<i>N</i> -Hydroxyphenylacetic acid	NHPA	Salicylates	Wasser	10	prepared	Yes	
<i>ortho</i> -Coumaric acid	oCuA	Salicylates	Ethanol	10	available	Yes	
Salicylic acid	SA	Salicylates	Ethanol	8	available	Yes	
Salicylic acid 2-O- β -D-glucoside	SA-G	Salicylates	Methanol	10	prepared	Yes	
Salicylic acid β -D-glucose ester	SA-GE	Salicylates	Methanol	10	prepared	Yes	
<i>trans</i> -Cinnamic acid	tCA	Salicylates	Ethanol	8	available	Yes	
($\pm$)5-Deoxystrigol	D-STR	Strigolactones	Methanol	10	prepared	Yes	
($\pm$)-Orobanchol	ORO	Strigolactones	Methanol	10	prepared	Yes	
($\pm$)Strigol	STR	Strigolactones	Methanol	10	prepared	Yes	
5-Hydroxy-3-methylbutenolide	HMB	Strigolactones	Methanol	20	prepared	Yes	

Table A-18: Internal standards tested for inclusion in the method, their stock solution compositions and, where applicable, reasons for non-inclusions in the final MRM method (highlighted in orange). ID, abbreviated analyte ID; LOD, limit of detection; RT, retention time.

Substance	ID	PH class	Solvent	Stock (mM)	Stock availability	In final method	Reason for exclusion
d6-(+)-Abscisic acid	d6-ABA	Abscisates	DMSO	10	prepared	Yes	
d2-Indole-3-acetic acid	d2-IAA	Auxins	Methanol	10	prepared	No	Interfering signals (UHPLC-MS + Sample prep.)
d5-Indole-3-acetic acid	d5-IAA	Auxins	DMSO	10	prepared	Yes	
d6- <i>N</i> 6-Isopentenyladenine	d6-iP	Cytokinins	Methanol	10	prepared	Yes	
d4-Aminocyclopropane-carboxylic acid	d4-ACC	Ethylene metabolites	Water	10	prepared	No	Interfering signals (UHPLC-MS + Sample prep.)
d2-Gibberellin A4	d2-GA4	Gibberellins	Methanol	1	prepared	Yes	
d4-Gibberellin A1	d4-GA1	Gibberellins	Methanol	1	prepared	Yes	
d5-($\pm$)-Jasmonic acid	d5-JA	Jasmonates	DMSO	10	prepared	Yes	
d4-Salicylic acid	d4-SA	Salicylates	Methanol	10	prepared	Yes	
($\pm$)-Strigolactone GR24	GR24	Strigolactones	Methanol	10	prepared	Yes	

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Table A-19: Transitions of the final MRM method as it was used during method validation (Chapter 3.3) and then applied in the analysis of tomato plants (Chapter 3.4.2). Transitions names ending in _1 are quantifier transitions, transition names ending in _2 qualifier transitions; _p and _n indicate the polarity. Transitions used for more than one analyte are marked in bold. IS, Internal standard; RT, Retention time; Q1, Quadrupole 1 (precursor ion); Q3, Quadrupole 3 (product ion); DP, Declustering potential in V; CE, Collision energy in V; CXP, Cell exit potential in V; wt, weight.

Analyte	Class	IS	Polarity	RT (min)	Window (s)	Transition	Q1 m/z	Q3 m/z	DP (V)	CE (V)	CXP (V)	wt
d5-Indole-3-acetic acid	Auxins	Yes	Positive	5.67	40	d5-IAA_p_1	181.1	133.9	60	25	7	1
						d5-IAA_p_2	181.1	132.9	60	26	20	1
d6-N6-Isopentenyladenine	Cytokinins	Yes	Positive	4.06	40	d6-iP_p_1	210.2	136.9	88	23	15	1
						d6-iP_p_2	210.2	118.9	88	45	15	1
Kinetin	Cytokinins	No ¹	Positive	3.55	40	KIN_p_1	216.1	80.9	104	26	16	1
						KIN_p_2	216.1	53.0	104	58	11	1
Kinetin riboside	Cytokinins	No ¹	Positive	4.56	40	KIN-R_p_1	348.1	216.0	88	25	8	1
						KIN-R_p_2	348.1	147.9	88	34	16	1
(±)-Strigolactone GR24	Strigolactones	Yes	Positive	8.86	40	GR24_p_1	299.1	185.1	64	13	6	1
						GR24_p_2	299.1	157.1	66	32	7	1
Dihydrophaseic acid	Abscisates	No	Positive	10.04	40	DHPA_p_1	283.2	173.2	100	20	10	1
						DHPA_p_2	283.2	209.1	100	13	6	1
4-Chloroindole-3-acetic acid	Auxins	No	Positive	6.72	40	4Cl-IAA_p_1	210.0	164.0	71	23	13	1
						4Cl-IAA_p_2	210.0	101.0	71	52	12	1
Indole-3-acetic acid	Auxins	No	Positive	5.72	40	IAA_p_1	176.1	103.0	83	44	11	2
						IAA_p_2	176.1	129.9	83	23	9	2
Indole-3-acetyl-L-aspartic acid	Auxins	No	Positive	9.15	60	IAA-Asp_p_1	291.1	129.9	84	31	12	1
						IAA-Asp_p_2	291.1	77.0	84	86	9	1
Indole-3-acetate methyl ester	Auxins	No	Positive	7.59	40	IAA-ME_p_1	190.1	129.9	76	22	10	1
						IAA-ME_p_2	190.1	103.0	76	48	11	1
Indole-3-acetonitrile	Auxins	No	Positive	6.92	40	IAN_p_1	157.1	116.9	93	31	17	1
						IAN_p_2	157.1	130.1	93	20	14	1
3-Methyloxindole	Auxins	No	Positive	5.70	40	Me-OxI_p_1	148.1	104.0	127	45	14	1
						Me-OxI_p_2	148.1	77.1	127	47	9	1
Tryptophol	Auxins	No	Positive	5.74	40	TPL_p_1	162.1	144.0	52	20	13	1
						TPL_p_2	162.1	143.1	52	34	15	1
cis- and trans-Zeatin	Cytokinins	No	Positive	2.40	120	Z_p_1	220.1	136.0	49	23	13	2
						Z_p_2	220.1	119.0	49	48	16	2
Dihydrozeatin	Cytokinins	No	Positive	2.27	90	DHZ_p_1	222.1	147.9	89	32	17	1
						DHZ_p_2	222.1	136.0	89	30	19	1
N6-Isopentenyladenine	Cytokinins	No	Positive	4.10	40	iP_p_1	204.1	136.0	84	23	13	2
						iP_p_2	204.1	119.0	84	45	19	2

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Analyte	Class	IS	Polarity	RT (min)	Window (s)	Transition	Q1 m/z	Q3 m/z	DP (V)	CE (V)	CXP (V)	wt
N6-Isopentenyladenine-N9-glucoside	Cytokinins	No	Positive	4.42	40	iP-9G_p_1	366.2	204.1	56	26	15	1
						iP-9G_p_2	366.2	136.1	56	40	14	1
N6-Isopentenyladenosine	Cytokinins	No	Positive	5.24	40	iP-R_p_1	336.2	204.0	75	24	9	1
						iP-R_p_2	336.2	136.0	75	42	11	1
meta-Topolin	Cytokinins	No	Positive	3.41	40	mT_p_1	242.1	107.0	122	30	12	1
						mT_p_2	242.1	77.0	122	61	13	1
ortho-Topolin	Cytokinins	No	Positive	4.29	40	oT_p_1	242.1	135.9	94	24	10	1
						oT_p_2	242.1	118.9	94	55	11	1
trans-Zeatin-N7-glucoside	Cytokinins	No	Positive	2.08	90	tZ-7G_p_1	382.2	220.2	80	28	4	1
						tZ-7G_p_2	382.2	136.0	80	48	11	1
trans-Zeatin riboside	Cytokinins	No	Positive	3.48	40	tZ-R_p_1	352.2	220.0	112	27	7	1
						tZ-R_p_2	352.2	136.0	112	42	13	1
cis-Jasmone	Jasmonates	No	Positive	9.20	40	JSMO_p_1	165.1	81.1	55	27	13	1
						JSMO_p_2	165.1	79.1	55	35	10	1
Methyl jasmonate	Jasmonates	No	Positive	9.42	40	MeJA_p_1	225.2	151.0	47	17	16	1
						MeJA_p_2	225.2	133.0	47	22	9	1
N-Hydroxypipelicolic acid	Salicylates	No	Positive	1.21	60	NHPA_p_1	146.1	70.0	80	26	10	1
						NHPA_p_2	146.1	109.9	80	18	11	1
(±)5-Deoxystriol	Strigolactones	No	Positive	11.32	40	D-STR_p_1	331.2	216.1	71	24	13	1
						D-STR_p_2	331.2	234.1	51	15	8	1
(±)-Orobanchol	Strigolactones	No	Positive	8.53	40	ORO_p_1	347.2	233.0	75	19	9	1
						ORO_p_2	347.2	135.0	75	34	17	1
(±)Strigol	Strigolactones	No	Positive	8.55	40	STR_p_1	347.2	215.1	76	20	7	1
						STR_p_2	347.2	163.0	84	25	10	1
d6-(+)-Absciscic acid	Abscisates	Yes	Negative	6.51	40	d6-ABA_n_1	269.2	158.7	-84	-17	-14	1
						d6-ABA_n_2	269.2	206.9	-84	-27	-5	1
Isohomobrassinolide	Brassinosteroids	No	Negative	10.38	40	IHBL_n_1	493.4	349.2	-78	-33	-10	1
						IHBL_n_2	493.4	143.1	-78	-37	-8	1
d2-Gibberellin A4	Gibberellins	Yes	Negative	8.32	40	d2-GA4_n_1	333.2	214.9	-83	-41	-19	1
						d2-GA4_n_2	333.2	271.1	-83	-31	-11	1
d4-Gibberellin A1	Gibberellins	Yes	Negative	5.01	40	d4-GA1_n_1	351.2	233.0	-77	-43	-7	1
						d4-GA1_n_2	351.2	289.1	-77	-33	-4	1
d5-(±)-Jasmonic acid	Jasmonates	Yes	Negative	7.42	40	d5-JA_n_1	214.2	62.0	-70	-17	-7	1
						d5-JA_n_2	214.2	109.0	-70	-27	-6	1
d4-Salicylic acid	Salicylates	Yes	Negative	6.65	90	d4-SA_n_1	141.1	78.0	-65	-42	-9	1
						d4-SA_n_2	141.1	69.0	-65	-39	-7	1
(+)Absciscic acid	Abscisates	No	Negative	6.53	40	ABA_n_1	263.1	203.9	-43	-26	-22	2
						ABA_n_2	263.1	201.1	-43	-19	-8	2

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Analyte	Class	IS	Polarity	RT (min)	Window (s)	Transition	Q1 m/z	Q3 m/z	DP (V)	CE (V)	CXP (V)	wt
(+)Absciscic acid b-D-glucose ester	Abscisates	No	Negative	5.14	40	ABA-GE_n_1	425.2	152.9	-83	-29	-10	1
						ABA-GE_n_2	425.2	263.0	-83	-17	-12	1
Absciscic aldehyde	Abscisates	No	Negative	6.70	40	ABAlde_n_1	247.1	124.9	-100	-27	-12	1
						ABAlde_n_2	247.1	203.1	-100	-31	-10	1
Indole-3-acetyl-L-alanine	Auxins	No	Negative	5.41	40	IAA-Ala_n_1	245.1	87.9	-66	-21	-6	1
						IAA-Ala_n_2	245.1	156.0	-66	-17	-10	1
Indole-3-acetyl b-D-glucose ester	Auxins	No	Negative	4.54	40	IAA-GE_n_1	336.1	173.9	-85	-14	-14	1
						IAA-GE_n_2	336.1	130.0	-85	-31	-14	1
Indole-3-acetyl-L- isoleucine & -leucine	Auxins	No	Negative	7.55	40	IAA-Ile_n_1	287.1	130.0	-74	-24	-17	1
						IAA-Ile_n_2	287.1	156.0	-74	-22	-8	1
Indole-3-acetyl-L-phenylalanine	Auxins	No	Negative	7.82	40	IAA-Phe_n_1	321.1	164.0	-60	-23	-6	1
						IAA-Phe_n_2	321.1	147.0	-60	-39	-18	1
Indole-3-acetyl-L-valine	Auxins	No	Negative	6.82	40	IAA-Val_n_1	273.1	116.0	-61	-25	-10	1
						IAA-Val_n_2	273.1	156.0	-61	-21	-10	1
Indole-3-acetamide	Auxins	No	Negative	4.38	40	IAC_n_1	173.1	130.0	-91	-22	-11	1
						IAC_n_2	173.1	127.9	-91	-29	-12	1
Indole-3-carboxylic acid	Auxins	No	Negative	5.29	40	ICA_n_1	160.0	115.9	-64	-21	-12	1
						ICA_n_2	160.0	65.8	-64	-64	-7	1
2-Oxindole-3-acetic acid	Auxins	No	Negative	4.33	40	OxIAA_n_1	190.1	130.8	-63	-33	-17	1
						OxIAA_n_2	190.1	146.0	-63	-20	-7	1
Phenylacetic acid	Auxins	No	Negative	5.64	40	PAA_n_1	135.1	90.9	-50	-10	-11	1
						PAA_n_2	135.1	90.9	-50	-25	-11	1
Brassinolide	Brassinosteroids	No	Negative	9.79	40	BL_n_1	479.3	129.1	-137	-34	-12	1
						BL_n_2	479.3	349.2	-137	-29	-5	1
Castasterone	Brassinosteroids	No	Negative	10.27	40	CS_n_1	463.3	129.0	-61	-34	-10	1
						CS_n_2	463.3	297.2	-61	-37	-10	1
Gibberellin A1	Gibberellins	No	Negative	5.07	40	GA1_n_1	347.2	229.0	-78	-41	-8	2
						GA1_n_2	347.2	259.2	-78	-26	-7	2
Gibberellin A12 aldehyde	Gibberellins	No	Negative	12.09	40	GA12-Ald_n_1	315.2	271.1	-131	-34	-15	1
						GA12-Ald_n_2	315.2	163.0	-131	-39	-12	1
Gibberellin A20	Gibberellins	No	Negative	6.76	40	GA20_n_1	331.2	287.1	-88	-29	-14	1
						GA20_n_2	331.2	225.0	-88	-36	-16	1
Gibberellin A3	Gibberellins	No	Negative	4.99	40	GA3_n_1	345.1	143.0	-52	-37	-11	1
						GA3_n_2	345.1	239.0	-52	-21	-6	1
Gibberellin A34	Gibberellins	No	Negative	5.07	40	GA34_n_1	347.2	259.1	-81	-23	-13	1
						GA34_n_2	347.2	199.1	-81	-37	-11	1
Gibberellin A4	Gibberellins	No	Negative	8.34	40	GA4_n_1	331.2	257.0	-74	-34	-7	2
						GA4_n_2	331.2	213.1	-74	-40	-11	2

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Analyte	Class	IS	Polarity	RT (min)	Window (s)	Transition	Q1 m/z	Q3 m/z	DP (V)	CE (V)	CXP (V)	wt
Gibberellin A7	Gibberellins	No	Negative	8.21	40	GA7_n_1	329.1	223.1	-58	-27	-13	1
						GA7_n_2	329.1	211.0	-58	-34	-8	1
Gibberellin A8	Gibberellins	No	Negative	3.85	40	GA8_n_1	363.1	275.1	-83	-25	-15	1
						GA8_n_2	363.1	257.0	-83	-23	-5	1
Gibberellin A9	Gibberellins	No	Negative	9.93	40	GA9_n_1	315.2	271.2	-74	-27	-5	1
						GA9_n_2	315.2	253.0	-74	-36	-12	1
(±)12-Hydroxyjasmonic acid	Jasmonates	No	Negative	4.49	40	12OH-JA_n_1	225.1	59.0	-66	-20	-6	1
						12OH-JA_n_2	225.1	165.0	-66	-16	-8	1
(±)12-Hydroxyjasmonic acid glucoside	Jasmonates	No	Negative	4.02	40	12OH-JA-G_n_1	387.2	207.0	-95	-29	-12	1
						12OH-JA-G_n_2	387.2	59.0	-95	-59	-8	1
Dihydrojasmonic acid	Jasmonates	No	Negative	8.24	40	DHJA_n_1	211.1	59.1	-73	-17	-4	1
						DHJA_n_2	211.1	111.0	-73	-29	-12	1
(±)Jasmonic acid	Jasmonates	No	Negative	7.44	40	JA_n_1	209.1	58.9	-62	-16	-8	2
						JA_n_2	209.1	96.8	-62	-26	-12	2
Jasmonic acid isoleucine	Jasmonates	No	Negative	8.75	40	JA-Ile_n_1	322.2	130.0	-91	-28	-14	1
						JA-Ile_n_2	322.2	128.0	-91	-27	-10	1
12-Oxo-Phytodienoic acid	Jasmonates	No	Negative	10.99	40	OPDA_n_1	291.2	165.1	-66	-25	-8	1
						OPDA_n_2	291.2	273.1	-66	-24	-12	1
2,5-Dihydroxybenzoic acid	Salicylates	No	Negative	4.13	90	2,5-DHBA_n_1	153.0	107.9	-50	-30	-12	1
						2,5-DHBA_n_2	153.0	109.0	-50	-19	-8	1
<i>ortho</i> -Coumaric acid	Salicylates	No	Negative	5.63	40	oCuA_n_1	163.0	118.9	-52	-18	-14	1
						oCuA_n_2	163.0	116.9	-52	-33	-10	1
Salicylic acid	Salicylates	No	Negative	6.73	120	SA_n_1	137.0	75.0	-62	-42	-9	2
						SA_n_2	137.0	65.0	-62	-43	-8	2
Salicylic acid 2-O-b-D-glucoside	Salicylates	No	Negative	3.38	40	SA-G_n_1	299.1	136.9	-63	-19	-8	1
						SA-G_n_2	299.1	92.9	-63	-49	-11	1
Salicylic acid-b-D-glucose ester	Salicylates	No	Negative	4.09	40	SA-GE_n_1	299.1	136.9	-66	-17	-13	1
						SA-GE_n_2	299.1	179.1	-66	-13	-22	1
<i>trans</i> -Cinnamic acid	Salicylates	No	Negative	6.92	40	tCA_n_1	147.1	77.0	-52	-30	-10	1
						tCA_n_2	147.1	103.1	-52	-15	-6	1
5-Hydroxy-3-methylbutenolide	Strigolactones	No	Negative	1.79	60	HMB_n_1	113.0	51.0	-48	-28	-6	1
						HMB_n_2	113.0	66.9	-40	-30	-8	1

¹ KIN and KIN-R were originally intended as internal standards, but reclassified after their detection in tomato samples.

