Faculty of Veterinary Medicine
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# A METABOLOMIC FRAMEWORK TO STUDY THE REGULATING ROLE OF PHYTOHORMONES TOWARDS CAROTENOIDS IN TOMATO FRUIT

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| | |
|---|---|
| ABA | Abscisic acid |
| ACC | Aminocyclopropane-1-carboxylic acid |
| AGC | Automatic gain control |
| AIF | All ions fragmentation |
| APCI | Atmospheric pressure chemical ionization |
| APPI | Atmospheric pressure photoionization |
| $A_s$ | Peak shape |
| au | Arbitrary units |
| BA | $N^6$-benzyladenine |
| BEH | Ethylene bridged hybrid |
| BHT | Butylated hydroxytoluene |
| BL | Epibrassinolide |
| CAR | Carotenoid |
| CD | Commission directive |
| CID | Collision-induced dissociation |
| CID (PubChem) | Compound identifier |
| $CIJF_{JK}$ | Jack-knifed confidence interval |
| CHYB | Carotene β-hydroxylase |
| CHYE | Carotene ε-hydroxylase |
| Corr | Correlation |
| Cov | Covariance |
| CRTISO | Carotenoid isomerase |
| CV | Coefficient of variance |
| DAA | Days after anthesis |
| dd-$MS^2$ | Data-dependent tandem mass spectrometry |
| EC | Electrical conductivity |
| EC | Electrical conductivity |
| EI | Electron impact |
| ELISA | Enzyme-linked immunoassay sorbent |
| ESI | Electrospray ionization |
| FAB | Fast atom bombardment |
| FT-ICR | Fourier transform ion cyclotron resonance |
| FWHM | Full width at half maximum |
| GA3 | Gibberellic acid |
| GC | Gas chromatography |
| GDDP | Geranyl-geranyl diphosphate |
| HCD | High energy collisional dissociation |

| | |
|---|---|
| HESI | Heated electrospray ionization |
| HPLC | High-performance liquid chromatography |
| HRMS | High-resolution mass spectrometry |
| Hz | Herz |
| IAA | Indole-3-acetic acid |
| IAC | Immunoaffinity chromatography |
| ILVO | Institute for agriculture and fisheries research |
| IP | Identification point |
| IT | Injection time |
| JA | Jasmonic acid |
| LCYB | Lycopene β-cyclase |
| LCYE | Lycopene ε-cyclase |
| LOD | Limit of detection |
| LOQ | Limit of quantification |
| LTQ | Linear ion trap quadrupole |
| *m/z* | Mass-to-charge ratio |
| MALDI | Matrix-assisted laser desorption ionization |
| MRM | Multiple reaction monitoring |
| MS | Mass spectrometry |
| MS/MS | Tandem mass spectrometry |
| NCE | Normalized collision energy |
| NXS | Neoxanthin synthase |
| OPLS | Orthogonal partial least squares |
| OPLS-DA | Orthogonal partial least squares discriminant analysis |
| p | Model contribution |
| PAR | Photoactive radiation |
| PCA | Principal component analysis |
| PDA | Photodiode array |
| PLS | Partial least squares |
| ppm | Part per million |
| PDS | Phytoene desaturase |
| PSY | Phytoene synthase |
| q | Model reliability |
| RDBE | Ring double bound equivalent |
| RF | Radio frequency |
| RIA | Radioimmunoassay |
| $R_s$ | Chromatographic resolution |
| RT | Retention time |
| S/N | Signal-to-noise ratio |
| SA | Salicylic acid |
| SPE | Solid-phase extraction |
| RT QPCR | Real time quantitative polymerase chain reaction |

| | |
|---|---|
| SRM | Selected reaction monitoring |
| SUS-Plot | Shared and unique structures plot |
| $tMS^2$ | Targeted tandem mass spectrometry |
| $t_O$ | Orthogonal model principal component |
| ToF | Time of flight |
| $t_p$ | Predictive model principal component |
| tSIM | Targeted selected ion monitoring |
| TSQ | Triple stage quadrupole |
| UHPLC | Ultra high-performance liquid chromatography |
| UV-VIS | Ultraviolet-visible |
| VIP | Variable Importance in Projection |
| VDE | Violaxanthin deepoxidase |
| Z | Zeatin |
| ZE | Zeaxanthin epoxidase |
| ZDS | ζ-desaturase |

# Chapter I

# General Introduction

# 1. THE AIM OF IMPROVING TOMATO FRUIT QUALITY

Tomato (*Solanum lycopersicum* L.) has been recognized as one of the most important horticultural crops in the world. This fact relates to the annual production of 160 million tons of fresh weight (1) and the average yearly consumption of around 21 kg per capita (2). As a consequence, tomato has established a key role in the human diet. Indeed, as a major component of daily meals in many countries, tomato constitutes a valuable source of diverse health beneficial constituents. Especially carotenoids, vitamins, phenolic compounds, minerals, and dietary fibers have been assigned significant beneficial effects on health (2). Regular consumption of fresh tomatoes or tomato-based foods has been evidenced to reduce the incidence of various chronic degenerative diseases such as certain types of cancer, age-related macular degeneration, and cardiovascular disorders (3-5). These protective effects may be mainly attributed to a number of biological functions that have been ascribed to the concerned dietary compounds, i.e. provitamin A activity, immune-response modulation, antioxidant effects and induction of gap-junction communication (6,7).

With this rationale, the interest of consumers has been focused on the presence of health-promoting substances and thus on the nutritional aspects of fresh tomato fruits. Moreover, a general strong focus of consumers towards overall tomato fruit quality has emerged. Appearance, firmness, texture, dry matter, flavor and nutritional value have all been defined as important quality traits. These findings have incited growers and breeders to redirect their aims. Improvement of tomato fruit quality has indeed become an urgent issue to meet the ever-increasing demands of consumers in a highly competitive fresh market (2). In contrast, previous efforts were predominantly made with the aim of improving yield, yield potential, uniformity of appearance, disease tolerance, and shelf life (8).

Many factors such as genetics (cultivar or variety), agronomic practices (ripening stage at harvest and irrigation), and environment (light, temperature, and air composition) have been found to significantly affect fruit chemical composition (9). In the field of tomato breeding, considerable genetic improvements have been established, thereby introducing new varieties and hybrids with enhanced tomato fruit quality. Unfortunately, traditional breeding is regarded as a very lengthy

process. Within this context, molecular techniques and genetic engineering have opened new horizons and will undoubtedly contribute towards further genetic improvements (10). Changing environmental conditions or cultural practices is easier and faster than producing new cultivars, and has therefore a more immediate impact on the quality of tomato fruit (11). Although diverse agronomic and environmental factors have been claimed to significantly influence the accumulation of the health-promoting constituents in tomato fruit, conflicting or less conclusive data have been reported as well (8,9). In addition, interactive effects between environment, agronomic setting, and genetic background are also assigned significant influence (8). Therefore, defining optimum growth conditions is difficult and hazardous (8,9). In this regard, further deepening of the knowledge is highly necessary.

An interesting starting point may originate from the fact that synthesis and accumulation of health-promoting constituents involves many of the primary and secondary pathways, which are mediated through developmental, physiological and environmental signals (10). Within this context, phytohormonal compounds are assigned significant value. These secondary metabolites are indeed key signaling biomolecules, which fulfill a regulating role in plant development and physiology (12). Moreover, phytohormones have been recognized to coordinate the complex processes associated with tomato fruit development and ripening (13,14). Since these processes are accompanied by quantitative and qualitative changes of fruit components, phytohormones and their regulating functions may enclose an interesting context regarding the aim of improving tomato fruit quality.

# 2. THE HEALTH-PROMOTING CONSTITUENTS OF TOMATO FRUIT

## 2.1 GENERAL DESCRIPTION OF THE MAIN HEALTH-PROMOTING CONSTITUENTS

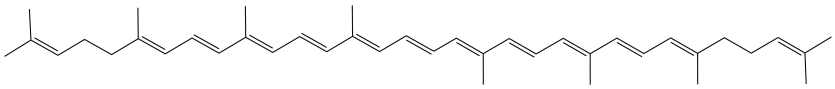
Tomato is generally recognized as an important source of substances with known beneficial effects on health. Especially carotenoids, vitamins and phenolic compounds have been allocated significant health-promoting properties. Indeed, their inherent antioxidative properties have been associated with a reduced incidence of various chronic and degenerative disorders (16). In this context, carotenoid compounds are defined as the main descriptors of tomato fruit nutritional value (11). Other health-promoting constituents of tomato fruit include dietary fibers and mineral elements (17).

### 2.1.1 CAROTENOIDS

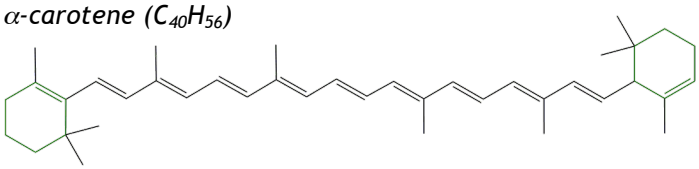
The main tomato fruit carotenoids include α-carotene, β-carotene, lycopene, lutein and zeaxanthin. However, low amounts of other carotenoids such as γ-, ε-, δ-carotene, β-cryptoxanthine, phytoene, phytofluene, and neurosporene have been detected as well (9,18). The total amount of carotenoids present in tomato fruit varies strongly from about 90 to 180 $\mu g\ g^{-1}$ fresh weight (10). Herewith, especially lycopene (80 to 90% of total carotenoid content) and β-carotene (7 to 10% of total carotenoid content) are important (19,20). The majority of all carotenoids includes a 40-carbon basal structure, which is typified by a system of conjugated double bonds. Modifications towards this polyisoprenoid basal structure are in essence determining the differences between the various carotenoids. These modifications encompass cyclization of the end-groups (cyclohexane and cyclopentane) and introduction of functional groups (hydroxy, keto, epoxy) (21). Based on the associated chemical composition, a carotenoid classification system has been introduced, hereby distinguishing between carotenes and xanthophylls (Figure 1.1). Carotenes are defined as highly unsaturated hydrocarbons, including amongst others α-carotene, β-carotene and lycopene. Xanthophylls are carotenoids with one or more oxygenated substituents at particular sites of the terminal rings. Within this class, lutein and zeaxanthin are the main representatives (23). In addition, due to the presence of conjugated double bonds, the formation of several *cis-trans* isomers in various positions along the carotenoid molecule is possible. However, the bulk of carotenoids in tomato occurs in all-*trans* configuration (24).

**Carotenes**

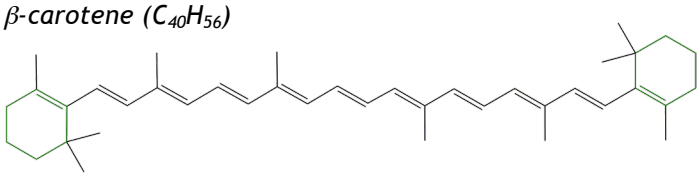
*Lycopene ($C_{40}H_{56}$)*



*α-carotene ($C_{40}H_{56}$)*

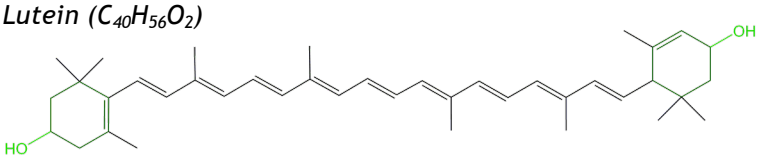


*β-carotene ($C_{40}H_{56}$)*



**Xanthophylls**

*Lutein ($C_{40}H_{56}O_2$)*




*Zeaxanthin ($C_{40}H_{56}O_2$)*

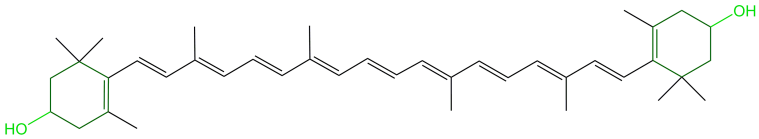



**FIGURE 1.1.** All-*trans* chemical structures of the major tomato fruit carotenoids. Structural modifications such as cyclic end-groups and oxygen functional groups have been highlighted (22). Based on these modifications, carotenoids are classified as carotene or xanthophyll.

Up to now, more than 700 different carotenoid structures have been isolated and identified from various natural sources (18). However, only a few of these carotenoids (~40 to 50) are present in the human diet. Moreover, because of selective uptake by the digestive tract, only about 25 different carotenoids are found in human blood (9). In this regard, tomato has been recognized as a valuable source of carotenoids and this with respect to both diversity and absolute amounts (25). Of the above-mentioned number of carotenoids found in human blood, up to 20 can be derived from fresh or processed tomato (9). In addition, tomatoes are ranked first as a source of lycopene (71.6%) and second as a source of β-carotene (17.2%) (26).

Several epidemiological studies have reported that the consumption of carotenoid-rich foods is associated with a reduced risk of several chronic and degenerative disorders (Figure 1.2). One of the main mechanisms by which carotenoids cause these beneficial health effects relates to their inherent antioxidant properties. In addition, some carotenoids are assigned provitamin A activity since they are able to be converted into vitamin A. However, carotenoids may mediate their protective effects by other mechanisms as well (Figure 1.2) (27).

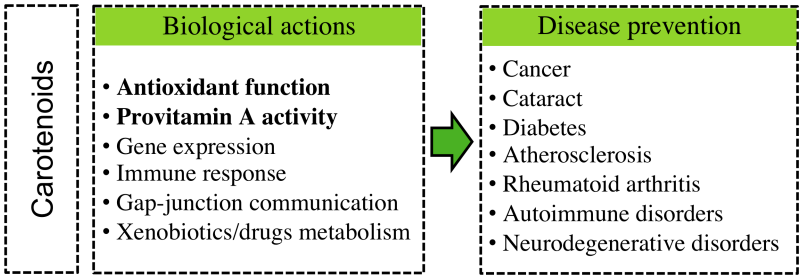



**FIGURE 1.2.** The biological actions of carotenoids and their role in the prevention of various diseases (27).

The antioxidant properties of carotenoids are mainly attributed to their ability of scavenging singlet molecular oxygen ($^1O_2$) and diverse peroxyl radicals. These reactive oxygen species are generated during normal cell metabolism or may originate from external sources such as pollution and radiation. They are capable of damaging biologically relevant molecules including DNA, proteins, carbohydrates, and lipids. The interaction of carotenoids with singlet molecular oxygen depends largely on physical quenching, in which the energy of the reactive oxygen species is transferred to the carotenoid molecule. As such, a harmless ground state oxygen molecule and triplet-excited carotenoid molecule are yielded (Figure 1.3) (28,29). The excited carotenoid molecule may return to its ground state as well, by dissipating its energy excess as heat. As a consequence, the carotenoid molecule remains intact and preserves its antioxidant properties (30).

$^1O_2 + CAR \rightarrow {}^3O_2 + {}^3CAR^*$ (a)

$^1O_2 + CAR \rightarrow CARO_2$ (b)

**FIGURE 1.3.** Physical (a) and chemical quenching (b) reactions between singlet molecular oxygen ($^1O_2$) and a carotenoid molecule (CAR) (29).

The efficacy of physical quenching relates to the number of conjugated double bonds, present in the carotenoid molecule. In this regard, lycopene is the most efficient quencher due to the acyclic structure, enclosing 11 conjugated double bonds (28). Although less frequently occurring, chemical quenching is a secondary mechanism by which singlet molecular oxygen can be neutralized (Figure 1.3). During this process the carotenoid molecule is modified and loses its antioxidant activity (28,29). The neutralization of peroxyl radicals is based on the formation of stable radical adducts such as carotenoid-epoxides and apo-carotenoids of various chain lengths (28).

Provitamin A activity has also been defined as one of the mechanisms by which carotenoids obtain their beneficial health effects. However, only carotenoid molecules with at least one unsubstituted β-ionone ring and a correct configuration of methyl groups are able to exhibit provitamin A activity. As a result, only α-carotene, β-carotene, β-cryptoxanthin, and γ-carotene are assigned provitamin A activity. Central or eccentric cleavage may yield retinal, which is a direct precursor of retinol (vitamin A) (Figure 1.4). Vitamin A is assigned a role in the formation and maintenance of healthy skin, teeth, skeletal and soft tissue, mucus membranes, and skin. In addition, this vitamin is also involved in the production of retina pigments in the eye (31).

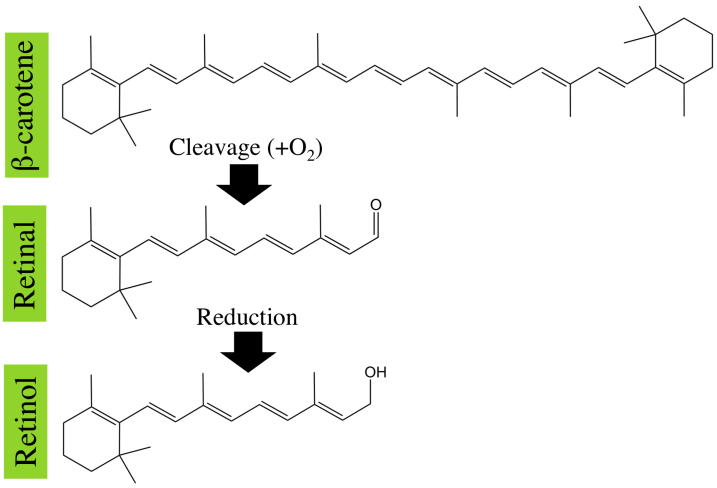



**FIGURE 1.4.** Formation of retinol (vitamin A) from β-carotene, which is assigned provitamin A activity (31).

### 2.1.2 VITAMINS

Although the protective effects of tomato fruit consumption towards human health are primarily attributed to carotenoids, other compounds such as vitamins are assigned important health-promoting effects as well. In this regard, especially vitamins B, C, and E are strongly represented in tomato fruit.

Vitamin E is actually referring to a group of various fat-soluble molecules, i.e. tocopherols (α, β, γ, and δ) and tocotrienols (α, β, γ, and δ). The classification of these molecules is based on the nature of the isoprenoid side chain, which is either fully saturated or unsaturated at three positions. Furthermore, the substituents at the chromanol ring are determining the α-, β-, γ-, or δ-form of the vitamin E molecules. This vitamin is recognized as a potent antioxidant and therefore assigned preventive actions towards various chronic degenerative diseases (11). In tomato fruits, levels of vitamin E are ranging between about 2 and 6 $\mu g\ g^{-1}$ fresh weight.

Vitamin C is defined as the generic term that enfolds both L-ascorbic and dehydroascorbic acid. These molecules are required for the prevention of scurvy and maintenance of healthy skin, gums and blood vessels. In addition, as an antioxidant, vitamin C also reduces the risk of arteriosclerosis, cardiovascular diseases and some types of cancer (32). In tomato fruit, concentration levels vary from about 100 to 200 $\mu g\ g^{-1}$ fresh weight (11).

Folates represent all forms of vitamin B, which is strongly involved in several physiological mechanisms with respect to andrology and gynecology. In particular, folates fulfill a role in one-carbon transfer reactions including purine and pyrimidine biosynthesis, amino acids metabolism, and methylation of nucleic acids, proteins, and lipids. For these reasons, folates are essential for fetal growth (11). Concentration levels in tomato fruit range between 40 and 350 $ng\ g^{-1}$ fresh weight (33).

### 2.1.3 PHENOLIC COMPOUNDS

Phenolic compounds constitute a very diverse group to which more than 8000 different compounds have been allocated. In general, the structure of a phenolic compound is characterized by the presence of at least one aromatic ring, having attached one or more hydroxyl groups (34). In tomato fruit, total phenolic content amounts about 450 to 600 $\mu g\ g^{-1}$ fresh weight. Herewith, the three main phenolic classes (flavonoids, phenolic acids and tannins) are represented (35). Phenolic compounds have been associated with therapeutic actions regarding obesity, type II diabetes, cardiovascular diseases, neurodegenerative diseases, cancer, rheumatoid arthritis, and aging. These health-beneficial effects are founded on the antioxidant activity and associated free radical scavenging potential (11).

## 2.2 BIOSYNTHESIS OF CAROTENOIDS AND ITS RELATION WITH FRUIT RIPENING

Tomato fruit ripening is a complex and highly coordinated developmental process, which is typically associated with significant changes in global fruit quality (36). These changes strongly depend on a wide range of synthetic and degradative biochemical reactions, being affected by altered fruit metabolism and gene expression. Regulated expression of thousands of genes during fruit ripening may indeed lead to fruit softening and accumulation of pigments, sugars, acids, and volatile compounds (37). Within this context, one of the most noticeable characteristics of ripening relates to the increased tomato fruit carotenoid content, which is reflected by distinct color changes (38). This process of intense pigmentation is mainly caused by the degradation of chlorophyll and massive accumulation of carotenoids (especially lycopene) within the cell plastids (39). The underlying mechanism for this phenomenon relates to the differentiation of chloroplasts into chromoplasts during fruit maturation (Figure 1.5).

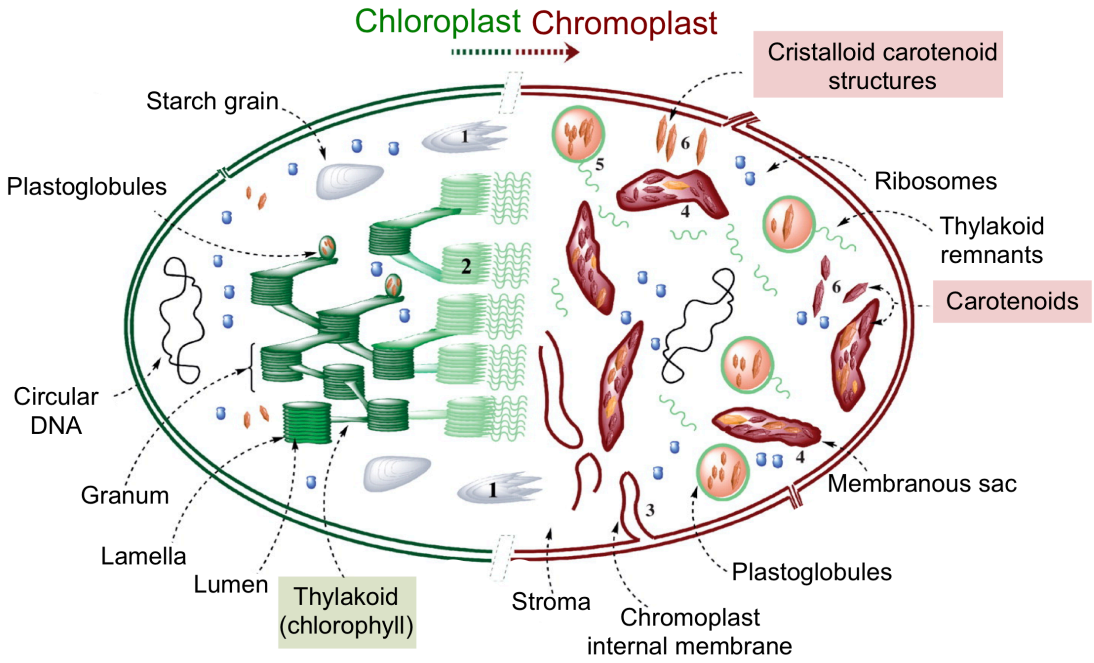



**FIGURE 1.5.** Schematic representation of a chloroplast, differentiating into a chromoplast plastid. The scheme represents the breakdown of starch granules (1) and thylakoid membranes (2), the synthesis of new membrane structures from the inner envelope of the plastid (3), the formation of carotenoid-rich membranous sacs (4), the increase in the number and size of plastoglobules (5), and the appearance of carotenoid-containing crystalloids (6) (adjusted from Egea *et al.* (2010) (40)).