Table A-20: Settings for MS and MS/MS optimization of analytes. Dimensionless settings use manufacturer-specific units.

Tab	Parameter	Setting
Syringe Pump Source/Gas	Syringe pump speed	7 μ L/min
	Curtain Gas	20
	Collision Gas	9
	Ion Spray Voltage	+4500 V / -4500V
	Temperature	0
	Ion Source Gas 1	20
	Ion Source Gas 2	0
Resolution	Ion Energy Q1	1 Unit
	Ion Energy Q3	1.6 Units
Detector	Channel electron multiplier	2500
Precursor Ion / Target Ion	Search Window	± 0.500 Da
Product Ion Selection	Number of most intense peaks	6
	Number of peaks in final method	4
	Exclusion range	± 5.000 Da of precursor <i>m/z</i>
	Minimal mass	45 Da
Optimizations ranges	Threshold	2000 cps
	Collision energy range	5 to 130 / -130 to -5 V
	Collision energy steps	2 V
	Collision exit potential range	0 to 55 / -55 to 0 V
	Collision exit potential steps	2 V
	Declustering potential range	1 to 300 / -300 to -1 V
	Declustering potential steps	5 V

Table A-21: Relevant settings of the final UHPLC-MS/MS targeted phytohormone analysis method. Dimensionless settings are manufacturer-specific.

Tab	Parameter	Setting
Pumps	Total Flow	0.5 mL/min
	Pumping Mode	Binary Flow
	Maximum Pressure	950 bar
Column Oven	Temperature	60°C
Diode Array Detector	Start/Stop Wavelength (Step)	220 nm / 400 nm (1 nm)
	Stop Wavelength	400 nm
	Sampling Frequency	6.25 Hz
	Sampling Period	0.24 s
	Slit Width	8 nm
	Cell Temperature	40°C
	Injection Volume	10 μ L
Autosampler	Needle Stroke	51 mm
	Sampling Speed	5 μ L/s
	Cooler Temperature	4°C
	Control Vial Stroke	51 mm
	Rinse Pump Mode	Rinse Port Only
	Rinse Pump Time	1 s
	Rinse Mode	Before and after aspiration
	Rinse Port Volume	300 μ L
	Curtain Gas	20
	Collision Gas	9
MS Source/Gas	Ion Source Gas 1	70
	Ion Source Gas 2	70
	Ion Spray Voltage	+5500 V / -4500V
	Temperature	550°C
	Entrance Potential	10
Scheduled MRM	Target scan time	0.25s

Table A-22: Concentration of phytohormone mixes used during method optimization and validation. Mixes were prepared separately for each class, using 30%ACN as solvent. ID, abbreviated analyte ID; conc., concentration.

Class	ID	Polarity	Mix 1 Base conc. at analysis (nM)	Mix 1 Conc. in mix (nM)	Mix 2 Base conc. at analysis (nM)	Mix 2 Conc. in mix (nM)
Internal Standards	d2-GA4	Negative	2	200	0.8	80
	d4-GA1	Negative	5	500	0.4	40
	d4-SA	Negative	50	5,000	10	1,000
	d5-IAA	Positive	25	2,500	5	500
	d5-JA	Negative	20	2,000	8	800
	d6-ABA	Negative	2	200	4	400
	d6-iP	Positive	0.2	20	0.4	40
	GR24	Positive	2	200	0.8	80
	IHBL	Negative	100	10,000	40	4,000
	KIN	Positive	1	100	0.1	10
	KIN-R	Positive	0.2	20	0.04	4
Abscisates	ABA	Negative	2	2,000	1.2	1,200
	ABA-GE	Negative	1	1,000	0.6	600
	ABAla	Negative	5	5,000	20	20,000
	DHPA	Positive	5	5,000	3	3,000
Auxins	IAA-Ala	Negative	20	2,000	4	4,000
	IAA-GE	Negative	5	500	10	10,000
	IAA-Ile	Negative	1	100	0.6	600
	IAA-Leu	Negative	1	100	0.6	600
	IAA-Phe	Negative	1	100	0.8	800
	IAA-Val	Negative	2	200	2	2,000
	IAC	Negative	5	500	2	2,000
	ICA	Negative	20	2,000	40	40,000
	OxIAA	Negative	1	100	2	2,000
	PAA	Negative	5	500	20	20,000
	4CI-IAA	Positive	50	5,000	2	2,000
	IAA	Positive	20	2,000	4	4,000
	IAA-ME	Positive	1	100	0.4	400
	IAN	Positive	5	500	2	2,000
	Me-OxI	Positive	2	200	4	4,000
	TPL	Positive	2	200	1.6	1,600
Brassinosteroids	BL	Negative	100	10,000	40	4,000
	CS	Negative	250	25,000	100	10,000
Cytokinins	cZ	Positive	2	2,000	4	4,000
	DHZ	Positive	2	2,000	4	4,000
	iP	Positive	0.5	500	1	1,000
	iP-9G	Positive	0.02	20	0.016	16
	iP-R	Positive	0.05	50	0.02	20
	mT	Positive	0.2	200	0.4	400
	oT	Positive	0.2	200	0.4	400
	tZ	Positive	5	5,000	10	10,000
	tZ-7G	Positive	1	1,000	2	2,000
	tZ-R	Positive	0.1	100	0.04	40
Gibberellins	GA1	Negative	1	1,000	0.8	800
	GA12-Ald	Negative	0.5	500	0.2	200
	GA20	Negative	2	2,000	1.6	1,600
	GA3	Negative	0.2	200	0.2	200
	GA34	Negative	0.2	200	0.8	800
	GA4	Negative	0.05	50	0.004	4
	GA7	Negative	5	5,000	3	3,000

Class	ID	Polarity	Mix 1 Base conc. at analysis (nM)	Mix 1 Conc. in mix (nM)	Mix 2 Base conc. at analysis (nM)	Mix 2 Conc. in mix (nM)
Jasmonates	GA8	Negative	1	1,000	0.8	800
	GA9	Negative	2	2,000	1.6	1,600
	12OH-JA	Negative	5	500	3	300
	12OH-JA-G	Negative	1	100	0.6	60
	DHJA	Negative	10	1,000	10	1,000
	JA	Negative	50	5,000	100	10,000
	JA-Ile	Negative	0.5	50	0.3	30
	OPDA	Negative	0.2	20	0.16	16
Salicylates	JSMO	Positive	1	100	1	100
	MeJA	Positive	1	100	0.8	80
	2,5-DHBA	Negative	200	20,000	40	4,000
	oCuA	Negative	2	200	2	200
	SA	Negative	20	2,000	8	800
	SA-G	Negative	2	200	0.4	40
	SA-GE	Negative	2	200	1.2	120
	tCA	Negative	50	5,000	100	10,000
Strigolactones	NHPA	Positive	300	30,000	1,200	120,000
	HMB	Negative	100	10,000	40	40,000
	D-STR	Positive	0.1	10	0.04	40
	ORO	Positive	0.2	20	0.2	200
	STR	Positive	2	200	1.6	1,600

Phytohormone Analysis Method Validation

Table A-23: Analyte and internal standard mixes used for method validation of quantified analytes and for the analysis of tomato plants. Mixes were prepared separately, using 30%ACN as solvent. ID, abbreviated analyte ID; conc., concentration.

Class	ID	Polarity	Mix 1 Base conc. at analysis (nM)	Mix 1 Conc. in mix (nM)	Mix 2 Base conc. at analysis (nM)	Mix 2 Conc. in mix (nM)
Internal standards	d6-ABA	Negative	10	1,000	20	2,000
	d2-GA4	Negative	10	1,000	5	500
	d4-GA1	Negative	20	2,000	40	4,000
	d5-JA	Negative	40	4,000	20	2,000
	d4-SA	Negative	100	10,000	200	20,000
	d5-IAA	Positive	40	4,000	20	2,000
	d6-iP	Positive	2	200	1	100
	GR24	Positive	/	/	0.8	80
Analytes	JA	Negative	0.4	800	/	/
	SA	Negative	12	24,000	/	/
	GA1	Negative	0.8	1,600	/	/
	GA4	Negative	0.3	600	/	/
	ABA	Negative	1.2	2,400	/	/
	tZ	Positive	4	8,000	/	/
	iP	Positive	0.08	160	/	/
	IAA	Positive	1.6	3,200	/	/

Table A-24: Analyte mixes used for spiking experiments in leaf and tomato samples. Spike mixes were prepared separately, using 30%ACN as solvent. ID, abbreviated analyte ID; conc., concentration; Lvl, level.

Tissue	ID	Spike Lvl 1 LLOQ factor at analysis	Spike Lvl 1 Base conc. at analysis (nM)	Spike Lvl 1 Conc. in mix (nM)	Spike Lvl 2 LLOQ factor at analysis	Spike Lvl 2 Base conc. at analysis (nM)	Spike Lvl 2 Conc. in mix (nM)
Leaf	JA	50	20	200	250	100	1,000
	SA	50	600	6,000	250	3,000	30,000
	GA1	50	40	400	250	200	2,000
	GA4	2.5	0.75	7.5	12.5	3.75	37.5
	ABA	50	60	600	250	300	3,000
	tZ	2.5	10	100	12.5	50	500
	iP	15	1.2	12	75	6	60
	IAA	20	32	320	100	160	1,600
Fruit	JA	5	2	20	25	10	100
	SA	50	600	6,000	250	3,000	30,000
	GA1	40	32	320	200	160	1,600
	GA4	2.5	0.75	7.5	12.5	3.75	37.5
	ABA	50	60	600	250	300	3,000
	tZ	2.5	10	100	12.5	50	500
	iP	2.5	0.2	2	12.5	1	10
	IAA	5	8	80	25	40	400

Phytohormone Analysis Method Application

Table A-25: Nutrient concentrations in nutrient solutions applied during plant cultivation. Micronutrient concentrations were the same for all three solutions. Conc., concentration.

Macronutrient	Solution 1 conc. (mM)	Solution 2 conc. (mM)	Solution 3 conc. (mM)	Micronutrient	Conc. (μ M)
NO ₃	10.75	12.25	13.75	Fe	15
NH ₄	1	1	0	Mn	10
H ₂ PO ₄	1.25	1.25	1.25	Zn	5
K	6.5	8.5	11	B	30
Ca	2.75	2.75	2.75	Cu	0.75
Mg	1	1	2	Mo	0.5
SO ₄	1.5	1.75	2.75		

8.2 Results

Potato Metabolome Analysis

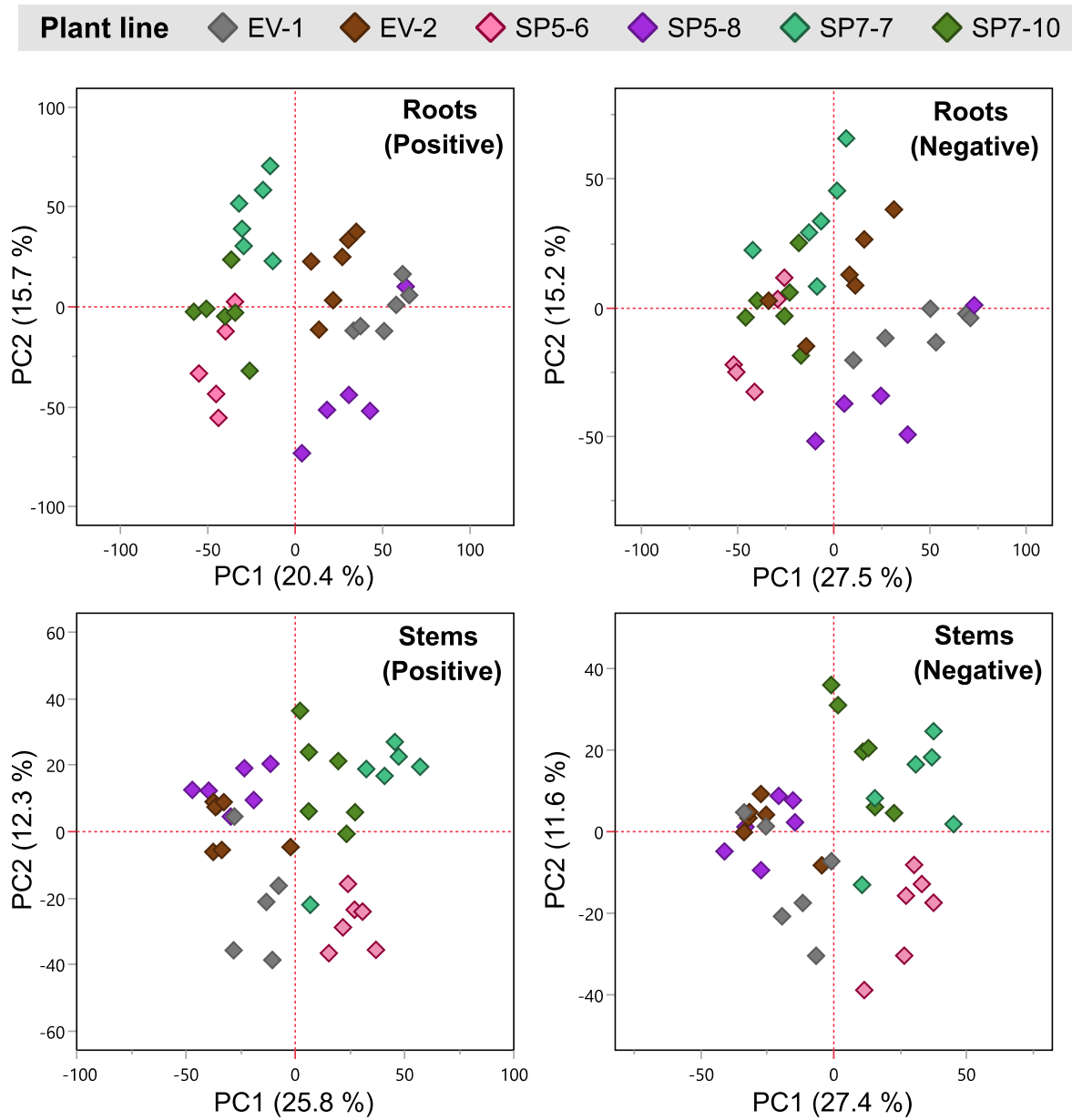


Figure A-1: Principal component analyses of potato root and stem samples. Samples are labelled according to their plant line. PC, principal component.

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Table A-26: Features identified at MSI level 1-3 after selection as highly relevant to SP7 expression in the potato leaves and stems. Distinct metabolites of the same type are distinguished by their suffix digit, features of the same metabolite are marked with FT1/2 as their suffix. Log2 FC values have been color coded on a fixed scale with negative values displayed in red and positive values displayed in blue. The color intensity maximum/minimum are fixed to the values +4 / -4. Substance class names were allocated based on the “ChemOnt” chemical taxonomy [290]. MSI, Metabolomics Standards Initiative, S/N, Signal/Noise, FC, Fold Change.