The plastid differentiation process is characterized by the disintegration of thylakoid membranes, degradation of chlorophylls and many other components of the photosynthetic apparatus, and accumulation of carotenoids in novel chromoplast structures (41,42). Hereby, carotenoids are typically linked with proteins and polar lipids to form sequestering substructures, which may prevent carotenoid end products from overloading the plastid membranes (the site of carotenoid biosynthesis) and avoid a negative feedback to the biosynthetic pathway (43).

Significant progress has been made in the understanding of carotenoid biosynthesis and catabolism in plants. Nearly all genes and encoded enzymes, involved in the biosynthetic pathway, have been isolated and characterized (43). An outline of the current understanding of this pathway is presented in Figure 1.6. The initial step within the carotenoid biosynthetic pathway enfolds the condensation of two geranyl-geranyl diphosphate molecules (GGDP), which are provided by the preceding and recently identified 1-deoxy-D-xylose-5-phosphate (DOXP) pathway. During the DOXP-pathway, isopentyl diphosphate is formed and subsequently isomerized to its allylic isomer dimethylallyl diphosphate, the activated substrate of GGDP. The condensation reaction between two GGDPs is catalyzed by phytoene synthase (PSY) and renders 15-*cis*-phytoene, the first $C_{40}$ carotenoid. Phytoene may then be converted in lycopene by means of phytoene desaturase (PDS) and carotene ζ-desaturase (ZDS), thereby introducing four double bonds (43,44). The cyclization of lycopene creates a series of carotenes that have one or two rings, being either of the β- or ε-type. Lycopene β-cyclase (LCYB) catalyzes a two-step reaction that leads to β-carotene (two β-rings), whereas ε-cyclase (LCYE) creates one ε-ring to produce δ-carotene. It is assumed that α-carotene (β,ε-carotene) is formed by the action of both enzymes (45). Eventually, carotenes may be oxygenated by addition of hydroxyl, epoxy or keto groups, mediated by hydroxylase enzymes. This may lead to the formation of xanthophylls such as lutein, zeaxanthin, violaxanthin and neoxanthin (43,44). Synthesized carotenoid end products may be catabolized through oxidative cleavage, thereby producing apocarotenoids. Among these catabolic compounds, a large structural diversity is noted, which relates to the variation in cleavage sites and subsequent modification and glycosylations of cleavage products. Some of the apocarotenoids are very potent aroma compounds (e.g. β-ionone) or important pigments of high industrial value (e.g. crocin and bixin). Other apocarotenoids may have significant biological activities as signaling molecules, phytohormones (e.g. abscisic acid), or vitamin A precursors (e.g. retinal).

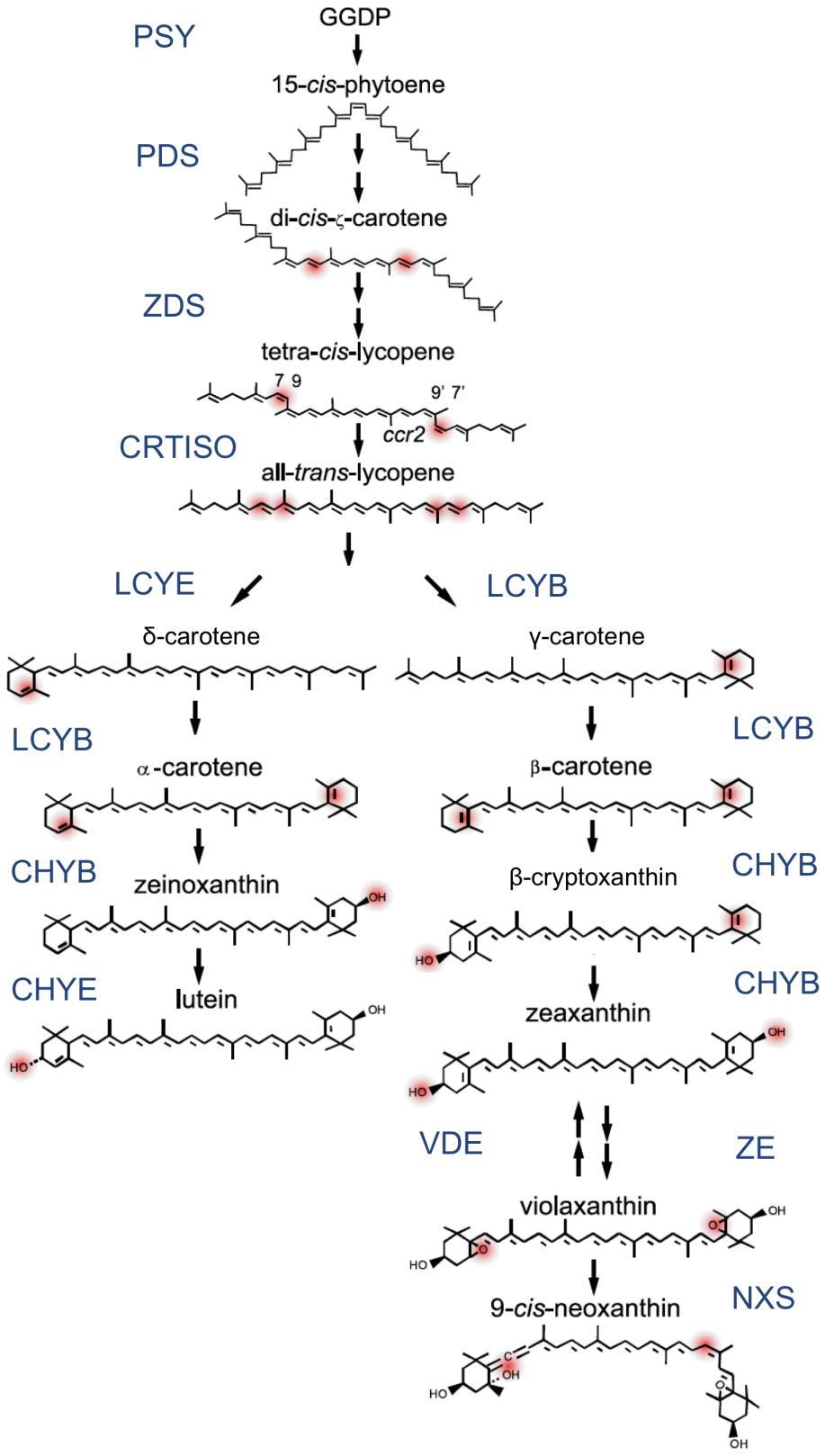



**FIGURE 1.6.** Schematic representation of the carotenoid biosynthetic pathway in plants, indicating the various steps and involved enzymes (PSY, phytoene synthase; PDS, phytoene desaturase; ZDS, ζ-desaturase; CRTISO, carotenoid isomerase; LCYE, lycope ε-cyclase; LCYB, lycopene β-cyclase; CHYE, carotene ε-hydroxylase; CHYB, carotene β-hydroxylase; VDE, violaxanthin deepoxidase; ZE, zeaxanthin epoxidase; NXS neoxanthin synthase) (adjusted from DellaPenna and Pogson, 2006) (50).

## 2.3 FACTORS AFFECTING TOMATO FRUIT CAROTENOID METABOLISM AND CONTENT

With respect to carotenoid metabolism, significant progress has been made in the understanding of biosynthesis and catabolism. Herewith, extensive knowledge has been gathered about the enzymes and associated genes that are involved in the established pathways. As a result, interest has been focused on the identification and deepening of those mechanisms, which regulate the expression of involved genes and thus carotenoid metabolism (38,44). In this regard, a lot of research has been conducted to study the impact of environmental factors and agricultural practices towards carotenoid metabolism. In addition, different tomato cultivars and associated genotypes have been extensively assessed for their influence on carotenoid content (9,10).

### 2.3.1 GENOTYPE

Carotenoid concentration levels in tomato fruit are strongly depending on the genetic background, making the selection of the cultivated variety a crucial aspect to achieve high nutritional value. The influence of the background has been assessed in the study of Kuti *et al*. (2005) (46), thereby determining the lycopene content of forty tomato cultivars, enclosing cluster-, round-, and cherry-type tomatoes. Significant differences in lycopene concentration on a fresh weight basis were noted between both tomato types and cultivars. Concentration levels ranged from 5.7 to 47.8 mg $kg^{-1}$ for the round tomato types, from 21.9 to 35.4 mg $kg^{-1}$ for the cluster tomato types, and from 48.9 to 63.9 mg $kg^{-1}$ for the cherry tomato types. In addition, interactive effects between genotype and environment (greenhouse or open field conditions) were observed. These findings indicate the importance of genetic background, which has been reported in other studies (47-49). Moreover, a relationship between carotenoid content and genetic background is of significant value in breeding programs (46), aiming for tomato cultivars with high nutritional value.

### 2.3.2 ENVIRONMENTAL FACTORS

The two best-known environmental factors, having a potential influence on tomato fruit carotenoid content, are light and temperature. The direct effects of other factors such as air humidity or carbon dioxide concentration are, however, less conclusive (9). Therefore, within this section, some general conclusions will be presented for light and temperature.

Although light is not required for the synthesis of carotenoids in ripening tomato fruits, this factor has been ascribed a fundamental and determining role towards final carotenoid content. Herewith, biosynthesis and accumulation of carotenoids are strongly affected by the intensity, duration and quality of the light, received by the tomato plant (9). Indeed, various studies have observed increased lycopene and β-carotene levels when fruits are exposed to higher light intensities (51-54). However, too intense radiation (650 W $m^{-2}$) may adversely affect the accumulation of these fruit constituents (55). Moreover, in a few studies (56,57), no effects on lycopene content were concluded when light intensity was altered. With respect to light quality, it has been indicated that red light (~580-700 nm) exerts a stimulating influence on carotenoid accumulation whereas far-red light (~700-800 nm) inhibits carotenoid biosynthesis (58-60). This finding has been demonstrated in the study of Adegoroye and Jolliffe (1987) (58) in which brief treatments of red light were found to stimulate lycopene accumulation in detached mature-green fruits by about 2-fold (from 37.2 to 86 mg $kg^{-1}$ fresh weight) and subsequent far-red light treatments were able to reverse this increase (51.5 mg $kg^{-1}$ fresh weight). Lastly, light duration has also been found to affect carotenoid content. The study of Cox *et al.* (2003) (61) observed that tomato fruits, which were exposed to an 8-h photoperiod failed to develop lycopene levels as high as those in 24-h exposed fruits.

Fruit temperature has been indicated to significantly affect general plant metabolism and respiration, as such altering carbon skeleton availability. This evidently may have a considerable impact on carotenoid biosynthesis and thus on tomato fruit nutritional value (62,63). In addition, temperature may also exert a direct influence on enzyme activity and carotenoid synthesis. In this context, it has been determined that lycopene synthesis is strongly inhibited at temperatures below 12 °C and even completely stopped at temperatures above 32 °C (64). As such, the optimum temperature for lycopene biosynthesis has been found to range between 20 and 24 °C (65), being dependent on genotype and interactions with other environmental and agricultural factors. In contrast to lycopene, β-carotene is only slightly affected by temperature, most probably due to the enhanced conversion of lycopene into β-carotene under high temperature conditions (66).

### 2.3.3 AGRICULTURAL TECHNIQUES

As with the environmental factors, various agronomic practices may also have a significant effect on tomato fruit nutritional quality, enfolding carotenoid content. In this respect, numerous studies have been conducted in which the specific impact of water management, mineral nutrients, growing system, plant management, ripening stage at harvest, etc. is investigated. In this particular section, however, we will solely focus on those practices that are believed to be relevant towards this research.

The amount of irrigated water is an important determinant of tomato fruit yield as well as quality. A precise irrigation scheduling to optimize water supply to the plant is thus of significant importance to achieve the intended aims (9). Increasing water supply is generally considered to positively affect tomato fruit yield, but to reduce carotenoid concentration levels due to higher fruit water content (62). On a fresh weight basis, carotenoid content is usually lower under high water supply (67). Under low water supply the concentration of carotenoids may be enhanced, being the result of impaired water uptake by the fruits and the activation of physiological plant (stress) responses. With respect to the latter, altered phytohormone concentration levels (i.e. abscisic acid and ethylene) have been suggested to exert an important regulating influence (9). Such positive effects on carotenoid content from reduced water supply have for example been revealed in the study of Mitchell *et al.* (1991) (68) and Veit-Köhler *et al.* (1999) (69).

Management of the irrigated nutrient solution electrical conductivity (EC) has intensively been studied as a strategy to enhance tomato fruit nutritional quality, including carotenoid content. Herewith, it is largely accepted that high nutrient solution EC-levels have the potential to improve tomato fruit quality. At the same time, salinization of the nutrient solution may reduce fruit size and marketable yield (3). In this respect, any improvement of tomato fruit carotenoid content was exclusively attributed to a concentration effect, being the result of osmotic stress and related to impaired water uptake. However, physiological plant responses may be activated as well (70). For example, De Pascale *et al.* (2001) (71) determined an optimum total carotenoid and lycopene content (on both a fresh and dry weight basis) for salinity EC-levels between 4.0 and 4.4 dS $m^{-1}$. A similar finding is described in the study of Wu *et al.* (2008) (72), in which enhanced lycopene levels were observed in hydroponically grown tomato plants using a nutrient solution of 4.8 dS $m^{-1}$

compared to standard EC-conditions of 2.4 dS $m^{-1}$. Hereby, the increase in lycopene concentration (34 to 85%) for the four cultivars tested was significantly higher than the increase in total soluble solids (12 to 22%). However, in the study of Krauss *et al.* (2006) (73), lycopene concentrations were enhanced by increased salinity on a fresh weight basis only. When expressed on a tomato fruit dry weight basis, no significant differences were observed. As such it may be stated that the effect of altered EC-levels depends on interaction between cultivar, environmental factors and cultural practices (3).

The effects of minerals on carotenoid concentration in tomato fruit are strongly depending on the specific mineral, the mineral form, the plant genotype, and any possible interactions with environmental conditions and agronomic practices (10). In general, the minerals that have been assigned the highest impact on carotenoid content are nitrogen, calcium, phosphorus and potassium (9). For example, in the study of Serio *et al.* (2007) (74), tomato fruit lycopene concentration on a fresh weight basis was increased by almost 35% when the potassium level was raised from 150 to 450 mg $L^{-1}$ nutrient solution. This positive effect from potassium has been confirmed by various other studies (75-77). However, because of the above-cited interactive effects, data about the effects of other minerals are often less conclusive (10).

### 2.3.4 INTERACTIVE EFFECTS

The final nutritional quality of tomatoes is strongly depending on the interactive effects between genotype, environmental factors, and agricultural techniques. Finding the best combination between these factors to maximize nutritional quality is therefore a challenging task. Although a single set of conditions would be optimal, such an outcome may not be possible. Indeed, the synergistic and antagonistic effects between factors may strongly complicate an unambiguous interpretation of the obtained results and constitute the main reason for the conflicting data, which have frequently been reported. New approaches (e.g. modeling) and more data would therefore be of great interest to understand more clearly how agronomic factors and techniques are liable for the effects they cause, as has been stated by Dumas *et al.* (2003) (10).

# 3. ANALYSIS OF CAROTENOIDS IN TOMATO

## 3.1 THE ANALYTICAL CHALLENGE

The health-promoting properties of carotenoids have been the underlying reason for the wide variety of methods that have been developed for their analysis. Characterization of carotenoids in organic biological matrices is indeed a crucial step in any research valorization trajectory, which pursues a further deepening of the physiological and metabolic functions of carotenoids (78,79). However, the development of analytical methods for these compounds is regarded as a complicated task because of the structural diversity among carotenoids and the existence of several geometric *cis-trans* isomers (21,78). In addition, the typical chemical structures of carotenoids are responsible for their instability with respect to oxygen, light, and heat. For this reason, a series of precautions should be taken, in particular during extraction (79).

The complexities, which have been associated with carotenoid analysis, are of critical significance and should be taken into consideration during optimization of analytical procedures. The stages that are typically included are extraction, chromatographic separation and compound detection.

## 3.2 EXTRACTION OF CAROTENOIDS, A MATTER OF PRECAUTIONS

Because of the large chemical diversity among carotenoids and the wide range of fruits and vegetables containing these compounds, there exists no standard extraction procedure for carotenoids (21). However, most procedures do follow a common path including solid-liquid or liquid-liquid extraction, purification by solvent partitioning and saponification, and extract enrichment by evaporation. During each of these steps, precautionary measures should be taken to avoid carotenoid degradation or transformation. The conjugated double bond system is indeed accountable for carotenoid instability. In addition, acidic or alkaline conditions may also have detrimental effects on certain carotenoids (21). As such, it is advisable to use antioxidants (e.g. butylated hydroxytoluene), subdued light conditions, and extraction solvents with low boiling points (79). The addition of magnesium or calcium carbonate to the extracting solvent mixture is also recommended in order to neutralize trace levels of organic acids (80).

The most widely accepted procedures involve carotenoid extraction with organic solvents including pentane, *n*-hexane, dichloromethane, chloroform, tetrahydrofuran, methanol, ethanol, acetone, ethyl acetate, and petroleum ether (21,79,81,82). Herewith, non-polar solvents such as hexane are a good choice for the extraction of non-polar (carotenes) or esterified carotenoids, while polar solvents such as ethanol and acetone are more appropriate for the more polar compounds (xanthophylls) (83). Therefore, solvent mixtures such as ethanol/hexane (4:3, v/v) and acetone/hexane (4:6, v/v) have been introduced to efficiently extract representatives of both carotenoid classes (84,85,86). This first extraction step is usually repeated several times to improve extraction efficiency (82). After initial extraction, the obtained extract may still contain some interfering compounds, which are the motive for purification. Partitioning of the extract against water or an aqueous salt solution has been used for the removal of some polar lipids (21). In addition, saponification (using potassium hydroxide) is performed to eliminate both chlorophylls and lipids. However, since alkaline conditions are imposed, substantial degradation of carotenoids might occur (21,79). The final step of the extraction procedure typically encloses extract enrichment by evaporation. Because of the thermolability of carotenoids, this step should be avoided if possible. Temperatures during evaporation should remain below 40 °C to limit degradation (82).

### 3.3 CHROMATOGRAPHIC SEPARATION OF CAROTENOIDS

Chromatography is a physical separation technique in which compounds are selectively distributed between two immiscible phases, i.e. a mobile phase that is flowing through a stationary phase. Based on the physical state of the mobile phase, a differentiation between gas and liquid chromatography has been made. However, gas chromatography is unsuited for carotenoid analysis because of the inherent thermal instability and low volatility of these compounds (87). As such, reversed-phase high-performance liquid chromatography (HPLC) has emerged as the reference method for chromatographic separation of carotenoids.

HPLC-columns with a monomeric octyl ($C_8$) or octadecyl ($C_{18}$) stationary phase are most intensively used (81-83). Unfortunately, these traditional reversed-phase chromatographic columns seldom have the ability to separate the geometric *cis-trans* isomers of a particular carotenoid (21). In contrast, the novel triacontyl silica $C_{30}$ stationary phase has shown superior chromatographic

resolution for isomer separation (21,83,88,89). The main hindrances that have been identified for HPLC include the extensive analysis times (> 30 min) and high organic solvent usages (85,90,91). Therefore, throughout the last years, there has been a trend towards ultra high-performance liquid chromatography (UHPLC). This technique retains the basic principles of HPLC but enables a faster separation due to the use of columns packed with sub-2 μm particles. UHPLC provides improved speed of analysis, as well as a better resolution, an increased sensitivity and a reduction of matrix effects (81). So far, UHPLC ethylene bridged hybrid (BEH) $C_{18}$ and high strength silica (HSS) $C_{18}$ and $T_3$ (trifunctional alkyl phase) have been successfully used to separate several carotenoids (92-95). $C_{30}$ stationary phases are not yet available for UHPLC columns (78).

With regard to the mobile phase, solvent systems are mostly modifications of acetonitrile and methanol. A small percentage of a less polar solvent is usually added to obtain the desired retention, increase solubility of the analytes and improve separation. The most frequently used modifiers are dichloromethane, tetrahydrofuran, methyl-tert-butyl ether, ethyl acetate, acetone, and water (83). A comprehensive overview of various methods for carotenoid chromatographic separation has been presented by Amorim-Carrilho *et al.* (2014) (83) and Rivera and Canela-Garayoa (2012) (81).

## 3.4 DETECTION OF CAROTENOIDS

### 3.4.1 ULTRAVIOLET-VISIBLE ABSORBANCE DETECTION

The detection of carotenoids has traditionally been carried out by ultraviolet-visible (UV-Vis) absorbance spectroscopy (96). After all, the majority of carotenoids exhibit absorption in the visible region of the spectrum, more particularly between 420 and 500 nm. Both fixed-wavelength and photodiode array (PDA) UV-Vis detectors have been applied for carotenoid detection (97). However, PDA-detectors are more often used because of their ability to record a specific spectral range, which is extremely important for identification purposes. Since the UV-Vis spectral patterns of many carotenoids are very similar and structurally related carotenoids may coelute, this approach may pose the risk of compound misidentification (94,96). Especially in the case of complex food matrices such as tomato, UV-Vis approaches may be inadequate for unambiguous identification of the present carotenoids, owing to the risk of spectral interferences (97).

### 3.4.2 MASS SPECTROMETRIC DETECTION

Mass spectrometric (MS) techniques have emerged as an interesting alternative to UV-Vis detection since they are much more selective (78). The MS operating principle consists of ionizing chemical compounds to generate charged molecules or molecule fragments, sorting and identifying the ions according to their mass-to-charge ratio (*m*/*z*), and measuring the abundance of the ions. As such, the mass spectrometer comprises an ionization source and mass analyzer (98).

#### *3.4.2.1 IONIZATION SOURCE*

Several ionization methods have been reported for MS analysis of carotenoids including electron impact (EI), fast atom bombardment (FAB), matrix-assisted laser desorption ionization (MALDI), electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), atmospheric pressure photoionization (APPI), and atmospheric pressure solids analysis probe (ASAP) (81). Among these techniques, ESI and APCI have been recognized as the most effective for carotenoid ionization (87,94,97,99-102). Overall, APCI has a greater ability to ionize non-polar and medium polar compounds, and has therefore become the preferred technique for the ionization of carotenoids (83,97). This ion source has indeed successfully been used for the ionization of xanthophylls, carotenes and carotenoid esters, providing excellent sensitivity and detector response linearity (78,81). Although carotenoid ionization in negative mode has been reported, most mass spectra have been acquired using the positive ionization mode. Herewith, protonated ($[M+H^+]^+$) and radical molecular ions ($M^{+\bullet}$) are predominantly formed (103). Moreover, significant amounts of in-source fragments resulting from loss of one ($[M+H^+-H_2O]^+$) or two water molecules ($[M+H^+-2H_2O]^+$) may be produced upon xanthophyll ionization (78).