Tissue	Polarity	Name	MSI level	Substance class	Adduct ion <i>m/z</i>	Adduct type	S/N ratio	log ₂ FC SP7-7	log ₂ FC SP7-10
Leaf	Positive	Pyridoxin	2	Alkaloids	170.0780	[M+H] ⁺	13	-1.56	-1.45
		Pyridoxin hexoside 2	2	Alkaloids	332.1342	[M+H] ⁺	224	-0.99	-1.15
		Betaine	1	Amino acid (derivatives)	118.0852	[M+H] ⁺	404	1.10	1.01
		Taxifolin hexoside 1	2	Flavonoids	305.0646	[M+H] ⁺	80	1.12	1.10
		Phosphocholine	2	Organonitrogen compounds	184.0717	[M+H] ⁺	69		
Leaf	Negative	Taxifolin hexoside 2	2	Flavonoid <i>O</i> -glycosides	465.1087	[M-H] ⁻	193	1.27	1.22
		Isorhamnetin-3-rutinoside	2	Flavonoids	623.1691	[M-H] ⁻	199	1.05	1.26
		Vanillic acid <i>O</i> -(sulfohexoside)	2	Hydrolysable tannins	409.0472	[M-H] ⁻	665	1.21	1.21
		Furofuranoid lignan glycoside	3	Lignan glycosides	579.2086	[M-H] ⁻	347	1.44	1.41
		Vanillic acid-4- <i>O</i> -glucoside	1	Phenolic acids	329.0895	[M-H] ⁻	562	1.15	0.94
		Homogentisic acid	2	Phenolic acids	167.0325	[M-H] ⁻	44	0.87	0.51
		Lysophosphatidylcholine 16:0	2	Phospholipids	540.3364	[M+HCOO] ⁻	1,649	1.00	0.59
Stem	Positive	Pyridoxin hexoside 1	2	Alkaloids	332.1339	[M+H] ⁺	440	1.74	1.71
		Pyridoxin hexoside 2	2	Alkaloids	332.1342	[M+H] ⁺	224	1.08	0.93
		Betaine	1	Amino acid (derivatives)	118.0852	[M+H] ⁺	404	-0.87	-0.89
		Quercetin-3,4'- <i>O</i> -diglucoside _ FT1	1	Flavonoids	627.1575	[M+H] ⁺	841	1.35	1.17
		Quercetin-3,4'- <i>O</i> -diglucoside _ FT2	2	Flavonoids	303.0513	[M+H] ⁺	145	1.40	0.98
		Quercetin disaccharide / dihexoside (<i>O</i> -dHex, <i>O</i> -Hex) _ FT2	2	Flavonoids	303.0514	[M+H] ⁺	316	1.69	1.51
		Taxifolin hexoside 1	2	Flavonoids	305.0646	[M+H] ⁺	80	1.24	1.11
		Kaempferol disaccharide / dihexoside	2	Flavonoids	611.1580	[M] ⁺	203	1.04	0.97
		Quercetin disaccharide / dihexoside (<i>O</i> -dHex, <i>O</i> -Hex) _ FT1	2	Flavonoids	611.1632	[M+H] ⁺	308	0.97	0.95
		Quercetin trisaccharide (<i>O</i> -Hex-dHex, <i>O</i> -Hex)	2	Flavonoids	773.2147	[M+H] ⁺	10,828	-0.94	-0.95
		Isoflavone conjugate	3	Flavonoids	271.0611	[M+H] ⁺	122	1.40	1.26
		Tyrosol hexoside	2	<i>O</i> -glycosyl compounds	318.1523	[M+NH ₄] ⁺	2,280	1.23	1.11
		Phenethyl disaccharide	2	<i>O</i> -glycosyl compounds	434.2049	[M+NH ₄] ⁺	2,554	2.00	1.77
		Ferulic acid conjugate	3	Phenylpropanoids	177.0525	[M-H ₂ O+H] ⁺	124	0.73	0.71
		Caffeic acid conjugate	3	Phenylpropanoids	181.0483	[M+H] ⁺	10	1.28	1.29
								1.74	1.71

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Tissue	Polarity	Name	MSI level	Substance class	Adduct ion <i>m/z</i>	Adduct type	S/N ratio	log ₂ FC SP7-7	log ₂ FC SP7-10
Stem	Negative	Taxifolin hexoside 2	2	Flavonoids	465.1087	[M-H]-	193	1.76	1.52
		Kaempferol-3- <i>O</i> -sophoroside	2	Flavonoids	609.1531	[M-H]-	1726	1.24	1.02
		Flavonoid hexoside 1	3	Flavonoids	449.1144	[M-H]-	27,141	0.89	0.92
		Flavonoid hexoside 2	3	Flavonoids	449.1152	[M-H]-	1,478	0.97	0.97
		Flavonoid hexoside 3	3	Flavonoids	495.1195	[M+FA-H]-	790	0.87	1.28
		<i>N-trans</i> -Feruloyltyramine	1	Phenylpropanoids	312.1260	[M-H]-	1,593	-1.99	-1.98
		Coumaric acid <i>O</i> -hexoside	2	Phenylpropanoids	325.0974	[M-H]-	110	1.46	1.29
		Vanillic acid <i>O</i> -(6-deoxy-6-sulfo)hexoside	2	Phenylpropanoids	409.0472	[M-H]-	665	0.55	0.68
		Tyrosol disaccharide (Hex + Pent)	3	Phenylpropanoids	477.1658	[M+FA-H]-	1,269	1.06	0.97

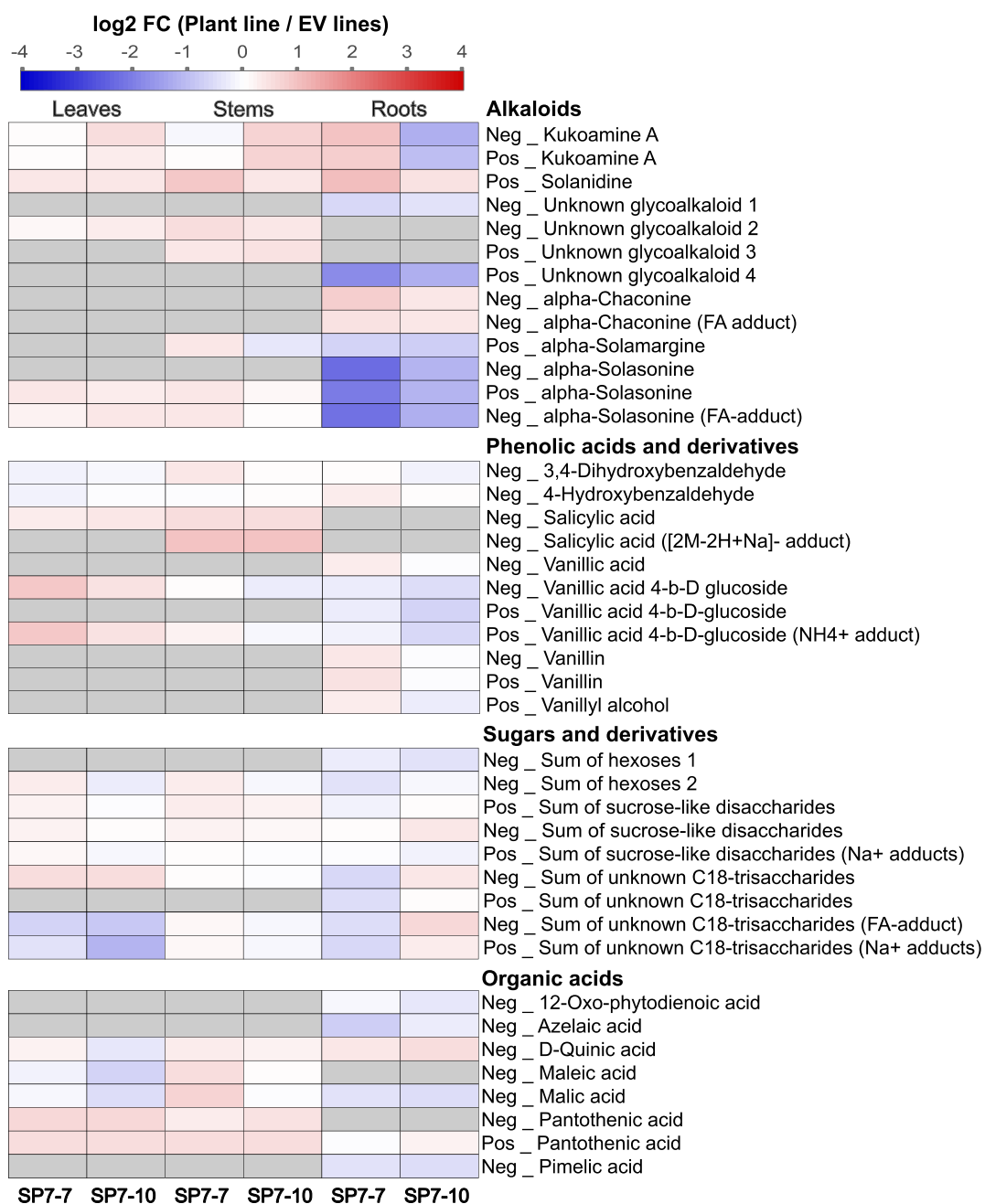


Figure A-2: Heatmap visualization of the log₂ FC of identified alkaloids, benzoic acids, sugars and other, not-classified analytes in the three analyzed potato tissues. Non-detected features are marked in grey. The mean signal intensity of both EV lines was used as the reference for FC calculation. FC, Fold change; EV lines, Empty vector (control) lines.

Phytohormone Analysis Method Development

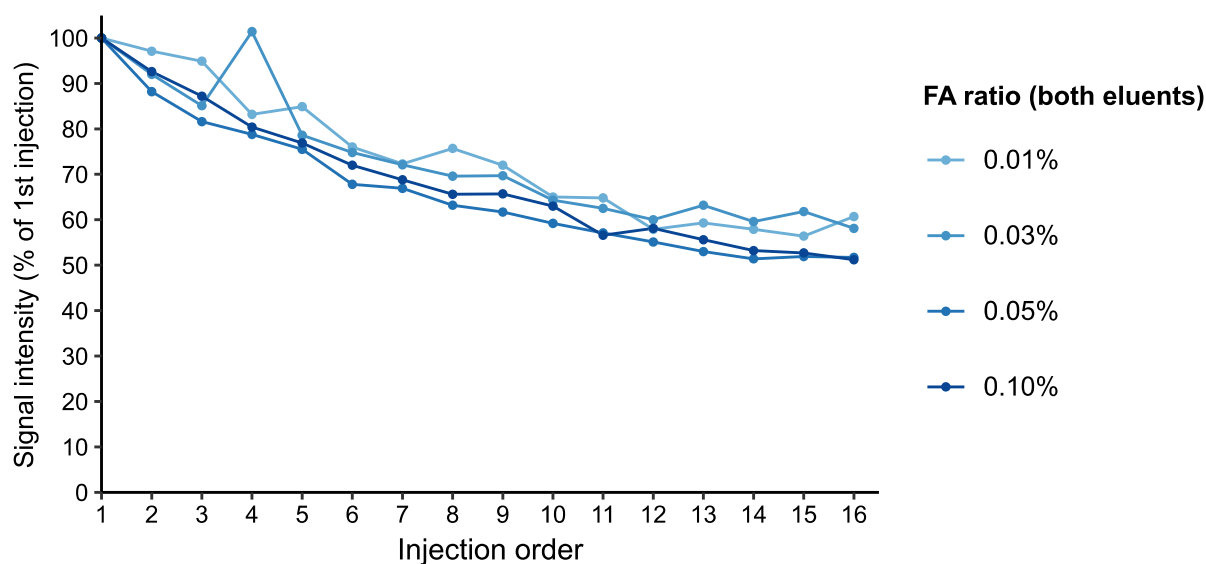


Figure A-3: Relative signal intensity of IAA (quantifier transition) with different FA contents of the eluents. The first 16 injections of the same solution on a freshly equilibrated column are shown. All percentages are volume percentages (v/v). FA, Formic acid; IAA, Indole-3-acetic acid.

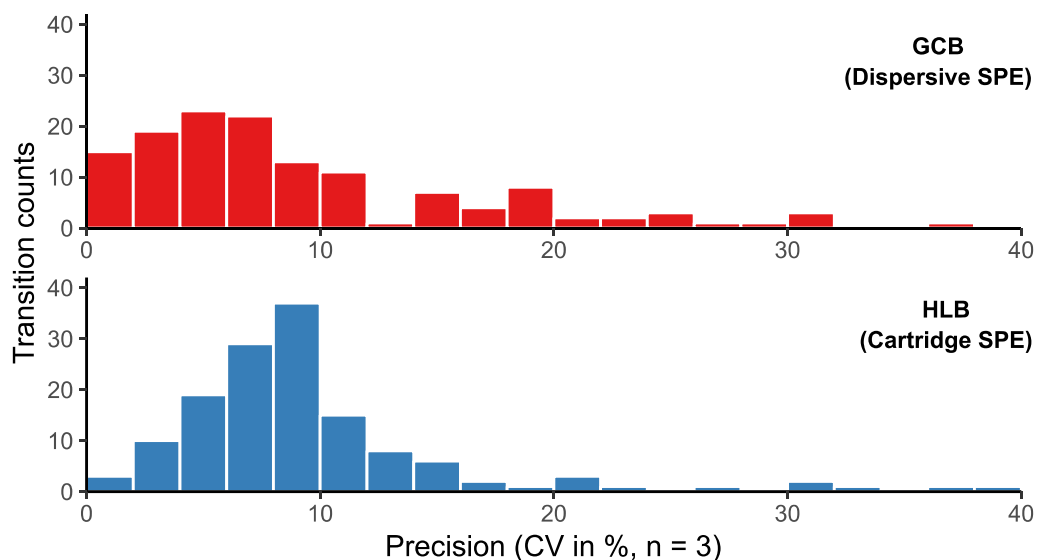


Figure A-4: Precision distributions of analyte mixes prepared using different purification workflows. It should be noted that the higher CVs observed for some analyte transitions prepared with GCB stem from low signal intensities (due to low recoveries). GCB, Graphitized carbon black; HLB, Hydrophilic-Lipophilic-Balanced; SPE, Solid phase extraction.

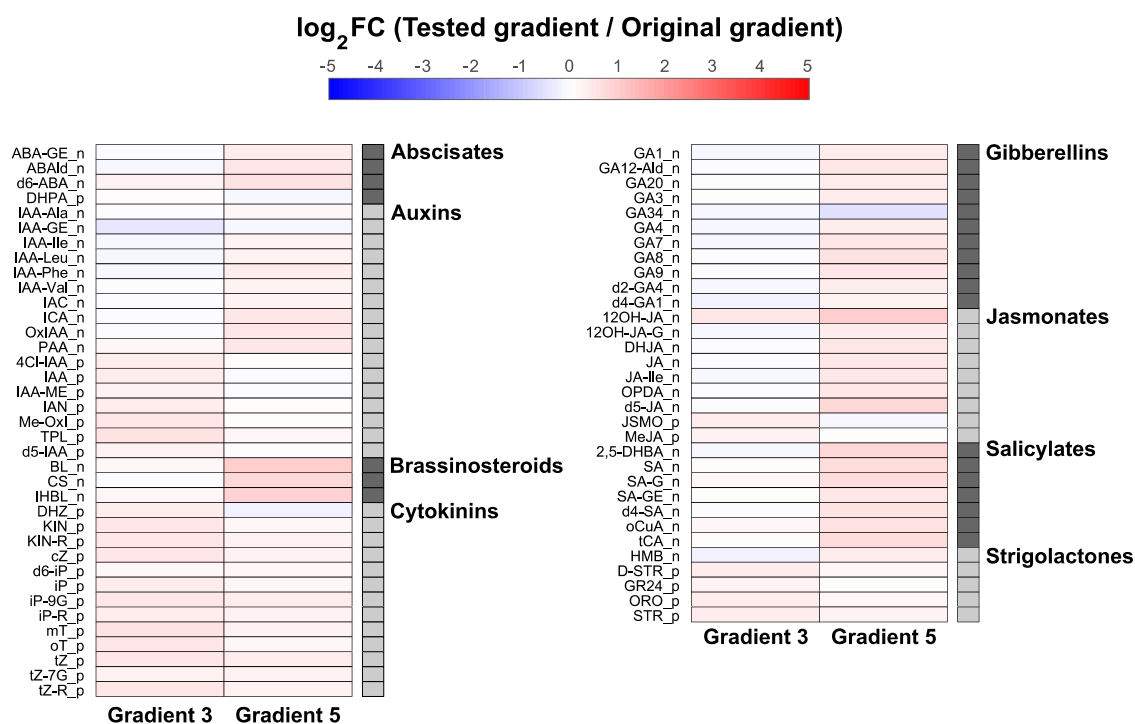


Figure A-5: Heatmap visualization of the log₂ FC of the phytohormone (metabolite) analytes measured with different UHPLC gradients. Metabolite phytohormone classes are annotated with colors. The best-performing eluent composition (0.03% FA in Eluent A only) was used for all experiments. Measurements with the original gradient were used as the reference for FC calculation. FC, Fold Change; FA, Formic acid.

Phytohormone Analysis Method Validation

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Table A-27: Instrumental validation parameters of all analyte transitions included in the final method. CVs were determined using eight replicates per concentration level. LODs and LLOQs of quantified analyte transitions (underlined) were re-determined separately (see Chapter 3.3.3.1). CV, Coefficient of variation. LOD, Limit of detection; LLOQ, Lower limit of quantification; n.d., Not determined; R², Coefficient of determination.