#### *3.4.2.2 MASS ANALYZER*

In literature, two types of mass analyzers have been primarily used for the detection of carotenoids, i.e. ion trap (99,104-106) and triple quadrupole mass spectrometers (94,96,107-109). Both instruments offer the possibility of collision-induced dissociation (CID) in order to generate ion fragments. The acquired fragmentation pattern may serve as an additional "fingerprint" for carotenoid identification, thereby enclosing valuable information about the type of functional groups, polyene backbone, and location of double bonds in the ring structure (78,99,103). It should be noted that fragmentation patterns might differ depending on the molecular ion species that are

generated during ionization (protonated or radical molecular ions). Characteristic fragments typically involve the cleavage of toluene, xylene, water, ionone ring moieties, and methyl-cyclopentadiene (78,103,110). Within the context of this thesis and because of its predominant use, only the triple quadrupole mass analyzer (tandem mass spectrometry) will be discussed in detail.

A triple quadrupole (QqQ) mass spectrometer typically enfolds a linear series of three quadrupole elements (Figure 1.7), which may function as either mass filter or collision cell. Herewith, a single quadrupole element is composed of four parallel rods (poles) that are equally arranged around a central axis. By applying accurately controlled voltages to opposing sets of rods, a mass filter may be created. Only ions with a particular mass-to-charge ratio will follow a stable resonance trajectory and pass the mass filter. This mass filtering mode is employed in the first (Q1) and third (Q3) quadrupole. In the middle element (Q2), collision-induced dissociation is performed by using argon, helium, or nitrogen gas. Although various operating modes are possible, the selected reaction monitoring (SRM) (Figure 1.7) mode is generally recognized as the most quantitative and sensitive (112). In such SRM experiments, the first and third quadrupole are used as static mass filters to monitor a particular fragment ion of a selected precursor ion. Alternatively, when multiple SRM transitions (pairs of *m*/z-values associated to the precursor and fragment ions) are measured within the same experiment, multiple reaction monitoring (MRM) is performed (111).

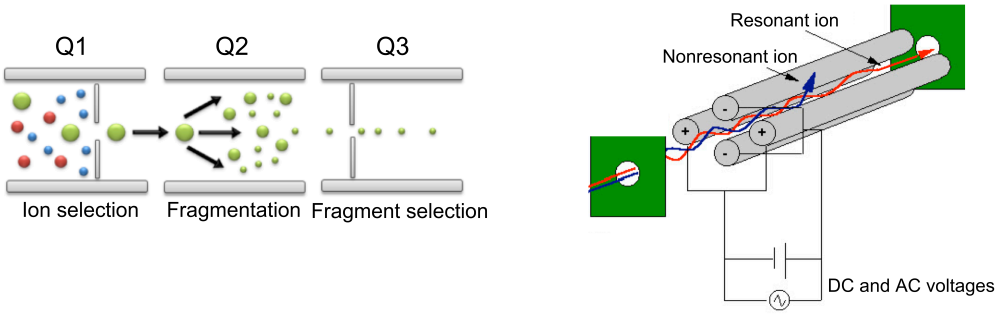



**FIGURE 1.7.** Schematic representation of the triple quadrupole mass analyzer, operating in SRM mode (113), and a focus on the operating principle of ion (or fragment ion) selection within a quadrupole element (114).

# 4. PHYTOHORMONES AND THEIR REGULATING ROLE IN PLANTS

## 4.1 WHAT ARE PHYTOHORMONES AND HOW DO THEY FUNCTION?

Phytohormones are defined as a group of naturally occurring secondary metabolites, which are of vital importance for the normal functioning of plants. As a minor component of the metabolome, these compounds are of particular interest because of their regulating role in numerous plant processes such as germination, growth, reproduction, and stress responses (13). These basic aspects of plant life are indeed delicately mediated by phytohormones, which are present and active at very low concentration levels, usually in the range of 0.1 to 50 ng $g^{-1}$ fresh weight (115). These structurally and chemically diverse compounds have been grouped into several phytohormonal classes including auxins, abscisates, cytokinins, gibberellins, ethylene, brassinosteroids, jasmonates, polyamines, salicylates, and strigolactones (116). Although to each class characteristic biological effects have been attributed, the concept has emerged that none of the crucial biological functions can be completely explained in a mono-causal manner. Indeed, it has been indicated that the plant response to a given stimulus results from the synergistic, antagonistic or additive actions between multiple phytohormones (13,117). Despite of these complex fundaments of phytohormonal regulation, the recent developments regarding chemical analysis and molecular genetics enable further elucidation of hormonal signaling networks, functions, and metabolic pathways (118). This is of particular interest with respect to agriculture development, for which understanding plant hormone physiology has become an important target. The phytohormonal actions that are associated with plant development and environmentally induced plant responses may indeed have a direct or indirect influence on crop yield and quality (119).

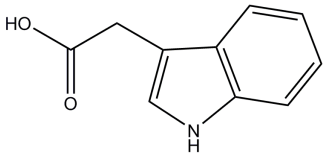
## 4.2 THE PHYTOHORMONAL CLASSES AND THEIR BIOLOGICAL FUNCTIONS

Auxins, cytokinins, gibberellins, abscisates and ethylene are recognized as the five classic hormonal classes. However, evidence has accumulated to extend this concept by incorporating brassinosteroids, jasmonates, and salicylates. In addition, it is likely that further newcomers such as strigolactones and polyamines may be added to this list in the future (116). A brief overview of the several hormonal classes regarding their structural characteristics and main biological activities is given below.

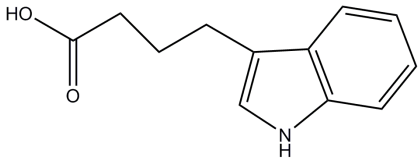
### 4.2.1 AUXINS

Auxins are defined as low-molecular weight organic acids containing an aromatic ring and a carboxyl group (Figure 1.8). The most abundant auxin is indole-3-acetic acid, which is assigned most of the recognized auxin biological actions. In addition to indole-3-acetic acid, only three other naturally occurring auxins have been ascribed biological activity in plants, namely indole-3-butyric acid, 4-chloroindole-3-acetic acid and phenylacetic acid (120). Both free acids and conjugated auxins may occur in plants. These conjugates serve as reservoirs of inactive auxins that can be hydrolyzed to provide plants with active hormone (121). The main conjugates are formed by auxin linkage with sugars, high molecular weight glycans, amino acids, and peptides (122). Although an extraordinarily broad variety of biological phenomena are regulated by auxins, their main regulating actions relate to cell enlargement, cell division, vascular tissue differentiation, root initiation, tropistic responses, apical dominance, leaf senescence, leaf and fruit abscission, fruit set and growth, assimilate partitioning, fruit ripening, flowering, and (pathogen) plant defense (13,123).

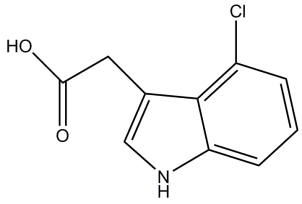
*Indole-3-acetic acid*




*Indole-3-butyric acid*




*4-Chloroindole-3-acetic acid*




*Phenylacetic acid*

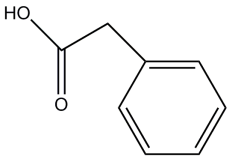
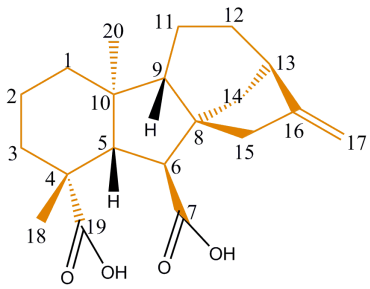



**FIGURE 1.8.** Chemical structures of the main naturally occurring active auxins (120).

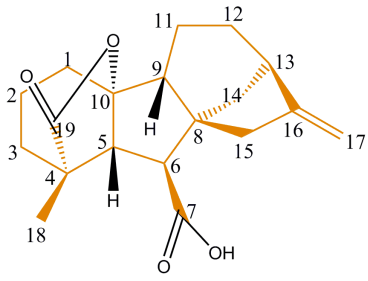
### 4.2.2 GIBBERELLINS

Gibberellins constitute a large family of naturally occurring tetracyclic diterpenoid structures, which enclose either an ent-gibberellane (20 carbon atoms) or ent-20-norgibberellane (19 carbon atoms) skeleton (Figure 1.9). Although up to 136 gibberellin derivatives have been identified so far, only a few are assigned intrinsic biological activity. The presence of an ent-20-norgibberellane carbon skeleton with a 4,10-lactone, a 3β-hydroxyl functional group on C3, and a carboxylic acid group on C6 are generally required for gibberellin activity. Gibberellin A1 (GA1), GA3, GA4, GA5, GA6, and GA7 fulfill these requirements and are regarded as biologically active. Other gibberellins are likely to be inactive precursors or deactivation products (124,125). The active gibberellins are now known to be regulating many phases of higher plant development, including seed germination, stem growth, induction of flowering, pollen development, and fruit growth (126). In addition, emerging evidence suggests that gibberellin-signaling components play major roles in plant diseases resistance and susceptibility (127).

**Ent-gibberellane based gibberellin** *(GA12)*

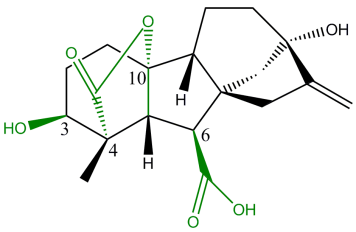



**Ent-20-norgibberellane based gibberellin** *(GA9)*

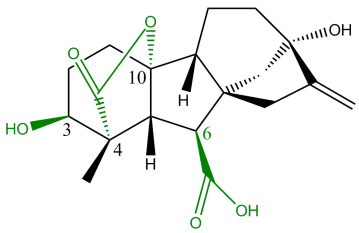



**Main bioactive gibberellins**

*GA1*




*GA3*




*GA4*

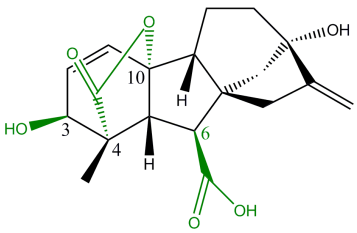


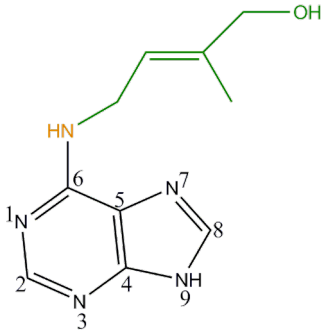

**FIGURE 1.9.** Ent-gibberellane and ent-20-norgibberellane carbon skeleton, respectively enfolded by GA12 and GA9. Chemical structure of the main bioactive gibberellins (GA1, GA3, and GA4) with indication of the structural elements, required for biological activity (besides the ent-20-norgibberellane skeleton) (126).

### 4.2.3 CYTOKININS

The natural cytokinins are adenine derivatives, which carry either an isoprene-derived or aromatic side chain at the $N^6$-terminus (Figure 1.10) (128). Cytokinins with an unsaturated isoprenoid chain are by far the most prevalent, in particular those with a *trans*-hydroxylated $N^6$-side chain (e.g. *trans*-zeatin and its derivatives). The best-known aromatic cytokinins are kinetin and $N^6$-benzyladenine, both containing ring substituents at the $N^6$-position (129). In both groups, there are small variations in the side-chain structure such as the absence or presence of hydroxyl groups and their stereoisomeric positions (128). This phytohormonal class has been found involved in cell proliferation and differentiation of plant cells, seed germination, control of the shoot/root balance, nutrient mobilization, increased crop productivity, floral development, breaking of bud dormancy, and delay of senescence (130). Moreover, there are indications that cytokinins are involved in the regulation of plant defense responses against some pathogens (127).