Class	Transition	LOD (fmol/ sample)	LLOQ (fmol/ sample)	Levels	CV (%) LLOQ	CV (%) 5x LLOQ	CV (%) 10x LLOQ	CV (%) 25x LLOQ	CV (%) 50x LLOQ	CV (%) 100x LLOQ	CV (%) 500x LLOQ	CV (%) 1000x LLOQ	R ²
Abscisates	d6-ABA_n_1	12	40	8	3.9	4.2	3.2	4.8	2.6	5.3	3.5	2.5	0.99504
	d6-ABA_n_2	80	400	6	4.2	5.3	6.4	2.4	2.5	3.3	n.d.	n.d.	0.99898
	<u>ABA_n_1</u>	<u>7</u>	<u>50</u>	8	10.0	5.1	6.2	4.5	4.5	1.7	2.4	1.0	0.99256
	<u>ABA_n_2</u>	<u>70</u>	<u>120</u>	8	8.5	5.2	2.4	1.9	3.1	2.4	5.7	2.8	0.98265
	ABA-GE_n_1	8	60	8	19.7	6.3	3.3	5.4	4.5	2.1	5.6	1.9	0.98910
	ABA-GE_n_2	10	60	8	10.8	5.6	6.7	3.1	4.9	3.2	3.9	2.9	0.98728
	ABAI_d_n_1	40	300	8	13.8	6.7	5.7	2.7	3.9	2.6	2.6	2.3	0.98006
	ABAI_d_n_2	300	2,000	8	9.2	5.8	6.0	2.5	3.2	2.7	6.4	2.9	0.95655
	DHPA_p_1	50	200	8	3.8	3.5	2.3	1.1	0.9	2.0	2.6	1.9	0.98659
Auxins	DHPA_p_2	100	300	8	3.6	2.5	1.9	0.7	3.1	3.7	2.1	1.3	0.90981
	d5-IAA_p_1	100	150	8	10.7	6.0	5.1	3.5	3.4	3.1	2.9	1.6	0.99188
	d5-IAA_p_2	150	500	6	12.2	6.1	7.6	4.5	3.4	4.2	n.d.	n.d.	0.96650
	4Cl-IAA_p_1	10	100	8	4.5	3.3	3.4	1.7	2.5	2.2	2.7	1.4	0.99657
	4Cl-IAA_p_2	100	200	8	8.0	3.5	4.4	3.2	3.7	6.1	2.9	1.5	0.89757
	<u>IAA_p_1</u>	<u>40</u>	<u>160</u>	8	11.5	6.7	4.8	4.5	2.7	2.3	3.5	2.1	0.98660
	<u>IAA_p_2</u>	<u>80</u>	<u>160</u>	8	6.4	3.7	4.0	3.1	3.9	5.6	2.5	1.4	0.79654
	IAA-Ala_n_1	2	16	8	12.6	7.7	5.8	4.4	5.1	5.9	4.7	3.6	0.99984
	IAA-Ala_n_2	40	400	8	12.5	6.6	4.0	4.1	3.0	2.2	4.2	2.9	0.96578
	IAA-Asp_p_1	6,000	40,000	4	9.5	2.6	1.8	1.3	n.d.	n.d.	n.d.	n.d.	0.85680
	IAA-Asp_p_2	20,000	80,000	4	7.3	1.5	0.9	1.1	n.d.	n.d.	n.d.	n.d.	0.84766
	IAA-GE_n_1	30	200	8	10.4	5.9	3.9	5.3	3.0	2.8	1.8	2.3	0.99900
	IAA-GE_n_2	300	1,000	8	7.2	7.1	4.2	2.1	2.9	1.2	4.2	2.8	0.95969
	IAA-Ile_n_1	0.8	4	8	14.0	9.4	4.9	11.2	6.3	5.6	5.3	4.7	0.99813
	IAA-Ile_n_2	6	60	8	7.9	10.6	7.5	6.0	5.7	3.3	6.3	2.4	0.95195
	IAA-Leu_n_1	0.8	4	8	10.6	7.5	9.9	5.4	4.3	3.6	3.6	4.6	0.99789
	IAA-Leu_n_2	6	60	8	14.0	10.2	5.8	3.8	4.4	5.3	7.4	4.0	0.94290
	IAA-ME_p_1	2	8	8	6.8	4.4	4.9	2.9	2.7	3.1	3.9	1.6	0.99568
	IAA-ME_p_2	8	40	8	8.9	4.7	3.4	3.8	3.5	5.3	2.8	1.9	0.87387
	IAA-Phe_n_1	0.4	2	8	9.4	7.1	4.0	3.3	2.7	1.4	1.3	0.9	0.99761
	IAA-Phe_n_2	40	80	6	8.3	5.6	6.5	2.0	3.3	2.8	n.d.	n.d.	0.99908
	IAA-Val_n_1	0.4	8	8	8.6	3.3	5.4	4.9	2.4	4.7	1.7	2.9	0.99742
	IAA-Val_n_2	80	200	8	7.7	10.4	5.1	5.7	4.3	2.4	6.2	3.6	0.95849
	IAC_n_1	10	100	8	6.2	5.0	2.1	5.2	2.9	3.5	3.2	1.5	0.99968

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Class	Transition	LOD (fmol/ sample)	LLOQ (fmol/ sample)	Levels	CV (%) LLOQ	CV (%) 5x LLOQ	CV (%) 10x LLOQ	CV (%) 25x LLOQ	CV (%) 50x LLOQ	CV (%) 100x LLOQ	CV (%) 500x LLOQ	CV (%) 1000x LLOQ	R ²
Brassinoster.	IAC_n_2	100	200	8	15.5	11.5	7.8	4.7	5.7	4.1	5.2	4.0	0.98240
	IAN_p_1	40	200	8	10.8	3.9	10.9	6.2	7.8	8.0	3.3	3.8	0.88959
	IAN_p_2	30	200	8	17.4	9.2	7.2	5.6	6.5	8.2	3.3	5.0	0.89676
	ICA_n_1	40	80	6	9.3	3.3	3.7	4.3	3.1	4.0	n.d.	n.d.	0.99923
	ICA_n_2	1,200	4,000	8	13.2	10.1	8.4	4.6	3.7	3.1	6.4	3.0	0.92589
	Me-Oxl_p_1	80	200	8	9.1	5.3	5.1	3.4	3.8	2.4	4.2	2.6	0.98703
	Me-Oxl_p_2	80	400	8	7.3	4.8	5.9	4.1	4.1	6.7	4.1	1.8	0.82551
	OxIAA_n_1	6	20	8	14.2	6.1	3.4	2.2	3.1	5.2	2.8	2.3	0.99905
	OxIAA_n_2	100	200	6	14.6	9.9	7.0	3.4	2.3	3.6	n.d.	n.d.	0.99961
	PAA_n_1	200	500	7	n.d.	9.6	3.4	6.8	2.9	2.3	2.1	1.2	0.99866
	PAA_n_2	1,000	2,000	7	n.d.	12.0	8.1	10.1	1.6	3.3	6.2	2.8	0.95538
	TPL_p_1	80	120	8	8.2	4.9	3.2	3.5	2.9	2.8	3.5	1.3	0.99295
	TPL_p_2	20	160	8	24.5	6.3	8.7	5.8	5.0	4.9	2.7	1.4	0.85494
	IHBL_n_1	600	4,000	6	10.6	10.1	8.1	4.9	4.8	2.7	n.d.	n.d.	0.95022
	IHBL_n_2	2,000	4,000	6	7.7	7.2	5.2	3.8	3.6	3.0	n.d.	n.d.	0.95168
	BL_n_1	1,000	4,000	6	6.4	6.8	4.4	3.1	3.5	1.6	n.d.	n.d.	0.92077
	BL_n_2	800	4,000	6	12.6	9.2	2.5	2.2	3.8	3.5	n.d.	n.d.	0.93635
	CS_n_1	500	10,000	6	12.8	7.3	7.4	6.7	4.9	8.2	n.d.	n.d.	0.86399
	CS_n_2	2,000	10,000	6	11.5	11.8	7.7	6.6	4.4	2.6	n.d.	n.d.	0.84648
Cytokinins	d6-iP_p_1	1.6	8	8	8.7	3.0	3.0	3.0	2.6	2.0	2.9	1.2	0.99763
	d6-iP_p_2	8	40	6	11.8	6.4	3.3	2.8	3.6	3.6	n.d.	n.d.	0.98455
	KIN_p_1	2	8	6	8.2	3.5	3.3	3.2	3.3	3.8	n.d.	n.d.	0.98256
	KIN_p_2	4	10	6	16.4	7.0	6.1	3.6	4.9	3.0	n.d.	n.d.	0.98050
	KIN-R_p_1	0.4	2	8	8.9	5.7	3.3	2.9	1.5	1.5	1.5	1.7	0.99855
	KIN-R_p_2	2	4	6	15.4	4.9	3.9	3.4	3.3	1.4	n.d.	n.d.	0.99608
	cZ_p_1	12	120	8	8.2	5.0	7.9	7.4	8.2	6.6	3.4	2.7	0.99793
	cZ_p_2	80	400	8	9.0	3.8	2.0	1.8	4.1	4.5	3.0	1.5	0.95103
	DHZ_p_1	80	400	8	5.6	3.1	4.5	1.0	2.6	4.8	4.1	1.7	0.95661
	DHZ_p_2	40	400	7	4.3	4.4	2.6	1.1	3.2	4.7	3.7	n.d.	0.93511
	iP_p_1	<u>1</u>	<u>4</u>	7	9.9	3.4	4.5	2.1	2.0	2.1	3.1	n.d.	0.99836
	iP_p_2	<u>4</u>	<u>8</u>	8	15.6	6.4	4.7	5.1	4.3	4.8	3.2	2.1	0.96133
	iP-9G_p_1	0.4	0.8	8	10.0	12.6	4.9	5.6	2.7	1.8	1.6	1.6	0.99934
	iP-9G_p_2	0.8	1.6	8	7.3	7.3	3.8	3.2	1.9	2.6	1.7	1.0	0.98979
	iP-R_p_1	0.5	2	8	9.2	2.9	3.2	3.6	2.7	2.7	0.7	1.1	0.98957
	iP-R_p_2	0.4	2	8	11.6	7.3	7.2	2.2	2.6	2.6	0.5	1.2	0.98992
	mT_p_1	1.2	8	8	8.2	5.3	5.3	1.6	1.6	0.9	2.4	0.8	0.99753
	mT_p_2	8	40	7	7.0	3.2	2.5	2.1	3.6	5.4	2.1	n.d.	0.95928
	oT_p_1	4	12	8	8.9	6.1	3.8	2.8	2.7	1.8	3.3	2.1	0.99849

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Class	Transition	LOD (fmol/ sample)	LLOQ (fmol/ sample)	Levels	CV (%) LLOQ	CV (%) 5x LLOQ	CV (%) 10x LLOQ	CV (%) 25x LLOQ	CV (%) 50x LLOQ	CV (%) 100x LLOQ	CV (%) 500x LLOQ	CV (%) 1000x LLOQ	R ²
Gibberellins	oT_p_2	8	40	8	3.8	7.3	4.3	2.3	4.7	3.7	3.6	2.0	0.97287
	tZ_p_1	<u>20</u>	<u>100</u>	8	5.5	7.2	4.7	3.0	4.7	3.2	3.1	1.9	0.99869
	tZ_p_2	<u>80</u>	<u>400</u>	8	8.0	5.6	3.3	3.1	3.9	5.5	3.9	2.3	0.96113
	tZ-7G_p_1	10	80	8	5.8	2.2	2.7	4.0	1.4	2.9	1.8	1.8	0.99880
	tZ-7G_p_2	40	200	8	6.8	7.6	3.9	1.6	1.7	2.8	1.2	1.6	0.98452
	tZ-R_p_1	0.6	4	8	5.8	4.0	2.2	1.1	2.3	2.7	1.3	1.2	0.98697
	tZ-R_p_2	2	4	8	8.5	2.4	1.9	2.7	2.2	2.8	1.2	1.4	0.98808
	d2-GA4_n_1	12	80	6	6.6	10.3	6.3	2.9	2.7	2.2	n.d.	n.d.	0.99916
	d2-GA4_n_2	16	80	6	9.0	7.3	7.6	3.0	2.7	2.3	n.d.	n.d.	0.99818
	d4-GA1_n_1	10	40	6	15.5	10.6	5.0	4.7	5.0	5.8	n.d.	n.d.	0.99913
	d4-GA1_n_2	5	40	6	8.5	9.5	3.6	4.4	3.2	5.5	n.d.	n.d.	0.99920
	GA1_n_1	<u>30</u>	<u>50</u>	8	10.6	6.6	4.9	2.4	3.2	2.2	2.6	2.0	0.98643
	GA1_n_2	<u>30</u>	<u>80</u>	8	12.4	6.6	4.3	2.9	2.9	1.3	2.4	2.6	0.98658
	GA12-Ald_n_1	4	20	8	2.0	6.1	2.9	1.8	2.5	2.0	3.2	2.9	0.97486
	GA12-Ald_n_2	10	20	8	11.2	4.5	6.8	2.6	2.2	1.1	3.3	3.0	0.97423
	GA20_n_1	8	20	8	13.3	9.8	1.9	5.4	5.1	4.6	3.6	5.1	0.99785
	GA20_n_2	80	160	8	17.4	11.4	7.6	7.7	3.3	4.4	2.3	3.6	0.96606
	GA3_n_1	2	12	8	11.4	5.5	5.1	2.4	3.2	5.6	2.2	2.4	0.99761
	GA3_n_2	12	20	8	11.1	10.0	6.5	5.5	3.6	4.6	3.7	4.4	0.97933
	GA34_n_1	12	20	3	n.d.	n.d.	n.d.	n.d.	n.d.	11.8	7.5	5.8	0.99990
	GA34_n_2	20	80	7	n.d.	11.7	14.8	6.7	4.2	6.9	2.9	1.6	0.98631
	GA4_n_1	<u>16</u>	<u>24</u>	7	7.2	4.3	2.5	1.9	2.4	1.0	2.3	n.d.	0.99730
	GA4_n_2	<u>16</u>	<u>32</u>	7	4.4	3.8	3.3	1.3	1.9	1.5	2.4	n.d.	0.99723
Jasmonates	GA7_n_1	20	40	8	6.6	6.9	5.8	2.7	2.2	2.0	1.7	1.5	0.99640
	GA7_n_2	100	300	8	8.5	8.7	5.8	5.4	1.3	2.1	3.7	2.3	0.96698
	GA8_n_1	4	20	8	8.3	4.6	2.8	2.1	1.9	2.4	2.6	1.3	0.99883
	GA8_n_2	40	80	8	5.3	4.4	3.7	4.9	3.1	1.8	2.5	2.4	0.97895
	GA9_n_1	12	40	8	5.6	3.1	1.5	2.3	1.2	2.1	1.5	1.5	0.99563
	GA9_n_2	16	160	8	12.4	4.8	3.7	2.2	3.1	1.2	1.9	2.0	0.96148
	d5-JA_n_1	8	16	8	5.3	4.1	4.5	3.3	3.1	2.2	3.6	3.6	0.99820
	d5-JA_n_2	120	800	6	10.6	6.1	7.5	3.4	3.5	1.7	n.d.	n.d.	0.99791
	12OH-JA_n_1	10	20	7	n.d.	10.8	4.8	7.2	5.2	3.7	3.8	1.8	0.99599
	12OH-JA_n_2	40	300	6	8.8	2.6	4.1	2.6	2.9	4.2	n.d.	n.d.	0.99686
	12OH-JA-G_n_1	10	60	6	8.3	3.9	5.2	2.5	3.0	2.5	n.d.	n.d.	0.99880
	12OH-JA-G_n_2	10	60	6	6.6	6.7	5.4	5.0	2.2	2.7	n.d.	n.d.	0.99837
	DHJA_n_1	n.d. ¹ .	40	8	7.5	7.3	2.9	3.2	3.1	2.0	2.2	1.7	0.99824
	DHJA_n_2	400	1,000	6	9.2	8.9	4.4	3.0	1.5	2.9	n.d.	n.d.	0.99269
	JA_n_1	<u>10</u>	<u>40</u>	8	4.3	5.3	3.4	3.6	2.7	3.0	2.7	2.1	0.99580

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Class	Transition	LOD (fmol/ sample)	LLOQ (fmol/ sample)	Levels	CV (%) LLOQ	CV (%) 5x LLOQ	CV (%) 10x LLOQ	CV (%) 25x LLOQ	CV (%) 50x LLOQ	CV (%) 100x LLOQ	CV (%) 500x LLOQ	CV (%) 1000x LLOQ	R ²
Salicylates	JA_n_2	2,000	10,000	6	11.6	5.2	6.1	3.8	3.0	1.4	n.d.	n.d.	0.98175
	JA-Ile_n_1	1	4	8	6.3	3.7	3.0	1.4	2.5	2.1	1.9	1.0	0.99800
	JA-Ile_n_2	10	30	6	6.9	8.1	6.6	2.6	0.8	2.1	n.d.	n.d.	0.99947
	JSMO_p_1	20	80	8	4.1	3.6	4.1	2.5	2.6	3.4	4.8	1.8	0.99387
	JSMO_p_2	40	100	6	9.0	6.1	6.7	8.7	3.8	5.6	n.d.	n.d.	0.96598
	MeJA_p_1	8	60	8	6.2	5.4	3.4	2.0	2.0	2.1	2.3	2.0	0.99801
	MeJA_p_2	20	80	6	6.7	3.9	5.1	5.1	3.0	2.2	n.d.	n.d.	0.98819
	OPDA_n_1	0.8	4	8	6.6	2.2	2.2	2.2	1.8	2.0	2.3	1.1	0.99752
	OPDA_n_2	8	16	6	8.1	5.4	3.7	3.6	1.6	1.8	n.d.	n.d.	0.99588
	d4-SA_n_1	50	1,000	6	14.4	4.0	4.4	3.4	3.4	3.1	n.d.	n.d.	0.99937
	d4-SA_n_2	200	1,000	6	3.9	3.2	1.9	2.0	2.2	1.7	n.d.	n.d.	0.99988
	2,5-DHBA_n_1	80	400	8	10.3	6.0	4.3	6.4	2.8	2.8	3.3	2.9	0.98400
	2,5-DHBA_n_2	2,000	4,000	6	16.2	7.0	5.2	2.0	3.8	1.1	n.d.	n.d.	0.99916
	NHPA_p_1	60,000	120,000	4	13.3	7.5	4.1	2.3	n.d.	n.d.	n.d.	n.d.	0.86247
	NHPA_p_2	60,000	120,000	4	15.1	6.9	4.2	2.6	n.d.	n.d.	n.d.	n.d.	0.86392
	oCuA_n_1	80	200	6	6.6	5.9	3.4	2.5	1.1	1.0	n.d.	n.d.	0.99889
	oCuA_n_2	80	200	6	5.0	8.1	7.0	4.5	3.3	1.9	n.d.	n.d.	0.99980
	SA_n_1	n.d. ¹	960	6	11.4	6.1	4.4	4.9	2.3	1.1	n.d.	n.d.	0.99936
	SA_n_2	n.d. ¹	1,280	6	7.8	3.8	3.4	2.8	1.7	1.6	n.d.	n.d.	0.99956
	SA-G_n_1	8	40	6	3.4	2.3	3.2	1.9	1.4	1.4	n.d.	n.d.	0.99925
	SA-G_n_2	8	40	6	6.2	4.7	2.3	1.6	1.8	0.9	n.d.	n.d.	0.99917
Strigolactones	SA-GE_n_1	8	40	8	5.4	5.5	4.9	3.3	5.0	4.7	2.1	2.8	0.99677
	SA-GE_n_2	40	120	6	16.7	6.5	4.1	3.0	3.2	2.6	n.d.	n.d.	0.99917
	tCA_n_1	1,000	2,000	8	7.4	7.5	5.9	3.1	6.0	4.4	1.9	3.5	0.99401
	tCA_n_2	4,000	10,000	5	12.5	5.7	6.3	5.2	3.5	n.d.	n.d.	n.d.	0.99912
	GR24_p_1	8	20	8	6.9	6.7	2.3	2.4	2.7	1.5	1.6	1.7	0.99945
	GR24_p_2	16	80	6	6.8	2.4	5.3	3.1	2.1	1.9	n.d.	n.d.	0.99117
	D-STR_p_1	1	4	8	3.4	5.0	2.9	2.4	2.4	2.5	1.4	1.1	0.98822
	D-STR_p_2	0.8	4	8	8.4	5.1	1.5	1.2	1.8	2.6	1.5	1.4	0.98566
	HMB_n_1	800	4,000	8	3.3	4.1	2.8	2.1	1.0	1.5	3.0	2.0	0.97547
	HMB_n_2	800	4,000	8	7.4	4.0	4.5	2.0	0.8	1.5	2.9	1.9	0.97461
	ORO_p_1	2	8	8	6.7	4.3	2.1	2.0	2.5	1.8	2.0	2.1	0.99842
	ORO_p_2	8	20	8	8.4	2.6	3.6	2.9	1.5	3.3	1.0	1.4	0.96745
	STR_p_1	20	80	8	11.8	4.0	4.1	2.8	1.3	1.2	2.0	1.4	0.99931
	STR_p_2	40	160	8	13.0	4.2	2.6	2.6	2.9	3.1	1.5	2.1	0.98202

¹ LODs could not be determined due to peaks in solvent blanks.

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Table A-28: Parameters of three separately prepared and measured calibration curves for each quantified analyte. Cal, Calibration number. ID, Abbreviated analyte ID (see Table A-17); IS, Internal standard; LLOQ/ULOQ, Lower/upper limit of quantification; R^2 , Coefficient of determination.

ID	IS	Cal	Levels	LLOQ (fmol)	ULOQ (fmol)	ULOQ (x-fold LLOQ)	Slope	Intercept	R^2	RMSE (%)	Level 1 (% bias)	Level >1 (max % bias)
ABA	d6-ABA	1	7	1.2	600	500	0.03898	0.00299	0.99934	2.7	4.8	7.9
		2	7	1.2	600	500	0.03919	0.00335	0.99962	2.3	2.8	6.7
		3	8	1.2	1,200	1,000	0.03844	0.00798	0.99950	2.4	13.0	7.3
GA1	d4-GA1	1	7	0.8	400	500	0.02623	-0.00065	0.99985	1.4	3.8	5.6
		2	7	0.8	400	500	0.02653	-0.00149	0.99963	1.7	11.3	6.6
		3	7	0.8	400	500	0.02691	-0.00464	0.99993	0.8	8.8	3.6
GA4	d2-GA4	1	6	0.3	30	100	0.03069	0.00205	0.99971	1.3	2.4	5.3
		2	6	0.3	30	100	0.03091	-0.00026	0.99963	2.0	4.3	2.9
		3	6	0.3	30	100	0.03082	0.00158	0.99972	1.5	3.8	3.5
IAA	d5-IAA	1	8	1.6	1,600	1,000	0.01703	-0.00114	0.99998	0.2	3.4	1.7
		2	8	1.6	1,600	1,000	0.01709	0.00018	0.99963	1.4	1.0	6.8
		3	8	1.6	1,600	1,000	0.01735	-0.00248	0.99897	4.9	13.7	12.9
iP	d6-iP	1	8	0.08	80	1,000	0.07156	-0.00228	0.99986	1.3	6.3	4.5
		2	8	0.08	80	1,000	0.07050	-0.00078	0.99988	1.0	4.0	3.9
		3	8	0.08	80	1,000	0.07200	-0.00967	0.99962	3.0	15.1	5.4
JA	d5-JA	1	7	0.4	200	500	0.00377	-0.00068	0.99958	2.5	17.3	6.7
		2	7	0.4	200	500	0.00387	-0.00094	0.99909	3.8	19.3	7.7
		3	6	0.4	40	100	0.00367	-0.00026	0.99921	3.6	9.5	3.9
SA	d4-SA	1	8	12	12,000	1,000	0.19527	0.03833	0.99871	6.0	12.8	6.8
		2	7	12	6,000	500	0.20393	-0.00177	0.99963	8.6	5.3	5.8
		3	8	12	12,000	1,000	0.20287	-0.00149	0.99969	2.6	0.1	6.7
tZ	d6-iP	1	8	4	4,000	1,000	0.25526	-0.02057	0.99976	2.1	4.2	4.7
		2	8	4	4,000	1,000	0.25184	-0.03002	0.99941	4.1	6.4	3.6
		3	8	4	4,000	1,000	0.25447	-0.03345	0.99970	2.8	8.0	6.1
	KIN	1	8	4	4,000	1,000	0.21764	-0.02655	0.99989	0.7	8.1	6.0
		2	8	4	4,000	1,000	0.21811	-0.03779	0.99994	0.5	13.0	5.8
		3	8	4	4,000	1,000	0.21951	-0.02145	0.99978	2.5	7.5	5.2

Table A-29: Intra- and interday precision and absolute recoveries of all semi-quantified analytes (qualifier transitions) at 2,5-fold concentration of the respective LLOQ. CV, coefficient of variation; n, number of datapoints.

Class	Transition	n (total)		Day 1	Day 2	Day 3	All days
Abscisates	ABA-GE_n_1	14	CV (%)	3.4	7.0	5.1	20.4
			Recovery (%)	79.3	80.3	118.0	90.7
	ABAla_n_1	14	CV (%)	3.4	4.8	3.7	21.4
Recovery (%)			68.8	71.1	105.9	80.2	
Auxins	DHPA_p_1	14	CV (%)	6.9	12.2	14.9	12.9
			Recovery (%)	45.8	43.4	51.2	46.5
	4Cl-IAA_p_1	14	CV (%)	4.1	8.2	2.1	6.8
			Recovery (%)	69.1	65.1	72.8	68.7
	IAA-Ala_n_1	14	CV (%)	5.0	15.5	2.4	10.5
			Recovery (%)	89.2	80.1	92.3	86.8
	IAA-Asp_p_1	14	CV (%)	4.5	14.3	4.2	23.9
			Recovery (%)	106.3	82.1	60.8	84.7
	IAA-GE_n_1	14	CV (%)	4.5	10.8	3.1	23.5
			Recovery (%)	76.8	73.6	117.5	87.3
	IAA-Ile_n_1	14	CV (%)	5.2	14.2	4.8	16.3
			Recovery (%)	81.6	74.2	102.9	85.0
	IAA-Leu_n_1	14	CV (%)	6.4	15.5	5.4	14.9
			Recovery (%)	80.0	73.6	97.0	82.6
	IAA-ME_p_1	14	CV (%)	3.2	12.4	4.2	16.7
			Recovery (%)	53.2	49.6	69.5	56.6
	IAA-Phe_n_1	14	CV (%)	5.7	15.4	3.0	14.3
			Recovery (%)	80.9	72.1	95.1	81.8
	IAA-Val_n_1	14	CV (%)	6.1	15.0	2.3	14.0
			Recovery (%)	81.9	74.1	96.8	83.4
	IAC_n_1	14	CV (%)	12.1	13.4	3.3	21.3
			Recovery (%)	78.9	72.8	110.7	85.8
	IAN_p_1	14	CV (%)	4.3	7.7	5.7	8.0
			Recovery (%)	63.2	63.2	71.2	65.5
	ICA_n_1	14	CV (%)	1.4	6.5	3.4	10.9
			Recovery (%)	78.4	75.2	94.3	81.8
	Me-OxI_p_1	14	CV (%)	4.7	7.5	4.2	7.0
			Recovery (%)	84.0	89.7	93.7	88.8
	OxIAA_n_1	14	CV (%)	43.2	52.3	12.8	37.5
			Recovery (%)	66.0	61.8	81.5	68.9
	PAA_n_1	14	CV (%)	5.4	20.1	5.4	35.3
			Recovery (%)	68.8	62.7	125.5	82.8
TPL_p_1	14	CV (%)	18.0	42.0	6.0	33.5	
		Recovery (%)	66.9	57.1	102.3	73.5	
Brassinoster.	BL_n_1	14	CV (%)	4.9	6.2	2.1	23.7
			Recovery (%)	61.1	58.0	94.1	69.4
Cytokinins	CS_n_1	14	CV (%)	3.5	5.5	5.2	29.1
			Recovery (%)	65.7	58.9	108.0	75.4
	cZ_p_1	14	CV (%)	7.1	4.2	6.2	7.4
			Recovery (%)	81.8	91.6	89.2	87.4
	DHZ_p_1	14	CV (%)	4.0	2.8	5.9	7.5
			Recovery (%)	79.9	91.0	91.2	87.1
	iP-9G_p_1	14	CV (%)	4.0	4.4	7.9	6.5
			Recovery (%)	79.0	77.8	85.1	80.3
	iP-R_p_1	14	CV (%)	10.1	4.3	3.5	7.2
			Recovery (%)	77.2	84.2	81.3	80.9
	KIN_p_1	14	CV (%)	11.5	6.5	5.9	13.8
			Recovery (%)	69.2	78.8	91.3	78.9
KIN-R_p_1	14	CV (%)	6.0	6.7	4.2	5.5	
		Recovery (%)	81.9	82.1	80.4	81.5	

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Class	Transition	n (total)		Day 1	Day 2	Day 3	All days
Gibberellins	mT_p_1	14	CV (%)	9.5	6.6	5.9	16.3
			Recovery (%)	49.0	56.1	69.5	57.4
	oT_p_1	14	CV (%)	9.2	6.3	2.8	8.5
			Recovery (%)	80.7	81.8	91.7	84.2
	tZ-7G_p_1	14	CV (%)	2.7	1.6	3.4	7.4
			Recovery (%)	87.8	97.8	103.7	95.9
	tZ-R_p_1	14	CV (%)	3.1	3.7	4.6	9.3
			Recovery (%)	45.5	50.5	55.9	50.2
	GA12-Ald_n_1	0	CV (%)	n.d.	n.d.	n.d.	n.d.
			Recovery (%)	n.d.	n.d.	n.d.	n.d.
	GA20_n_1	14	CV (%)	1.6	5.6	3.2	12.8
			Recovery (%)	82.0	81.6	105.0	88.4
	GA3_n_1	14	CV (%)	5.0	2.0	7.5	13.2
			Recovery (%)	70.3	80.0	94.2	80.6
	GA34_n_1	14	CV (%)	5.4	6.8	6.4	13.9
			Recovery (%)	81.9	79.8	104.5	87.6
	GA7_n_1	14	CV (%)	1.3	3.8	2.4	14.0
			Recovery (%)	77.6	75.8	101.3	83.7
	GA8_n_1	14	CV (%)	2.3	4.4	4.0	13.9
			Recovery (%)	87.3	89.1	115.8	96.1
	GA9_n_1	14	CV (%)	8.7	16.8	3.6	19.6
			Recovery (%)	49.3	44.6	65.6	52.3
Jasmonates	12OH-JA_n_1	14	CV (%)	3.2	4.0	1.6	12.7
			Recovery (%)	100.3	103.9	131.0	110.4
	12OH-JA-G_n_1	14	CV (%)	12.7	16.8	6.8	13.1
			Recovery (%)	94.5	93.7	105.6	97.4
	DHJA_n_1	14	CV (%)	1.4	5.1	2.2	15.6
			Recovery (%)	78.0	75.3	104.2	84.5
Salicylates	JA-Ile_n_1	14	CV (%)	2.2	5.0	2.8	11.6
			Recovery (%)	85.0	84.6	106.4	91.0
	JSMO_p_1	4	CV (%)	n.d.	n.d.	46.1	46.1
			Recovery (%)	n.d.	n.d.	14.2	14.2
	MeJA_p_1	14	CV (%)	5.5	22.1	13.3	27.1
			Recovery (%)	42.4	41.1	65.9	48.7
	OPDA_n_1	14	CV (%)	13.1	11.5	57.0	65.1
			Recovery (%)	13.9	11.8	29.9	17.7
	2,5-DHBA_n_1	12	CV (%)	57.5	45.7	57.1	53.1
			Recovery (%)	50.3	46.3	34.2	43.9
	NHPA_p_1	2	CV (%)	100.5	n.d.	n.d.	100.5
			Recovery (%)	17.3	n.d.	n.d.	17.3
	oCuA_n_1	14	CV (%)	5.5	6.3	4.3	11.6
			Recovery (%)	70.5	69.8	86.9	74.9
	SA-G_n_1	14	CV (%)	7.8	6.1	3.0	16.9
			Recovery (%)	82.2	81.0	112.6	90.5
	SA-GE_n_1	14	CV (%)	2.7	4.1	0.8	18.7
			Recovery (%)	78.6	80.1	114.8	89.5
Strigolactones	tCA_n_1	14	CV (%)	7.0	7.0	4.8	30.4
			Recovery (%)	69.0	66.0	120.0	82.5
	D-STR_p_1	14	CV (%)	9.2	6.7	13.4	18.8
			Recovery (%)	15.1	12.2	18.0	14.9
	HMB_n_1	14	CV (%)	7.2	16.4	8.5	20.9
			Recovery (%)	69.7	65.9	97.4	76.2
	ORO_p_1	14	CV (%)	3.0	7.1	2.0	9.6
			Recovery (%)	77.3	80.2	93.7	83.0
	STR_p_1	14	CV (%)	1.5	5.4	0.9	7.6
			Recovery (%)	79.5	85.5	94.0	85.8

Table A-30: Endogenous phytohormone concentrations determined in pooled tomato leaves and fruits, measured in triplicate. CV, Coefficient of variation; ID, Abbreviated analyte ID (see Table A-17).

Matrix	ID	Sample mass (mg)	Samples >LOD	Sample content (fmol/mg)	CV (%)	Amount per sample (x-fold LLOQ)
Leaves	ABA	3	3	10,160	9.8	254
		10	3	10,540	5.0	878
	GA1	3	3	2,227	3.8	84
		10	3	1,601	1.4	200
	GA4	3	3	15.7	20.4	1.6
		10	3	12.3	11.4	4.1
	IAA	3	3	869	17.4	16
		10	3	567	2.0	35
	iP	3	3	21.4	9.6	8.0
		10	3	20.9	10.3	26
	JA	3	3	368	10.1	28
		10	3	378	2.0	95
	SA	3	3	12,821	14.0	32
		10	3	12,626	9.6	105
	tZ	3	2	166	7.8	1.2
		10	3	147	11.7	3.7
Fruits	ABA	10	3	4,143	1.7	345
		30	3	4,062	4.2	1,016
	GA1	10	3	357	14.0	45
		30	3	409	8.9	153
	GA4	10	1	0.692	n.d.	0.2
		30	3	1.101	64.3	1.1
	IAA	10	3	78.1	9.6	4.9
		30	3	92.3	5.9	17
	iP	10	3	1.60	9.1	2.0
		30	3	1.36	5.1	5.1
	JA	10	3	27.1	8.4	6.8
		30	3	25.6	10.1	19
	SA	10	3	43,451	3.3	362
		30	3	46,748	10.5	1,169
	tZ	10	0	n.d.	n.d.	n.d.
		30	0	n.d.	n.d.	n.d.