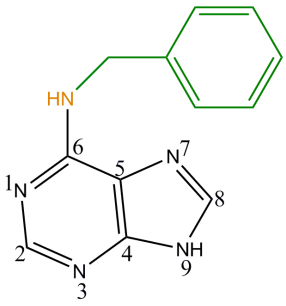
**Isoprenoid cytokinins**

*Trans-zeatin*




**Aromatic cytokinins**

*$N^6$-benzyladenine*




*Kinetin*

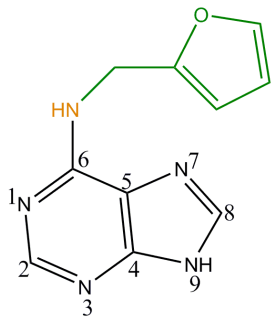



**FIGURE 1.10.** Chemical structures of the major cytokinins, representing both isoprenoids and aromatics (129).

### 4.2.4 ABSCISATES

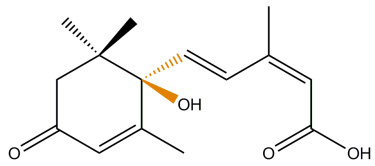
Abscisates are synthesized by cleavage of $C_{40}$ carotenoids and are thus categorized as isoprenoid compounds (131). The main representative is abscisic acid, which contains two chiral centra (Figure 1.11). Since these chiral centra may have either an R or S configuration, various stereoisomers of abscisic acid exist. Abscisic acid is typically defined as a 'stress hormone', named for its regulating role in responses to abiotic stress environments such as drought, salinity and cold. One notable effect of abscisic acid concerns the stomatal closure and associated prevented

loss of water by transpiration. In addition, this phytohormone has also been assigned a regulating role in the plant defense response against pathogens, including both negative and positive regulation (132,133). Besides the traits of stress response, abscisic acid is also involved in normal physiological manipulations like storage of compounds, dehydratation at later stages of embryogenesis, seed maturation, dormancy formation, and abscission (134). Abscisic acid is also circulated throughout the plant as an inactive glucosyl ester that can be rapidly released into the active form and contributes to the maintenance of abscisic acid homeostasis (Figure 1.11) (135,136).

**Abscisic acid**

*R,R-cis, trans-abscisic acid*

*(-)-cis, trans-abscisic acid*




**Abscisic acid glucosyl ester**

*S,S-cis, trans-abscisic acid glucosyl ester*

*(+)-cis, trans-abscisic acid glucosyl ester*

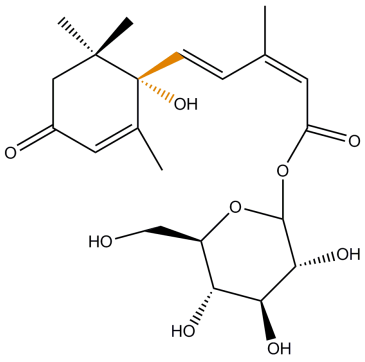



**FIGURE 1.11.** Chemical structures of the main abscisates, i.e. active abscisic acid and its inactive conjugate abscisic acid glucosyl ester. The presence of chiral centra may generate various enantiomers. R,R and S,S are herewith denoted as (-) and (+), respectively. A combination of R and S chirality (not presented) would be noted as (±) (131,135).

### 4.2.5 ETHYLENE

Ethylene is a gaseous hormone, chemically characterized by the simplest unsaturated hydrocarbon structure (i.e. $C_2H_4$). In the broadest of terms, ethylene triggers senescence of plant organs, acts as a stress hormone during biotic (necrotropic pathogens and herbivorous insects) and abiotic stress conditions, influences plant growth, and exhibits various morphogenetic effects. Among senescence processes, ethylene-induced ripening of so-called climacteric fruits has been most intensively investigated because of its great agronomic impact (127,137,138).

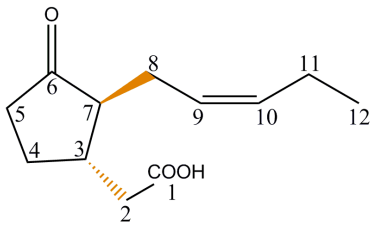
### 4.2.6 JASMONATES

Jasmonic acid and its methyl ester are defined as the principal representatives of the jasmonate hormonal class. These phytohormones are characterized by a linolenic acid derived cyclopentanone based structure, containing two chiral centers. Since these centers have either an R or S configuration, various stereoisomers may be generated (Figure 1.12) (139). Jasmonate functioning has mainly been associated with plant defense responses towards wounding by herbivore or insect attack. Other biological activities relate to vegetative development, fruit development, and pollen viability (139,140).

**Jasmonic acid**

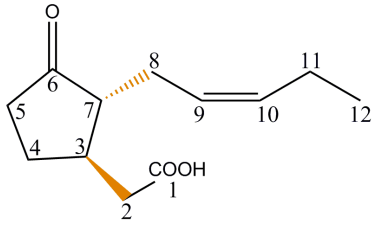
*3S, 7S-jasmonic acid*

*(+)-jasmonic acid*




*3R, 7R-jasmonic acid*

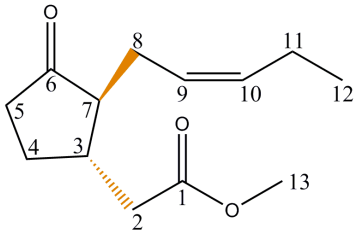
*(-)-jasmonic acid*




**Jasmonic acid methyl ester**

*3S, 7S-jasmonic acid methyl ester*

*(+)-jasmonic acid*




*3R, 7R-jasmonic acid methyl ester*

*(-)-jasmonic acid methyl ester*

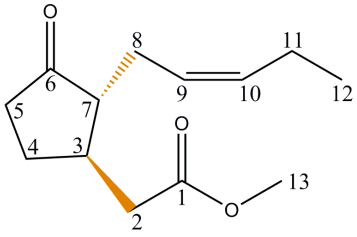



**FIGURE 1.12.** Chemical structures of jasmonic acid and its methyl ester. S and R chirality at C3 and C7 are defining the isomeric form, whereby 3R, 7R and 3S, 7S are noted as (+) and (-), respectively (139).

### 4.2.7 SALICYLATES

The hormonal class of salicylates encloses salicylic acid and its functional derivatives. As the main representative, salicylic acid is a phenolic compound, characterized by an aromatic ring and hydroxyl group (Figure 1.13). This phytohormone is known to form conjugates with a number of molecules by glycosylation and less frequently by esterification. These conjugates may function as

an inactive storage form that can release free salicylic acid (141,142). The biological activity of this particular phytohormone involves the mediation of plant response to abiotic stresses such as drought, chilling, heavy metal tolerance, and osmotic stress. Most of the research on salicylic acid has, however, focused on its role in the local and systemic response against microbial pathogens. In addition, there is evidence that this phytohormone fulfills a regulating role in seed germination, vegetative growth, photosynthesis, respiration, thermogenesis, stomatal closure, flower formation, fruit yield, and seed production (142,143).

*Salicylic acid*

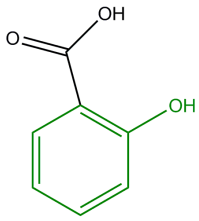
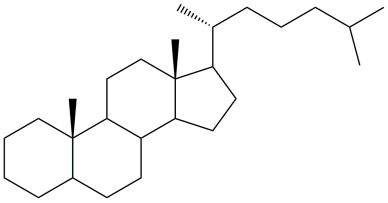



**FIGURE 1.13.** Phenolic-based structure of salicylic acid, the main representative of the salicylates (141).

### 4.2.8 BRASSINOSTEROIDS

Brassinosteroids enclose a wide variety of steroidal compounds, more particularly hydroxylated derivatives of cholestane. More than fifty compounds have been identified so far and are classified as $C_{27}$, $C_{28}$, or $C_{29}$ brassinosteroids, depending on the alkyl-substitution pattern of the side chain (144). These phytohormones are assigned a regulating role in cell expansion and division, stress resistance, reproductive development, tissue differentiation, and fruit size, quantity and quality (145-147). Brassinolide is regarded as the brassinosteroid with the highest biological activity (Figure 1.14).

**Cholestane basal skeleton**



*Brassinolide*

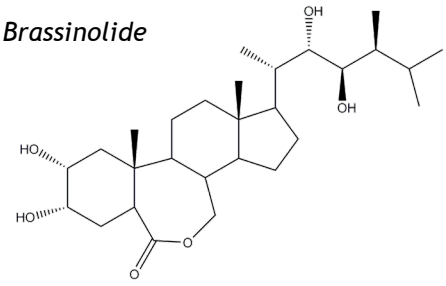



**FIGURE 1.14.** Chemical structure of the brassinosteroid cholestane basal skeleton and brassinolide (144).

### 4.2.9 POLYAMINES

In plants, the diamine putrescine, triamine spermidine, and tetramine spermine constitute the major polyamines (Figure 1.15). They occur in a free form or as conjugates, bound to phenolic acids and other low-molecular weight compounds or macromolecules such as proteins and nucleic acids. As such, they stimulate DNA replication, transcription and translation. They have been implicated in a wide range of biological processes including senescence, environmental stress and infection by fungi and viruses (148).

*Putrescine* *Spermidine* *Spermine*

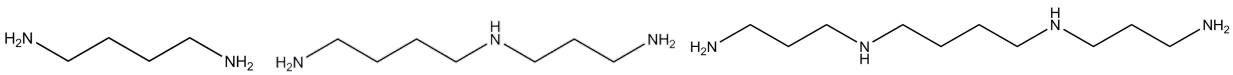


**FIGURE 1.15.** Chemical structure of the major polyamines in plants (148).

### 4.2.10 STRIGOLACTONES

Strigolactones have only recently been revealed as new hormonal players. This group of putatively carotenoid-derived terpenoid lactones (Figure 1.16) was isolated from root exudates and found to stimulate seed germination of root parasitic plants including *Striga* spp.; hence the name strigolactones was given. These phytohormones are also involved in the control of shoot branching and stimulation of plant symbiosis with myccorrhizal fungi (149,150). However, novel roles are continuously emerging. For example, a role in plant defense against fungal pathogens has recently been evidenced (151).

**5-Deoxystrigol**

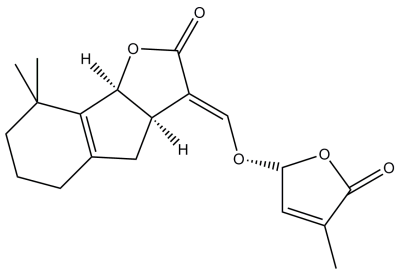


**FIGURE 1.16.** Chemical structure of 5-deoxystrigol, which is considered as the simplest strigolacton and therefore proposed to be the common precursor of other strigolactones (150).

## 4.3 HORMONAL REGULATION OF TOMATO FRUIT RIPENING

Fruit development and ripening is a well-coordinated complex process that involves the interplay of various biotic and abiotic factors. Within this context, the development of a fruit is generally separated into four phases, with growth and maturation following a single sigmoidal growth curve (15). A first phase comprises floral development, pollination, fertilization, and fruit set. The second phase enfolds cell division during which most fruit cells are established. The third phase primarily represents cell expansion, being responsible for attainment of the maximum fruit size. The final phase involves ripening, which is characterized by slow growth and intense metabolic changes (152). Hereby, phytohormones are essential for successful completion of each developmental stage and progression into the next stage. Given the specific research aims of this study, following description on hormonal regulation of tomato fruit development will focus on the latter developmental phase, enveloping ripening and carotenoid accumulation.