Table A-31: Analyte amounts added (spiked) and determined in tomato fruit and leaf samples. Samples spiked at extraction were analyzed with five replicates, samples spiked at reconstitution with four. CV, Coefficient of variation; ID, Abbreviated analyte ID (see Table A-17);

Tissue	ID	Level	Endogenous amount (fmol)	Spiked amount (fmol)	Spiked at	Determined amount (fmol)	Recovery (%)	CV (%)
Fruits	ABA	1	36,153	6,000	Extraction	7,518	125.3	35.2
					Reconstitution	1,838	30.6	91.2
		2	36,153	30,000	Extraction	33,456	111.5	7.1
					Reconstitution	23,069	76.9	8.2
	GA1	1	n.d.	3,200	Extraction	3,641	113.8	15.4
					Reconstitution	3,447	107.7	3.3
		2	n.d.	16,000	Extraction	19,062	119.1	4.0
					Reconstitution	17,406	108.8	3.8
	GA4	1	n.d.	75.0	Extraction	73.3	97.8	4.8
					Reconstitution	70.9	94.6	3.7
		2	n.d.	375	Extraction	397	105.8	3.2

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Tissue	ID	Level	Endogenous amount (fmol)	Spiked amount (fmol)	Spiked at	Determined amount (fmol)	Recovery (%)	CV (%)
Leaves	IAA	1	725	800	Reconstitution	372	99.1	2.0
					Extraction	881	110.2	3.7
		2	725	4,000	Reconstitution	813	101.6	13.8
					Extraction	4,709	117.7	6.4
	iP	1	1.00	20.0	Reconstitution	4,192	104.8	2.4
					Extraction	23.5	117.5	3.4
		2	1.00	100	Reconstitution	22.8	114.2	4.8
					Extraction	118	117.8	4.9
	JA	1	238	200	Reconstitution	117	116.6	4.5
					Extraction	202	101.0	14.4
		2	238	1,000	Reconstitution	181	90.6	48.8
					Extraction	1,004	100.4	5.3
	SA	1	396,797	60,000	Reconstitution	893	89.3	2.7
					Extraction	100,369	167.3	50.6
		2	396,797	300,000	Reconstitution	8,540	14.2	683.2
					Extraction	409,288	136.4	24.2
	tZ	1	n.d.	1,000	Reconstitution	347,591	115.9	20.3
					Extraction	1,496	149.6	5.9
		2	n.d.	5,000	Reconstitution	1,420	142.0	7.7
					Extraction	7,287	145.7	6.3
	ABA	1	52,227	6,000	Reconstitution	7,492	149.8	4.7
					Extraction	6,316	105.3	44.9
		2	52,227	30,000	Reconstitution	2,036	33.9	279.4
					Extraction	30,567	101.9	14.9
	GA1	1	n.d.	4,000	Reconstitution	27,926	93.1	17.8
					Extraction	4,188	104.7	5.2
		2	n.d.	20,000	Reconstitution	4,103	102.6	4.2
					Extraction	21,080	105.4	2.6
	GA4	1	n.d.	75.0	Reconstitution	22,997	115.0	13.0
					Extraction	88.4	117.9	9.5
		2	n.d.	375	Reconstitution	88.9	118.5	7.8
					Extraction	412	110.0	3.0
	IAA	1	3,611	3,200	Reconstitution	419	111.7	6.5
					Extraction	3,526	110.2	16.6
		2	3,611	16,000	Reconstitution	3,340	104.4	19.8
					Extraction	16,614	103.8	6.5
	iP	1	121	120	Reconstitution	16,285	101.8	4.3
					Extraction	122	101.4	11.5
		2	121	600	Reconstitution	122	101.8	17.1
					Extraction	583	97.2	5.1
	JA	1	2,095	2,000	Reconstitution	610	101.6	4.5
					Extraction	1,875	93.8	4.2
		2	2,095	10,000	Reconstitution	1,442	72.1	3.7
					Extraction	9,010	90.1	3.0
	SA	1	73,024	60,000	Reconstitution	8,655	86.6	2.0
					Extraction	54,411	90.7	16.3
		2	73,024	300,000	Reconstitution	44,740	74.6	11.2
					Extraction	278,969	93.0	11.9
	tZ	1	370	1,000	Reconstitution	276,596	92.2	6.5
					Extraction	1,733	173.3	7.9
		2	370	5,000	Reconstitution	1,818	181.8	15.5
					Extraction	8,444	168.9	6.4
					Reconstitution	9,046	180.9	9.0

Phytohormone Analysis Method Application

Table A-32: Quantification results of the targeted analysis of tomato leaves and fruits. ID, abbreviated analyte ID; HS, Heat stress treatment; HT/LT, High/Low temperature; TD, *Trichoderma* treatment; C/T, *Trichoderma* control/treatment; QC, Quality control; LLOQ; Lower limit of quantification.; CV, Coefficient of Variation; FW; Fresh weight; n.d.; not determined.

ID	HS	TD	Tomato fruits				Tomato leaves					
			QC x-fold LLOQ	Content (fmol / mg FW)	Content (pg / mg FW)	CV (%)	QC CV (%)	QC x-fold LLOQ	Content (fmol / mg FW)	Content (pg / mg FW)	CV (%)	QC CV (%)
ABA	HT	C	598	94,140	24,883	14.0	2.75	872	86,415	22,841	7.68	2.80
	LT			95,728	25,303	14.1			76,835	20,309	20.6	
	HT	T		74,840	19,782	15.0			96,580	25,528	6.63	
	LT			99,572	26,319	19.4			98,465	26,026	6.00	
GA1	HT	C	9.8	801	279	28.9	6.62	154	12,358	4,305	15.1	13.1
	LT			1034	360	22.8			10,751	3,746	19.2	
	HT	T		763	266	21.8			10,925	3,806	14.2	
	LT			886	309	29.7			8,492	2,958	35.2	
GA4	HT	C	n.d.	n.d.				n.d.	n.d.			
	LT			n.d.					n.d.			
	HT	T		n.d.					n.d.			
	LT			n.d.					n.d.			
IAA	HT	C	13	2,121	372	64.2	13.1	34	6,330	1,109	14.2	7.81
	LT			1,576	276	18.7			3,814	668	27.7	
	HT	T		4,278	749	59.9			6,211	1,088	25.9	
	LT			1,346	236	10.4			3,572	626	25.0	
iP	HT	C	1.4	15.6	3.17	15.3	6.31	2.5	18.5	3.76	25.3	5.51
	LT			8.59	1.75	24.8			15.9	3.24	22.7	
	HT	T		21.2	4.32	21.2			21.5	4.37	20.6	
	LT			8.66	1.76	23.8			18.2	3.71	38.5	
JA	HT	C	5.3	206	43.4	34.0	9.65	127	4,646	977	25.0	4.60
	LT			147	31.0	29.3			2,471	519	68.4	
	HT	T		451	94.9	15.7			7,404	1557	36.8	
	LT			216	45.5	48.1			2,775	584	53.8	
SA	HT	C	230	281,950	38,943	34.5	2.77	265	280,572	38,753	65.9	3.41
	LT			365,744	50,517	20.4			310,748	42,920	44.0	
	HT	T		338,800	46,795	21.4			273,018	37,709	51.2	
	LT			323,444	44,674	18.4			264,365	36,514	38.3	

Table A-33: Adjusted signal intensities of all reproducibly detected phytohormone analytes in tomato leaves and fruits. Signal intensities were corrected for sample masses, blank subtracted and, where applicable, IS-corrected. Transition data is provided in Table A-19 and analyte names in Table A-17. Transitions ending in _2 indicate that these transitions are usually intended as the qualifier transitions, but were used here as the quantifier transition due to analytical performance. n, number of samples where the transitions was detected. Other abbreviations are equal to the previous table.

Class	Transition	HS	TD	Fruits Signal intensity	n	CV (%)	QC CV (%)	Leaves Signal intensity	n	CV (%)	QC CV (%)
Abs	ABA_n_1	LT	C	8.1E+06	6	18.9	2.8	1.1E+07	6	21.3	2.8
			T	8.5E+06	6	21.2		1.3E+07	6	6.1	
		HT	C	7.4E+06	6	11.6		1.2E+07	6	8.3	
			T	6.0E+06	6	12.2		1.2E+07	6	6.5	

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Class	Transition	HS	TD	Fruits Signal intensity	n	CV (%)	QC CV (%)	Leaves Signal intensity	n	CV (%)	QC CV (%)
Aux	ABA-GE_n_1	LT	C	6.6E+06	6	14.2	4.3	1.2E+05	6	63.6	5.5
			T	6.9E+06	6	14.3		1.0E+05	6	18.4	
		HT	C	2.0E+06	6	11.8		9.1E+04	6	17.0	
			T	1.7E+06	6	13.6		1.0E+05	6	28.4	
	IAA_p_1	LT	C	7.7E+04	6	16.1	13.1	2.5E+05	6	27.4	7.8
			T	6.7E+04	6	9.8		2.3E+05	6	20.7	
		HT	C	9.7E+04	6	62.1		4.2E+05	6	14.6	
			T	2.0E+05	6	60.3		3.8E+05	6	23.5	
	IAA-Asp_p_1	LT	C	5.9E+04	5	23.8	28.5	n.d.	0	n.d.	n.d.
			T	1.4E+05	6	110.3		n.d.	0	n.d.	
		HT	C	1.3E+05	6	29.4		n.d.	0	n.d.	
			T	1.7E+05	6	68.6		n.d.	0	n.d.	
	IAA-GE_n_1	LT	C	1.7E+03	3	13.3	69.0	4.1E+03	6	19.0	24.8
			T	2.2E+03	5	86.2		5.0E+03	6	43.9	
		HT	C	3.5E+03	6	39.8		9.4E+02	1	n.d.	
			T	6.3E+03	6	33.2		n.d.	0	n.d.	
	IAC_n_2	LT	C	3.1E+03	6	24.6	10.5	5.1E+02	1	n.d.	n.d.
			T	3.5E+03	6	50.7		n.d.	0	n.d.	
		HT	C	1.3E+03	6	21.3		7.8E+02	2	5.7	
			T	1.2E+03	5	16.5		6.3E+02	2	11.4	
	IAN_p_2	LT	C	n.d.	0	n.d.	19.5	3.2E+04	6	15.1	5.7
			T	1.6E+04	1	n.d.		3.3E+04	6	24.1	
		HT	C	9.3E+03	2	88.8		2.1E+04	6	18.6	
			T	2.7E+04	1	n.d.		2.2E+04	6	19.6	
	ICA_n_1	LT	C	2.4E+04	6	27.2	14.6	1.8E+05	6	74.1	14.8
			T	1.5E+04	6	35.0		1.4E+05	6	63.7	
		HT	C	2.0E+04	6	25.1		1.1E+06	6	26.6	
			T	2.1E+04	6	17.2		1.2E+06	6	37.5	
	Me-Oxl_p_1	LT	C	1.6E+04	6	15.0	8.2	2.3E+04	6	19.7	6.0
			T	1.5E+04	6	19.6		2.3E+04	6	29.7	
		HT	C	1.6E+04	6	24.6		2.2E+04	6	25.0	
			T	1.6E+04	6	19.6		2.2E+04	6	23.3	
Cyt	OxIAA_n_1	LT	C	n.d.	0	n.d.	n.d.	6.0E+03	6	35.0	4.9
			T	n.d.	0	n.d.		7.8E+03	6	25.6	
		HT	C	n.d.	0	n.d.		1.4E+04	6	35.3	
			T	n.d.	0	n.d.		9.1E+03	6	59.0	
	PAA_n_1	LT	C	2.1E+04	6	43.7	8.3	1.8E+04	6	32.2	11.2
			T	1.9E+04	6	32.6		1.4E+04	6	41.3	
		HT	C	2.0E+04	6	23.0		1.5E+04	6	15.9	
			T	1.4E+04	6	20.0		1.4E+04	6	31.9	
	TPL_p_1	LT	C	1.8E+05	6	27.5	3.2	1.6E+05	6	50.7	4.2
			T	2.2E+05	6	22.3		1.2E+05	6	37.4	
		HT	C	1.2E+05	6	17.2		4.9E+05	6	37.8	
			T	1.2E+05	6	17.4		3.4E+05	6	25.4	
	cZ_p_1	LT	C	1.8E+04	6	4.8	8.5	1.9E+04	6	26.4	13.7
			T	1.6E+04	6	12.9		1.5E+04	6	13.6	
		HT	C	2.5E+04	6	19.7		2.3E+04	6	12.8	
			T	2.2E+04	6	13.9		3.0E+04	6	54.8	
	DHZ_p_2	LT	C	1.5E+04	5	19.1	14.5	5.7E+04	6	16.1	8.4
			T	2.0E+04	5	38.3		5.7E+04	6	14.2	
		HT	C	2.0E+04	6	33.8		5.1E+04	6	17.1	
			T	2.0E+04	6	26.2		6.0E+04	6	6.7	
	iP_p_1	LT	C	1.5E+04	6	32.8	7.0	4.1E+04	6	20.7	5.8
			T	1.5E+04	6	33.3		4.4E+04	6	30.1	
		HT	C	2.7E+04	6	19.2		4.7E+04	6	24.0	
			T	3.9E+04	6	22.2		5.0E+04	6	16.4	
	iP-9G_p_1	LT	C	1.9E+04	6	38.4	7.0	7.7E+03	6	15.9	7.4
			T	2.3E+04	6	26.7		8.6E+03	6	19.8	
		HT	C	2.2E+04	6	14.5		1.8E+04	6	14.9	
			T	2.2E+04	6	23.4		2.0E+04	6	14.9	

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Class	Transition	HS	TD	Fruits Signal intensity	n	CV (%)	QC CV (%)	Leaves Signal intensity	n	CV (%)	QC CV (%)
Gib	iP-R_p_1	LT	C	2.1E+05	6	10.2	4.2	1.6E+05	6	18.3	2.4
			T	1.8E+05	6	40.0		1.3E+05	6	28.2	
		HT	C	2.6E+05	6	37.8		3.2E+05	6	32.3	
			T	5.7E+05	6	56.7		2.5E+05	6	19.0	
	KIN_p_1	LT	C	n.d.	0	n.d.	n.d.	2.3E+05	6	30.1	4.9
			T	n.d.	0	n.d.		2.3E+05	6	47.2	
		HT	C	n.d.	0	n.d.		1.8E+05	6	39.5	
			T	n.d.	0	n.d.		2.0E+05	6	24.1	
	KIN-R_p_1	LT	C	7.4E+02	5	24.5	34.7	6.5E+03	5	26.2	n.d.
			T	5.9E+02	5	28.7		6.2E+03	5	36.6	
		HT	C	5.7E+02	5	26.1		n.d.	0	n.d.	
			T	5.4E+02	4	31.8		5.7E+03	1	n.d.	
	mT_p_1	LT	C	3.5E+03	4	33.7	79.8	n.d.	0	n.d.	n.d.
			T	3.6E+03	3	10.9		n.d.	0	n.d.	
		HT	C	2.4E+03	1	n.d.		n.d.	0	n.d.	
			T	n.d.	0	n.d.		n.d.	0	n.d.	
	tZ_p_1	LT	C	n.d.	0	n.d.	n.d.	5.8E+04	6	54.8	14.0
			T	n.d.	0	n.d.		3.8E+04	6	27.0	
		HT	C	n.d.	0	n.d.		3.6E+04	5	22.7	
			T	n.d.	0	n.d.		4.0E+04	6	23.7	
	tZ-7G_p_1	LT	C	9.1E+05	6	11.6	8.0	3.2E+06	6	27.5	4.3
			T	1.1E+06	6	17.1		3.3E+06	6	6.7	
		HT	C	4.8E+06	6	193.9		1.5E+06	6	6.3	
			T	1.2E+06	6	11.4		1.6E+06	6	12.0	
	tZ-R_p_1	LT	C	8.4E+05	6	42.5	4.4	2.3E+05	6	72.5	7.8
			T	7.6E+05	6	47.9		1.3E+05	6	45.3	
		HT	C	8.8E+05	6	64.8		2.1E+05	6	30.5	
			T	1.1E+06	6	34.0		1.9E+05	6	19.8	
	GA1_n_2	LT	C	6.9E+04	6	20.3	6.5	1.1E+06	6	18.5	13.1
			T	6.0E+04	6	29.5		8.2E+05	6	35.4	
		HT	C	5.0E+04	6	27.6		1.2E+06	6	13.9	
			T	4.9E+04	6	21.6		1.0E+06	6	17.9	
	GA3_n_1	LT	C	7.6E+04	6	7.3	5.9	6.0E+03	5	41.1	16.3
			T	8.1E+04	6	19.2		7.2E+03	6	23.2	
		HT	C	1.1E+05	6	39.2		8.5E+03	6	17.1	
			T	9.4E+04	6	6.8		1.1E+04	6	9.1	
	GA34_n_1	LT	C	1.4E+04	6	19.3	13.2	1.1E+05	6	20.7	10.1
			T	1.3E+04	6	16.0		9.7E+04	6	28.9	
		HT	C	1.1E+04	6	14.8		9.8E+04	6	10.6	
			T	1.2E+04	6	19.0		9.1E+04	6	24.6	
	GA8_n_1	LT	C	1.1E+04	6	16.8	7.0	n.d.	0	n.d.	n.d.
			T	1.3E+04	6	23.7		n.d.	0	n.d.	
		HT	C	3.9E+04	6	53.7		n.d.	0	n.d.	
			T	3.3E+04	6	32.2		n.d.	0	n.d.	
Jas	12OH-JA_n_1	LT	C	9.8E+04	6	23.1	15.2	8.3E+04	6	37.6	7.7
			T	7.5E+04	5	18.4		8.3E+04	6	51.3	
		HT	C	7.6E+04	6	31.4		1.9E+05	6	43.2	
			T	1.0E+05	6	35.1		1.8E+05	6	33.1	
	12OH-JA-G_n_1	LT	C	1.8E+07	6	26.4	4.1	1.1E+06	6	51.9	2.7
			T	2.3E+07	6	20.0		2.0E+06	6	164.3	
		HT	C	1.2E+07	6	22.6		9.7E+05	6	31.9	
			T	1.4E+07	6	29.0		1.3E+06	6	91.6	
	DHJA_n_1	LT	C	n.d.	0	n.d.	106.6	5.9E+03	4	58.5	38.9
			T	5.6E+02	1	n.d.		3.3E+03	4	99.0	
		HT	C	1.9E+03	5	82.5		1.8E+03	6	69.5	
			T	1.1E+03	4	26.7		2.8E+03	6	94.5	
	JA_n_1	LT	C	1.3E+04	6	27.7	9.7	3.8E+05	6	66.8	4.6
			T	2.0E+04	6	51.9		4.1E+05	6	52.9	
		HT	C	1.7E+04	6	33.7		7.1E+05	6	26.2	
			T	3.9E+04	6	13.5		1.0E+06	6	37.5	