The series of modifications that are associated with the transition of a green fruit into a ripe fruit involve many different metabolic pathways, which indicates the primary importance of those factors that strictly control this process. In this context, tomato fruit has been defined as a climacteric fruit whereby a peak in the rate of respiration can be measured before the visible onset of ripening (153). In such climacteric fruits, a peak of ethylene production is observed concomitant with the climacteric respiration. Accordingly, this phytohormone has been assigned a prominent role in tomato fruit ripening. The availability of many well-characterized mutants such as *Never-ripe* (*Nr*), *Green-Ripe* (*Gr*), *ripening-inhibitor* (*rin*), *non-ripening* (*nor*), and *Colorless non-ripening* (*Cnr*) have strongly contributed to the knowledge about ethylene-based regulation of ripening and physiological effects (36). In this context, a regulating role of ethylene in the rapid accumulation of lycopene and β-carotene has been revealed, which is most likely due to an up-regulation influence on the expression of the phytoene synthase enzyme (154). Although ethylene is generally considered as fulfilling a predominant regulating role in ripening and carotenoid biosynthesis, other phytohormones have been assigned significance as well. Especially indole-3-acetic acid and abscisic acid have frequently been documented to be involved in the control of ripening and carotenoid accumulation (15,152). The levels of free indole-3-acetic acid decline prior to the onset of tomato fruit ripening whereas the levels of some associated amino acid conjugates increase. This supports the role of indole-3-acetic acid in controlling the onset of fruit

ripening by modulating the level of its conjugations (155). In addition, down-regulation of specific auxin response factors resulted in dark-green fruits with increased chloroplasts (156). Abscisic acid is implicated in tomato fruit development and ripening, but detailed knowledge of its regulating role in carotenoid accumulation is rather limited. Suppression of abscisic acid biosynthesis has been associated by increased ethylene production and enhanced levels of β-carotene and lycopene. A possible reason for the enhanced accumulation of these carotenoids may relate to an increased carbon flow to carotenoid production and reduced carbon flux into free abscisic acid and metabolites. On the other hand, this phytohormone has also been considered to control carotenoid biosynthesis by regulating plastid development and thus carotenoid storage capacity (157).

Although the phytohormones, cited above, are considered as essential modulators in tomato fruit ripening, the roles of other phytohormones are just emerging. More specifically, jasmonates (jasmonic acid and jasmonic acid methyl ester), brassinosteroids (2,4-epibrassinolide), and polyamines have been shown to regulate carotenoid accumulation (15,155).

# 5. ANALYSIS OF PHYTOHORMONES IN TOMATO

## 5.1 THE ANALYTICAL CHALLENGE

Because of the complex crosstalk among hormone signaling pathways and the multifaceted roles of these signaling molecules in the regulation of a biological process, simultaneous analysis of multiple classes of phytohormones has become critically important (158). However, this analytical aim is regarded as an extremely challenging task because of diverse reasons. Phytohormones and their metabolites are typically present and active at very low concentration levels (~1.0 to 50 ng $g^{-1}$ fresh weight), in a background of a wide variety of more abundant primary and secondary plant metabolites. Even more, these potentially interfering metabolites might be up to ten thousand times more abundant than the compounds of interest (115,119). A secondary difficulty within this context relates to the mostly low quantities of plant material that are available for phytohormone quantification (119). Apart from these low concentration levels, widely differing chemistries have been described for the various phytohormonal classes as well. Herewith, phytohormones enclose several forms with different side groups, which is reflected by neutral, acidic, and basic functionalities (159,160). As a consequence, the simultaneous and comprehensive analysis of phytohormones entails the use of a highly efficient extraction procedure and an analytical platform that is both sensitive and selective. Moreover, the analytical method should be able to accommodate the wide range of chemicals, represented by the different hormonal classes (161).

## 5.2 SAMPLE PREPARATION AS A CRITICAL STARTING POINT

Sample preparation is considered as a critical first step towards the unambiguous detection and accurate quantification of phytohormones in plant tissue. After all, in response to mechanical wounding, which includes the removal of tissue from the plant, various defense and healing mechanisms are activated through hormonal control (162,163). As such, plant material should be kept cold to avoid chemical degradation or enzymatic induction of metabolic changes (119). In addition, during homogenization of the plant material, appropriate measures should be taken to preserve the initial metabolite composition. This homogenization step is essential for representative sampling and can be performed by grinding with mortar and pestle, ball grinders, and knife homogenizers (115).

## 5.3 GENERIC EXTRACTION

An effective generic extraction would encompass the extraction of all phytohormones and their metabolites, present in the plant sample under consideration. Such an approach would be particularly helpful to elucidate the complex crosstalk and signaling pathways between multiple phytohormones in their regulation of various plant responses. Although a real generic extraction is likely to remain utopic, considerable progress has been made.

Solvent extraction is the most widely applied method for the extraction of phytohormones from plant tissue. Herewith, the main intention is to efficiently extract the phytohormonal compounds and their metabolites without removing too much interfering substances from the plant matrix (115,119). Diverse solvents and mixtures of solvents such as methanol, methanol/water, acetone, acetone/water, propanol, propanol/water, and neutral or acidic buffers have been broadly used (164-167). Of these organic solvents, methanol has become the preferred solvent because of its ability to efficiently penetrate into the plant cells during extraction (119,168-170). Non-polar solvents such as ether are rather rarely used to extract phytohormones. After all, the major phytohormonal classes comprise polar compounds (171).

The risk of chemical or enzymatic degradation of phytohormones during extraction may be minimized by performing the extraction at low temperatures (4 °C or even lower for cytokinins), adding antioxidants, or using suitable solvents (115). With respect to the latter, modified Bieleski solvent (methanol/water/formic acid, 15/4/1, v/v/v) has been found to be extremely suited for phytohormone extraction since the degradation of phytohormones is prevented and the extraction of lipophilic compounds is blocked (119,172).

## 5.4 PURIFICATION OF CRUDE EXTRACTS

Purification of the crude plant extract is a crucial step in which substances such as sugars, pigments, and proteins are removed (115). Determination of phytohormone concentration levels could indeed be hindered by the presence of these more abundant primary and secondary metabolites. For this purpose, various methods and techniques have been reported.

### 5.4.1 LIQUID-LIQUID EXTRACTION

Liquid-liquid extraction is a purification strategy in which compounds are secluded on the basis of their relative solubility in two different immiscible liquids (119,172). To purify phytohormones, various organic solvents such as ethyl acetate, dichloromethane and diethyl ester have been used, and this in combination with water (173-175). This traditional liquid-liquid extraction approach, however, involves many steps, which makes it rather time and solvent consuming. In addition, the extraction efficiency of water-soluble phytohormones may be strongly reduced because of serious emulsification (119). Therefore, liquid-phase microextraction (e.g. hollow fiber-based liquid-liquid-liquid microextraction) may enfold a promising alternative for traditional liquid-liquid extraction (176).

### 5.4.2 SOLID-PHASE EXTRACTION

Solid-phase extraction is a sample clean-up technique in which the target compounds, dissolved in a liquid mixture, are separated from other potentially interfering compounds in function of their physical and chemical properties (119). More specifically, phytohormonal compounds are trapped on a solid-phase extraction column while the interfering compounds are eluted or vice versa. This purification strategy is widely used for the analysis of phytohormones because of its high reproducibility and its relatively fast nature (119). The main separation modes (and sorbents) include ion-exchange (DEAE-cellulose, SCX) (177,178), reversed-phase ($C_{18}$, Oasis HLB) (168,179) and mixed mode (Oasis Max, Oasis MCX) (180,181) cartridges.

### 5.4.3 IMMUNOAFFINITY CHROMATOGRAPHY PURIFICATION

Immunoaffinity chromatography (IAC) has a very broad potential for purification of phytohormones because of its high selectivity and sensitivity. Indeed, by using antibodies that are specific for the targeted phytohormones, specific capture and efficient enrichment of these compounds is allowed. However, the high specificity of the antibodies confines the analytes that are retained on the IAC-column to one class of phytohormones (119,172).

## 5.5 ANALYTICAL DETECTION APPROACHES

The very low endogenous phytohormone concentration levels and the presence of more abundant interfering metabolites in the plant extract points towards an analytical method that is both highly selective and sensitive for the targeted phytohormones. Indeed, even with the application of a multistep purification strategy, a large number of interfering substances might still be present in the plant extract to be analyzed (119). Although a number of methods have been used in the field of phytohormone analysis, two principal approaches have emerged over the past few years: immunoassays and mass spectrometry (MS) hyphenated to an appropriate separation technique such as high-performance liquid chromatography or gas chromatography (161,182).

### 5.5.1 IMMUNOASSAYS

Immunoassays represent an attractive strategy for the quantitation of phytohormones because of the associated high specificity and sensitivity. After all, the phytohormone concentration is determined based on the unique binding interactions between the target analyte (acting as antigen) and a complementary antibody. Herewith, the interaction is monitored by detection of labeled units, which are conjugated to the antigen or antibody (183).

Both enzyme and radioimmunoassays have been implemented for phytohormone quantitation (183-187). However, the extensive application of immunoassays remains rather limited because of a number of obstacles. Indeed, besides the time-consuming process of antibody preparation, quantification can be misleading due to cross-reactivity of antibodies and their inhibition or activation by interfering substances (160). Additionally, the specificity of antibodies conflicts with the demand for simultaneous detection of multiple classes of phytohormones (172).

### 5.5.2 GAS CHROMATOGRAPHY COUPLED TO MASS SPECTROMETRY

Gas chromatography (GC) coupled to mass spectrometry is a powerful tool for the sensitive analysis of different classes of phytohormones. This chromatographic separation technique depends on the physiochemical properties of the phytohormones, which includes boiling point and molecular polarity (118). In addition, the interaction with the column is also of significant importance for successful GC-MS analysis. Since the use of GC is limited to the analysis of volatile compounds, derivatization of phytohormones is inevitable. This technique is primarily performed to modify the

functionality of a compound and to induce volatility and stability (119). Traditionally, the carboxyl groups of phytohormones are methylated to render the volatile and more stable methyl esters. However, this derivatization process is time-consuming and some phytohormones are insufficiently volatilized for analysis (118). In addition, as phytohormones are thermally instable, the high temperatures in the gas chromatographic system may induce thermal disintegration (160,172). Despite these shortcomings, several studies have used GC coupled to mass spectrometry as an effective strategy for the analysis of phytohormones from diverse classes. Both single stage (MS) and tandem mass spectrometric (MS/MS) approaches have been employed, whereby the latter provides a higher level of selectivity (164,188-190). Herewith, chemical (APCI and ESI) and electron impact (EI) ionization techniques have been applied (118). A highly sensitive and accurate multiple GC-MS/MS technique has been reported for the analysis of indole-3-acetic acid, abscisic acid, jasmonic acid, salicylic acid, and 12-oxo-phytodienoic acid (190).

### 5.5.3 LIQUID CHROMATOGRAPHY COUPLED TO TANDEM MASS SPECTROMETRY

High-performance liquid chromatography coupled to mass spectrometry has emerged as an effective alternative for the analysis of the polar and thermally labile phytohormones (160). This analytical strategy has indeed the potential to monitor diverse phytohormones without the necessity of complex sample purification or derivatization (191).

With respect to the chromatography, columns with an octadecyl $C_{18}$ stationary phase are most intensively used to effectuate phytohormone separation. Herewith, the mobile phase is predominantly composed of methanol and water, both containing a small volume of formic acid (~0.05 to 0.2%) (117,174,182,192,193). Manipulation of the pH value is indeed one of the key strategies for optimizing chromatographic resolution and ionization efficiency (160).

Ionization of the polar phytohormonal compounds is mainly established by electrospray (ESI) or heated electrospray (HESI) ionization, which have been associated with high sensitivity and low background noise upon mass spectrometric analysis (119). In recent years, tandem mass (MS/MS) spectrometry has become the key technology for monitoring multiple phytohormones in plant tissue with high sensitivity, selectivity, accuracy, and reproducibility (162). Within this specific analytical setting, especially the use of triple quadrupole (in MRM mode) and quadrupole-linear ion trap instruments has frequently been reported (191).