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Class	Transition	HS	TD	Fruits Signal intensity	n	CV (%)	QC CV (%)	Leaves Signal intensity	n	CV (%)	QC CV (%)
Sal	JA-Ile_n_1	LT	C	5.7E+03	6	56.6	22.5	4.6E+05	6	72.7	3.3
			T	7.5E+03	6	54.5		4.5E+05	6	45.7	
		HT	C	7.6E+03	6	59.7		1.3E+06	6	18.7	
			T	3.5E+04	6	43.3		2.0E+06	6	24.9	
	JSMO_p_1	LT	C	n.d.	0	n.d.	n.d.	1.1E+04	6	37.7	14.5
			T	n.d.	0	n.d.		1.1E+04	6	29.1	
		HT	C	n.d.	0	n.d.		1.8E+04	6	21.7	
			T	n.d.	0	n.d.		2.1E+04	6	31.1	
	MeJA_p_1	LT	C	n.d.	0	n.d.	n.d.	2.4E+03	3	39.3	10.1
			T	n.d.	0	n.d.		2.0E+03	1	n.d.	
		HT	C	n.d.	0	n.d.		5.4E+03	6	34.8	
			T	n.d.	0	n.d.		6.1E+03	6	17.7	
	OPDA_n_1	LT	C	9.9E+03	6	25.7	13.3	1.8E+06	6	51.5	16.3
			T	1.0E+04	6	29.5		1.4E+06	6	53.3	
		HT	C	1.1E+04	6	30.8		4.3E+06	6	41.6	
			T	1.8E+04	6	46.2		2.8E+06	6	34.9	
	2,5-DHBA_n_1	LT	C	3.7E+05	6	29.4	5.0	n.d.	0	n.d.	n.d.
			T	4.4E+05	6	37.7		n.d.	0	n.d.	
		HT	C	5.7E+05	6	57.8		n.d.	0	n.d.	
			T	5.1E+05	6	36.9		n.d.	0	n.d.	
	NHPA_p_1	LT	C	n.d.	0	n.d.	n.d.	3.5E+03	5	38.8	8.0
			T	n.d.	0	n.d.		6.0E+03	5	30.1	
		HT	C	n.d.	0	n.d.		9.8E+03	6	7.9	
			T	n.d.	0	n.d.		1.2E+04	6	11.5	
	SA_n_1	LT	C	2.8E+06	6	17.3	2.8	3.3E+06	6	41.7	3.4
			T	2.6E+06	6	20.2		2.8E+06	6	42.3	
		HT	C	2.0E+06	6	30.3		3.0E+06	6	68.8	
			T	2.5E+06	6	23.4		2.7E+06	6	56.4	
	SA-G_n_1	LT	C	2.7E+07	6	27.1	4.3	4.5E+07	6	19.3	1.6
			T	3.6E+07	6	16.9		4.0E+07	6	26.2	
		HT	C	1.8E+07	6	32.5		3.5E+07	6	7.9	
			T	2.0E+07	6	10.5		3.7E+07	6	29.3	
	SA-GE_n_1	LT	C	2.8E+05	6	75.5	4.8	4.0E+05	6	53.6	3.3
			T	3.7E+05	6	47.4		4.2E+05	6	38.2	
		HT	C	7.6E+04	6	25.3		4.3E+05	6	26.1	
			T	1.2E+05	6	20.0		6.6E+05	6	32.3	
	tCA_n_1	LT	C	n.d.	0	n.d.	n.d.	3.0E+03	6	18.9	9.9
			T	n.d.	0	n.d.		3.8E+03	6	34.3	
		HT	C	n.d.	0	n.d.		2.4E+03	6	30.3	
			T	n.d.	0	n.d.		3.0E+03	6	23.2	
Str	ORO_p_2	LT	C	6.1E+03	6	17.8	7.3	n.d.	0	n.d.	n.d.
			T	4.5E+03	4	23.4		n.d.	0	n.d.	
		HT	C	1.1E+04	6	39.1		n.d.	0	n.d.	
			T	8.8E+03	6	22.0		n.d.	0	n.d.	
	STR_p_2	LT	C	9.5E+03	6	22.9	4.4	n.d.	0	n.d.	n.d.
			T	6.1E+03	6	17.3		n.d.	0	n.d.	
		HT	C	1.4E+04	6	31.7		n.d.	0	n.d.	
			T	1.2E+04	6	22.4		n.d.	0	n.d.	

Table A-34: Two-factor ANOVA results of all reproducibly detected phytohormone analytes in tomato leaves and fruits. Analytes and abbreviations correspond to the previous table; for star codes see Figure 44.

Analyte	Tissue	TD n	TD p-value	TD stars	HS n	HS p-value	HS stars	TD x HS p-value	TD x HS stars
ABA	Fruits	C:12 T:12	4.0E-01		HT:12 LT:12	6.5E-03	**	1.0E-01	
	Leaves	C:12 T:12	1.6E-02	*	HT:12 LT:12	8.6E-01		6.1E-02	
ABA-GE	Fruits	C:12 T:12	9.7E-01		HT:12 LT:12	2.0E-13	***	2.2E-01	
	Leaves	C:12 T:12	7.3E-01		HT:12 LT:12	3.9E-01		3.8E-01	
IAA	Fruits	C:12 T:12	1.0E-01		HT:12 LT:12	1.2E-02	*	5.1E-02	
	Leaves	C:12 T:12	2.4E-01		HT:12 LT:12	1.7E-05	***	7.7E-01	
IAA-Asp	Fruits	C:12 T:12	1.4E-01		HT:12 LT:12	3.9E-01		5.3E-01	
	Leaves		n.d.	n.d.		n.d.	n.d.	n.d.	n.d.
IAA-GE	Fruits	C:9 T:11	5.6E-02		HT:12 LT:8	1.0E-03	**	1.7E-01	
	Leaves	C:7 T:6	1.8E-01		HT:1 LT:12	n.d.	n.d.	n.d.	n.d.
IAC	Fruits	C:6 T:6	n.d.	n.d.	LT:12	n.d.	n.d.	n.d.	n.d.
	Leaves	C:10 T:11	1.1E-02	*	HT:10 LT:11	2.7E-04	***	7.7E-01	
IAN	Fruits	C:2 T:2	n.d.	n.d.	HT:3 LT:1	n.d.	n.d.	n.d.	n.d.
	Leaves	C:12 T:12	7.2E-01		HT:12 LT:12	7.2E-05	***	9.0E-01	
ICA	Fruits	C:12 T:12	1.0E-01		HT:12 LT:12	6.1E-01		2.8E-02	*
	Leaves	C:12 T:12	7.8E-01		HT:12 LT:12	3.1E-08	***	5.8E-01	
Me-Oxl	Fruits	C:12 T:12	7.5E-01		HT:12 LT:12	9.5E-01		5.2E-01	
	Leaves	C:12 T:12	9.3E-01		HT:12 LT:12	5.7E-01		9.9E-01	
OxIAA	Fruits		n.d.	n.d.		n.d.	n.d.	n.d.	n.d.
	Leaves	C:12 T:12	3.3E-01		HT:12 LT:12	7.9E-03	**	4.4E-02	*
PAA	Fruits	C:12 T:12	9.9E-02		HT:12 LT:12	2.5E-01		3.5E-01	
	Leaves	C:12 T:12	2.3E-01		HT:12 LT:12	4.9E-01		3.2E-01	
TPL	Fruits	C:12 T:12	1.8E-01		HT:12 LT:12	3.2E-05	***	1.9E-01	
	Leaves	C:12 T:12	5.0E-02	*	HT:12 LT:12	6.8E-06	***	2.3E-01	
DHZ	Fruits	C:11 T:11	3.1E-01		HT:12 LT:10	3.3E-01		3.0E-01	
	Leaves	C:12 T:12	2.1E-01		HT:12 LT:12	7.5E-01		1.5E-01	
iP	Fruits	C:12 T:12	2.4E-02	*	HT:12 LT:12	6.4E-07	***	3.6E-02	*
	Leaves	C:12 T:12	4.0E-01		HT:12 LT:12	1.6E-01		9.6E-01	
iP-9G	Fruits	C:12 T:12	3.6E-01		HT:12 LT:12	8.8E-01		4.2E-01	
	Leaves	C:12 T:12	2.2E-01		HT:12 LT:12	2.0E-10	***	7.2E-01	
iP-R	Fruits	C:12 T:12	5.7E-02		HT:12 LT:12	5.6E-03	**	2.5E-02	*
	Leaves	C:12 T:12	5.7E-02		HT:12 LT:12	1.6E-05	***	3.8E-01	
KIN	Fruits		n.d.	n.d.		n.d.	n.d.	n.d.	n.d.
	Leaves	C:12 T:12	8.1E-01		HT:12 LT:12	2.0E-01		9.1E-01	
KIN-R	Fruits	C:10 T:9	2.7E-01		HT:9 LT:10	1.8E-01		4.5E-01	
	Leaves	C:5 T:6	7.4E-01		HT:1 LT:10	n.d.	n.d.	n.d.	n.d.
mT	Fruits	C:5 T:3	7.2E-01		HT:1 LT:7	n.d.	n.d.	n.d.	n.d.
	Leaves		n.d.	n.d.		n.d.	n.d.	n.d.	n.d.
tZ	Fruits		n.d.	n.d.		n.d.	n.d.	n.d.	n.d.
	Leaves	C:11 T:12	2.6E-01		HT:11 LT:12	2.3E-01		1.3E-01	
tZ-7G	Fruits	C:12 T:12	3.8E-01		HT:12 LT:12	3.1E-01		3.3E-01	
	Leaves	C:12 T:12	7.1E-01		HT:12 LT:12	2.1E-08	***	8.1E-01	
tZ-R	Fruits	C:12 T:12	8.1E-01		HT:12 LT:12	3.4E-01		4.6E-01	
	Leaves	C:12 T:12	1.4E-01		HT:12 LT:12	7.3E-01		3.4E-01	
Z	Fruits	C:12 T:12	6.2E-02		HT:12 LT:12	7.8E-05	***	5.2E-01	
	Leaves	C:12 T:12	7.6E-01		HT:12 LT:12	1.6E-02	*	1.6E-01	
GA1	Fruits	C:12 T:12	4.3E-01		HT:12 LT:12	1.8E-02	*	5.4E-01	
	Leaves	C:12 T:12	1.6E-02	*	HT:12 LT:12	7.5E-02		8.6E-01	
GA3	Fruits	C:12 T:12	5.1E-01		HT:12 LT:12	2.3E-02	*	2.4E-01	
	Leaves	C:11 T:12	1.9E-02	*	HT:12 LT:11	2.0E-04	***	3.3E-01	
GA34	Fruits	C:12 T:12	8.9E-01		HT:12 LT:12	1.8E-02	*	2.6E-01	
	Leaves	C:12 T:12	2.1E-01		HT:12 LT:12	2.3E-01		6.4E-01	
GA8	Fruits	C:12 T:12	7.2E-01		HT:12 LT:12	7.9E-05	***	3.7E-01	

8 - Appendix

Analyte	Tissue	TD n	TD p-value	TD stars	HS n	HS p-value	HS stars	TD x HS p-value	TD x HS stars
GA8	Leaves		n.d.	n.d.		n.d.	n.d.	n.d.	n.d.
12OH-JA	Fruits	C:12 T:11	8.1E-01		HT:12 LT:11	9.3E-01		3.5E-02	*
	Leaves	C:12 T:12	9.4E-01		HT:12 LT:12	2.6E-04	***	9.4E-01	
12OH-JA-G	Fruits	C:12 T:12	5.2E-02		HT:12 LT:12	3.8E-04	***	3.6E-01	
	Leaves	C:12 T:12	4.1E-01		HT:12 LT:12	5.5E-01		6.5E-01	
DHJA	Fruits	C:5 T:5	2.8E-01		HT:9 LT:1	n.d.	n.d.	n.d.	n.d.
	Leaves	C:10 T:10	7.0E-01		HT:12 LT:8	7.6E-02		1.5E-01	
JA	Fruits	C:12 T:12	4.5E-05	***	HT:12 LT:12	4.7E-04	***	1.4E-02	*
	Leaves	C:12 T:12	1.2E-01		HT:12 LT:12	3.4E-04	***	2.0E-01	
JA-Ile	Fruits	C:12 T:12	3.6E-04	***	HT:12 LT:12	3.4E-04	***	1.2E-03	**
	Leaves	C:12 T:12	2.0E-02	*	HT:12 LT:12	2.7E-08	***	1.7E-02	*
JSMO	Fruits		n.d.	n.d.		n.d.	n.d.	n.d.	n.d.
	Leaves	C:12 T:12	3.7E-01		HT:12 LT:12	4.9E-04	***	3.5E-01	
MeJA	Fruits		n.d.	n.d.		n.d.	n.d.	n.d.	n.d.
	Leaves	C:9 T:7	1.5E-01		HT:12 LT:4	2.4E-03	**	5.4E-01	
OPDA	Fruits	C:12 T:12	9.6E-02		HT:12 LT:12	3.3E-02	*	1.2E-01	
	Leaves	C:12 T:12	5.7E-02		HT:12 LT:12	7.9E-04	***	2.8E-01	
DHBA	Fruits	C:12 T:12	9.0E-01		HT:12 LT:12	1.4E-01		4.6E-01	
	Leaves		n.d.	n.d.		n.d.	n.d.	n.d.	n.d.
NHPA	Fruits		n.d.	n.d.		n.d.	n.d.	n.d.	n.d.
	Leaves	C:11 T:11	5.0E-04	***	HT:12 LT:10	2.5E-09	***	9.8E-01	
SA	Fruits	C:12 T:12	6.0E-01		HT:12 LT:12	8.3E-02		1.1E-01	
	Leaves	C:12 T:12	5.3E-01		HT:12 LT:12	7.6E-01		8.7E-01	
SA-G	Fruits	C:12 T:12	2.0E-02	*	HT:12 LT:12	2.1E-05	***	1.6E-01	
	Leaves	C:12 T:12	7.0E-01		HT:12 LT:12	7.1E-02		3.1E-01	
SA-GE	Fruits	C:12 T:12	2.3E-01		HT:12 LT:12	6.8E-04	***	6.9E-01	
	Leaves	C:12 T:12	1.1E-01		HT:12 LT:12	8.5E-02		1.7E-01	
tCA	Fruits		n.d.	n.d.		n.d.	n.d.	n.d.	n.d.
	Leaves	C:12 T:12	7.0E-02		HT:12 LT:12	6.7E-02		8.2E-01	
ORO	Fruits	C:12 T:10	2.3E-01		HT:12 LT:10	6.2E-04	***	8.3E-01	
	Leaves		n.d.	n.d.		n.d.	n.d.	n.d.	n.d.
STR	Fruits	C:12 T:12	3.7E-02	*	HT:12 LT:12	1.6E-04	***	5.6E-01	
	Leaves		n.d.	n.d.		n.d.	n.d.	n.d.	n.d.

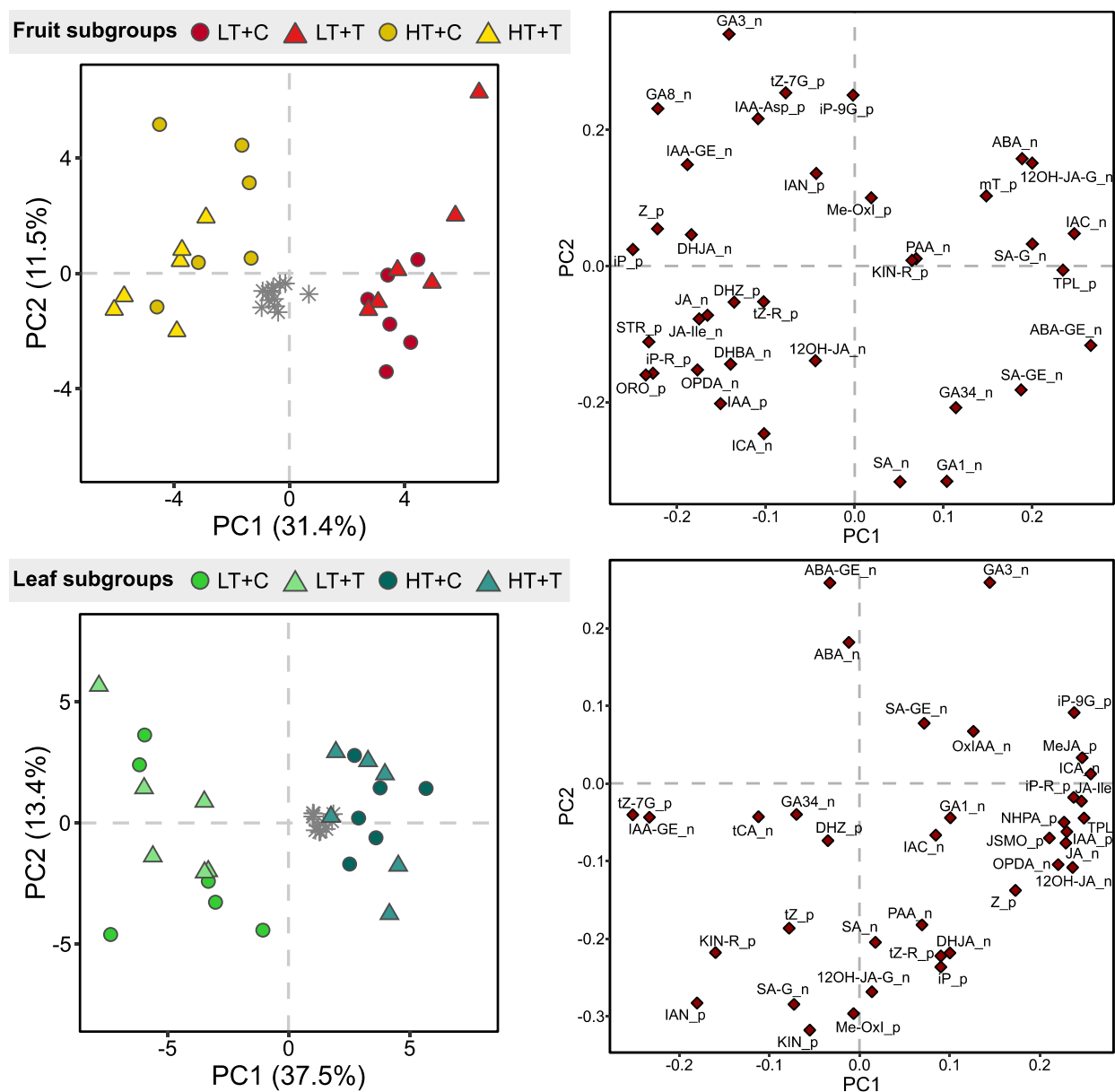


Figure A-6: 2D score (left) and loading (right) plots of the PCA of the phytohormone analytes reproducibly detected in tomato fruits (top) and leaves (bottom). Only the first two PCs are shown. Quality control samples are marked by star shapes. Total variance explained by each PC is provided in parentheses. PC(A), Principal component (analysis); LT/HT, Low/High temperature cultivation; C/T, *Trichoderma* control/treatment groups; For analyte abbreviations see Table A-17.

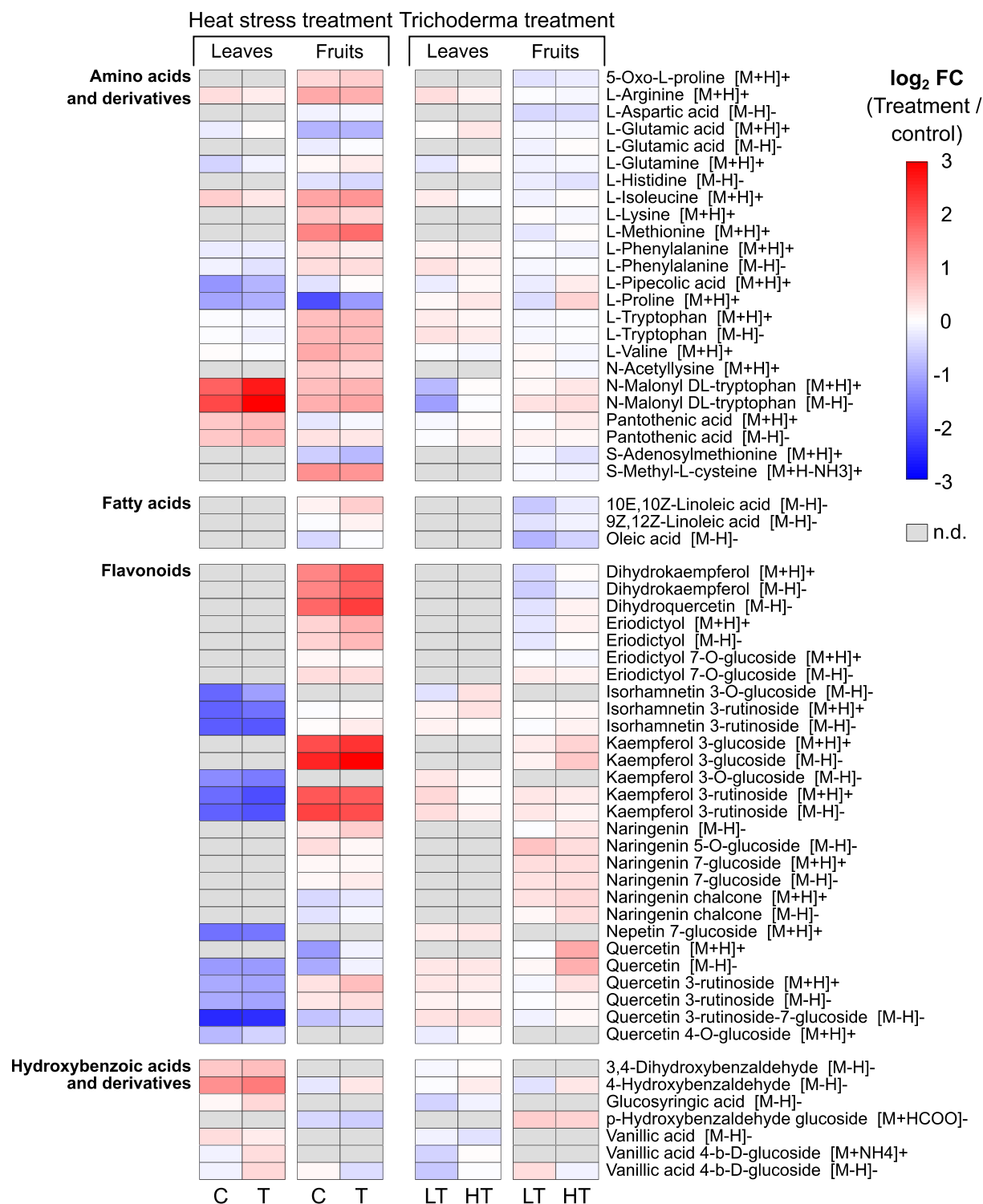


Figure A-7: Log₂ FC heatmaps of one half of the of metabolites identified with the in-house database in tomato fruit and leaf samples in tomato fruit and leaf samples. FC calculations were performed between means of treatment and control groups (T / C and HT / LT), which was done separately for the groups of the non-observed factor, resulting in two columns per plant tissue. Results of the statistical analysis are not presented for these metabolites. FC, Fold change, C/T, *Trichoderma* control/treatment; LT/HT, Low/High temperature cultivation; n.d., not determined (in at least one group of the comparison).

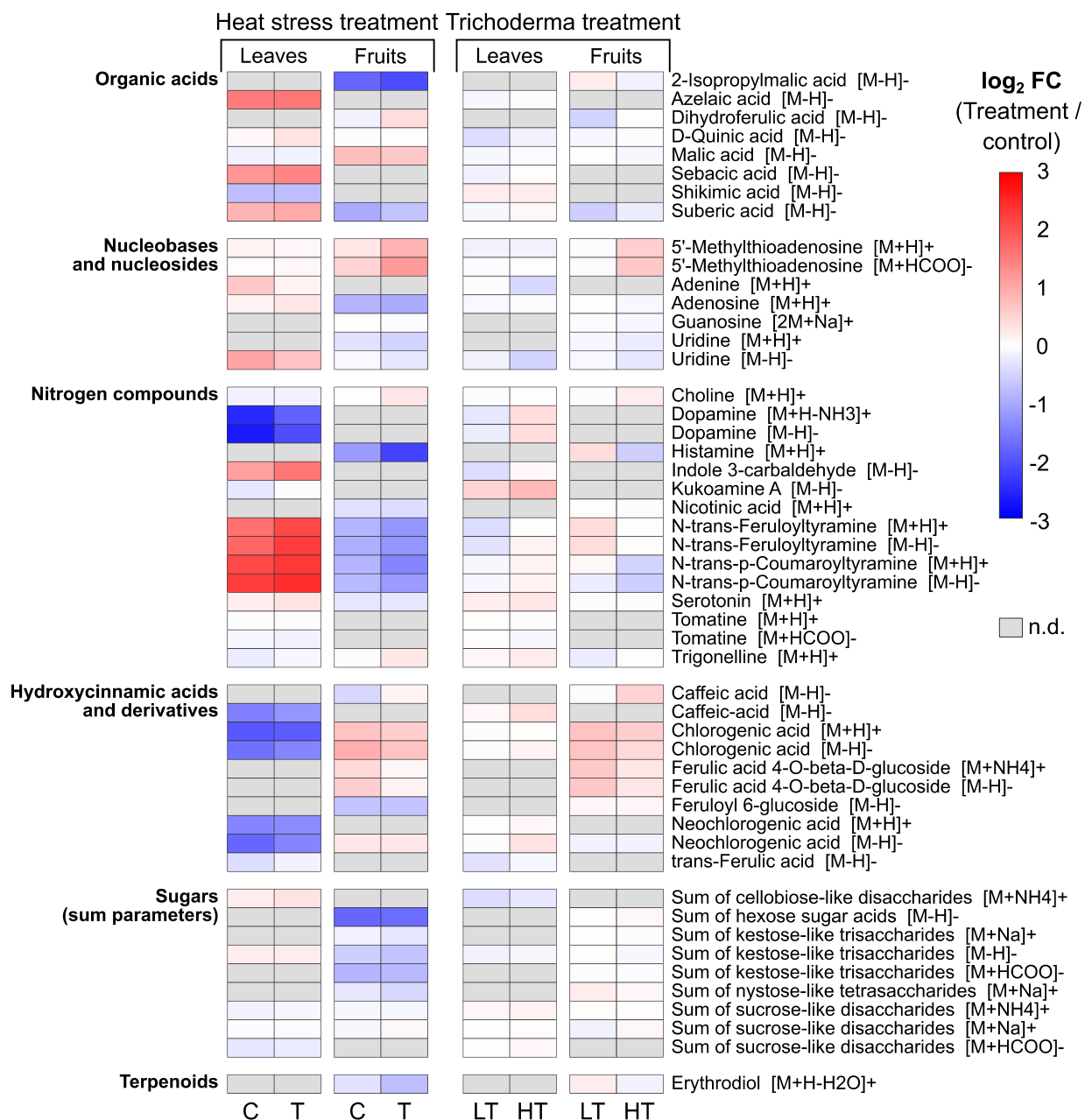


Figure A-8: Log₂ FC heatmaps of one half of the of metabolites identified with the in-house database in tomato fruit and leaf samples in tomato fruit and leaf samples. FC calculations were performed between means of treatment and control groups (T / C and HT / LT), which was done separately for the groups of the non-observed factor, resulting in two columns per plant tissue. Results of the statistical analysis is not presented for these metabolites. FC, Fold change, C/T, *Trichoderma* control/treatment; LT/HT, Low/High temperature cultivation; n.d., not determined (in at least one group of the comparison).

9 List of Publications (Publikationsliste)

Vorträge auf Fachtagungen

Knauth, J. (2023). Metabolic analyses of genetically engineered plants. JKIP Inaugurational Symposium – „Plants Fit for Future“, Karlsruhe.

Posterbeiträge auf Fachtagungen

Knauth, J., Soukup, S., Weinert, C., Pressler, P., Egert, B., Kulling, S., Bunzel, D. (2022). Phytochemical quality of Brassicaceae microgreens grown on different organic substrates: A deep dive into the metabolome. International Horticultural Congress, Angers (France).

Knauth, J., Beer, F., Soukup, S., Weinert, C., Egert, B., Huch, M., Stoll, D., Kulling, S., Bunzel, D. (2022). Einfluss des Anzuchtsubstrats auf das Metabolom und die mikrobiologische Qualität und Sicherheit von *Raphanus sativus*- Microgreens. 55. Jahrestagung DGQ: Qualität 2030: Produktqualität in Zeiten des globalen Wandels, Hohenheim.

Acknowledgements (Danksagung)

Mein Dank gilt zuallererst Prof. Dr. Sabine Kulling, für die Betreuung meiner Arbeit und insbesondere für das Vertrauen, das mir durchweg entgegengebracht wurde. Insbesondere bedanke ich mich auch für alle fachliche Anregungen, und die Möglichkeiten zur Weiterbildung, die mir gegeben wurden.

An Dr. Diana Bunzel richte ich meinen Dank für die Betreuung der ersten Monate meiner Promotion und für die Grundsteine, die sie für meine Arbeit gelegt hat.

Bei Prof. Dr. Mirko Bunzel möchte ich mich für die Übernahme des Korreferats bedanken, und für Einbindung in seinen Arbeitskreis, auch bezüglich Lehrveranstaltungen. Allen DoktorandInnen des Arbeitskreises möchte ich dabei ebenfalls ausdrücklich danke sagen.

Prof. Dr. Natalia Requena bin ich zu Dank verpflichtet für die Organisation des Plant Fit For Future Projekts, sowie für die Erzeugung und Kultivierung der Kartoffelpflanzen dieser Arbeit. Ebenso danke ich Dr. David Figueira-Galán für die überaus angenehme Zusammenarbeit.

Prof. Dr. Peter Nick und Dr. Michael Riemann danke ich für den fachlichen Input zu Phytohormonmetabolismen und -analytik, sowie für die Koordination und fachliche Betreuung bei der Kultivierung der Reispflanzen für diese Arbeit.

Bei Prof. Dr. Holger Puchta bedanke ich mich für die Ermöglichung eines einmonatigen Aufenthalts in seiner Arbeitsgruppe und die spannenden Einblicke in die CRISPR-Cas-Editierung von Pflanzen bedanken. Seiner gesamten Arbeitsgruppe und vor allem Dr. Fabienne Gehrke danke ich für die wunderbare Einbindung und Betreuung während dieser Zeit.

Darüber hinaus gilt mein Dank Dr. Sebastian Soukup für die organisatorische und fachliche Betreuung in der LC-MS-Analytik. Dem ganzen LC-Labor gilt dabei gleichermaßen mein höchster Dank, namentlich Dr. Falco Beer, Volker Müller, Bettina Schindler, Anastasia Schreiber, Michelle Urbach und Sabine Beissmann. Vielen Dank für die alltägliche Unterstützung und die große Hilfsbereitschaft. Patrick Pressler danke ich für seinen Einsatz bei der Kultivierung der Tomatenpflanzen. Auch allen anderen MitarbeiterInnen des Instituts OG möchte ich von ganzem Herzen für ihre Hilfsbereitschaft und Unterstützung danken.

Meinen MitdoktorandInnen, KollegInnen, und vor allem FreundInnen Elisa Streitenberger, Lea Böckstiegel, Oliver Wittek, Rebecca Rüscher, Melinda Sattler, Adetoye Adeyemo, Katharina Neumeier, Carina Mack, Katja Kaltenbach und Lea Michaelis, sowie Frederic Wall, kann ich nicht genug danken für die stetige Ermutigung, den spannenden Austausch und die vielen gemeinsam erlebten Momente. Und natürlich für den ganzen Kuchen.

Auch all meinen anderen FreundInnen danke ich zutiefst für ihren mentalen Support, ihr Verständnis und allgemein für ihre Präsenz in meinem Leben. Zudem möchte ich meiner Familie und vor allem meinen Eltern danken für ihre fortwährende Unterstützung, nicht zuletzt auch finanziell, während meines gesamten Studiums.