Traditionally, LC-MS/MS has been reported for the analysis of a single compound, a single hormonal class, or a few classes (~2 to 3) with similar characteristics (117,165,194-197). However, the enormous evidence of the complex interactions between phytohormones in their regulation of plant physiological and developmental processes has been the incentive to develop methods, which are capable of analyzing multiple hormonal classes. The study of Chiwocha *et al*. (2003) (159) was the first to effectuate the simultaneous analysis of multiple hormonal classes, significantly differing in physicochemical properties (acid-base functionalities). The presented analytical method was able to simultaneously quantify auxins, abscisates, gibberellins, and cytokinins in lettuce seeds. Subsequently, other methods for multiplex phytohormone analysis have been reported as well (160,192,198). However, it was only recently accomplished by Pan *et al*. (2010) (162) to analyze phytohormones from all seven major classes within a single run, thereby using HPLC-MS/MS. Although the presented methodology undeniably encloses valuable potential for phytohormone research, some limitations are still encountered. There is no possibility for post-acquisition re-interrogation of acquired data (when for example a new type of phytohormone would be discovered), only a limited number of compounds can be measured during a single run, and it is impossible to screen for unidentified, unknown compounds (199). These limitations are the reason for the observed current trend towards full-scan mass spectrometric experiments, which are considered of crucial importance to bridge the gap between the still more targeted analyses and the broader 'metabolomic' profiling strategies (200).

### 5.5.4 LIQUID CHROMATOGRAPHY COUPLED TO HIGH-RESOLUTION MASS SPECTROMETRY

Full-scan high-resolution mass spectrometry has become an effective strategy for global metabolome analyses and associated metabolomics experiments. Within this context, Fourier transform ion cyclotron resonance (FT-ICR), time of flight (ToF), and Orbitrap have emerged as key technologies. Given the importance of this type of mass spectrometry towards our research aims, a detailed description of the features, possibilities and limitations of the outlined technologies is provided. In this regard, a concise definition for some associated key concepts is given.

*MASS RESOLUTION* is generally defined as the ability of a mass analyzer to distinguish between narrow mass spectral peaks. The strict interpretation of this parameter is, however, depending on the considered approach. In the case of the valley based definition, mass resolution is indicated by the

ratio between the mass of the second peak and the mass difference between peaks. Alternatively, mass resolution may also be determined based on an isolated peak whereby the mass difference now relates to the peak width at a specified peak height. This peak width based approach is usually applied at peak half maximum (full width at half maximum, FWHM) (201).

*FULL-SCAN* mass spectrometric instruments provide a full mass spectrum of all analytes that were introduced into and ionized by the ion source. This approach offers the advantage of the simultaneous analysis of a virtually unlimited number of analytes. As a result, retrospective evaluation of acquired data for non-"a priori" selected compounds becomes possible by reconstructing any desired ion chromatogram (202).

*MASS ACCURACY* refers to the accuracy of an ion's *m*/*z*-value, provided by the mass analyzer. This parameter is thus indicative for the difference between the theoretical and measured *m*/*z*-value, which may be expressed in millimass units (mmu) or in parts per million (ppm). Mass accuracy is largely linked to the stability and resolving power of the used mass analyzer. In the case of very high mass accuracy, mass differences may be below 1 ppm (sub-ppm errors) (203).

*MASS CALIBRATION* is a crucial aspect within high-resolution mass spectrometry since the established high mass accuracy is the main driver towards adequate selectivity and reliable data analysis. Within this context, both internal and external mass calibration strategies have been suggested. External calibration of the mass analyzer is generally based on the infusion of calibration mixtures, which allow to verify and correct for potential mass measurement shifts. In the context of internal mass calibration, the use of a lock mass may improve mass accuracy measurements. This approach utilizes some continuously measured mass signals, related to the mobile phase, column bleeding or ubiquitous plasticizers such as phthalate (204).

*THE DYNAMIC RANGE* over which accurate measurements of mass can be made is a key analytical parameter for any type of accurate mass analyzer. Mass accuracy is indeed limited by too few detected ions or by mass spectral peak position shifts due to too many ions. In the first case, the relative high presence of noise may cause imprecision of peak centroiding and thus insufficient mass accuracy. In the second case, repulsion between the charged ions at high intensities may produce mass shifts. The dynamic range is as such defined as the intensity range within the generated ion population for which accurate mass measurements can be achieved (205).

The inherent ability of high-resolution mass spectrometers to accurately measure mass with sub-ppm errors contributes to an improved detection of metabolites and a facilitated identification or structural characterization (206). Currently, the best results in terms of accuracy (< 1.5 ppm) and mass resolving power (up to 1,000,000) are still achieved with FT-ICR instruments. However, this type of mass spectrometer is less popular because of the time-consuming signal optimization, rather long acquisition times, and the expensive price. For these reasons, Orbitrap and ToF based instruments are more widely used in metabolomics (206). With modern ToF instruments, mass resolving powers of up to 60,000 have been reported and mass accuracies of less than 2 ppm are normally achieved. The main difficulties, associated with these instruments, include the limited dynamic range and dependence of lock mass or internal calibration to maintain high mass accuracy. In contrast, external calibration can be performed with Orbitrap mass analyzers to obtain excellent mass accuracy (< 3 ppm) and resolving power (up to 240,000). In addition, Orbitrap instruments offer better dynamic range and sensitivity (206,207). The operating principle of the Orbitrap system is mainly founded on its ability to trap ions and allow them to start spinning around a central electrode (Figure 1.17). Trapping of ions is established in a radio frequency (RF)-only quadrupole (C-trap) and effectuates the momentary injection of a compact package of ions into the orbitrap mass analyzer. Inside the orbitrap, ions are electrostatically trapped while rotating around the central electrode and performing axial oscillation. These oscillating ions induce an image current, which is detected by a differential amplifier and converted into an *m/z*-spectrum by a Fast Fourier Transformation. This system is also capable of generating structural information in a non-selective manner (all ions fragmentation) by using a High Energy Collisional Dissociation (HCD) cell without precursor ion selection (208).

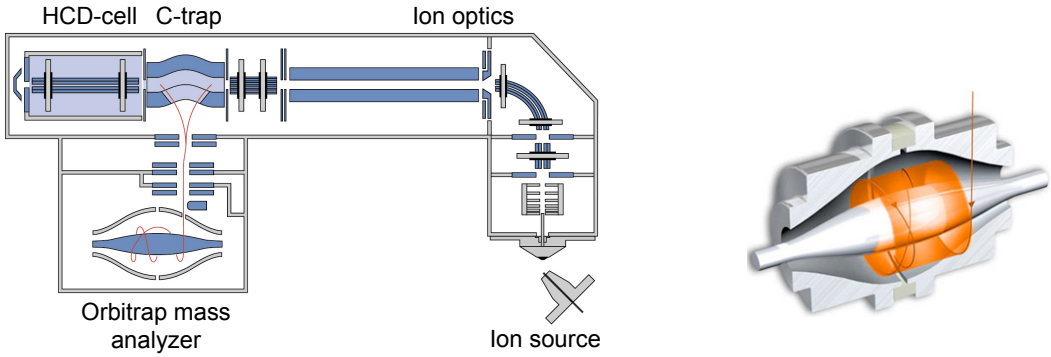



**FIGURE 1.17.** Schematic representation of a bench-top Exactive™ Orbitrap mass spectrometer and the orbitrap mass analyzing element.

In recent years, enormous progress has been made with respect to high-resolution mass spectrometers. Herewith, the combination of an orbitrap mass analyzer with a linear ion trap (LTQ-Orbitrap Velos) or a quadrupole mass filter (Q-Exactive) have become valuable hybrid instrument configurations to acquire structural information in a selective manner (Figure 1.18). With this, the tandem mass spectrometry capabilities and the strengths of high-resolution mass accuracy are combined within a single instrument (209,210).

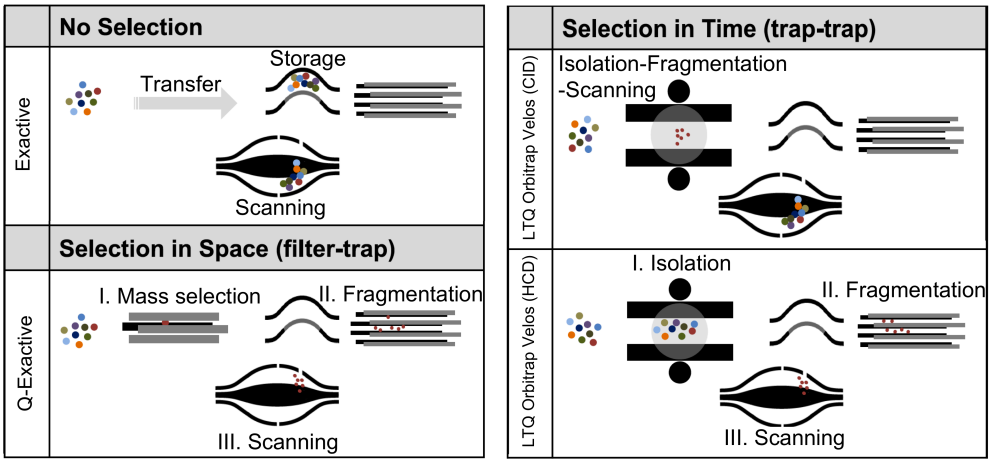



**FIGURE 1.18.** Various mass spectrometer configurations possible with orbitrap mass analyzers (211). Hereby, the colored dots represent the ion population.

The LTQ-Orbitrap Velos instrument represents a multistage trap combination by which both MS and MS/MS can be performed. In the MS mode, the ion population is collected by the linear trap and passed to an intermediate C-trap for injection and analysis in the orbitrap mass analyzer at high resolution. Alternatively, in the MS/MS mode, the linear ion trap retains ions within a specified mass window, which are subsequently activated by a supplemental RF-field, leading to fragmentation of the trapped precursor ions. The signals associated with this mass dependent scan are recorded at low resolution (Figure 1.18, LTQ Orbitrap Velos (CID)). In addition, there is the possibility of isolating precursor ions in the linear trap and performing fragmentation in the HCD-cell, which is interfaced directly to the C-trap. The generated fragments are subsequently analyzed with high mass accuracy in the orbitrap mass analyzer (211) (Figure 1.18, LTQ Orbitrap Velos (HCD)). The mode of data acquisition with the Q-Exactive (Figure 1.18, Q-Exactive) is quite similar to that of the LTQ-Orbitrap Velos using HCD. However, its overall effectiveness (selectivity) is far more advanced by its ability to isolate a more narrow population of precursor ions (212).

# 6. THE BASIC PRINCIPLES OF PLANT METABOLOMICS

## 6.1 GENERAL STRATEGIES FOR METABOLOMIC ANALYSIS

In plants, the metabolome is a compilation of all primary and secondary metabolites, which are regarded as the final recipients of genetic information (213). The plant metabolome is quite complex with current estimates of about 15,000 metabolites within a given species and over 200,000 different metabolites within the plant kingdom (214). As such, the metabolome embraces a wide variety of metabolites, prevailing in plants at very different concentration levels and with huge physicochemical diversity (215). In addition, the metabolome constituents have been assigned a significant role in a plethora of biological plant processes (213). Metabolic changes are therefore seen as a potent answer of an organism towards genetic alterations, diseases or environmental influences (216). In this regard, the presence and relative abundance of metabolites in plants are the best indicators of an organism's phenotype (217). Hence, the qualitative and quantitative measurement of intracellular metabolites by a metabolomics approach has become a valuable tool for advancing our understanding of primary and secondary metabolism in plants (213).

Metabolomics is generally defined as a holistic qualitative and quantitative analysis of all metabolites that are present within a biological system, surveyed at a given time point and under specific physiological conditions (213). As such, the generated data enclose the potential to identify patterns or metabolite markers that are characteristic for a species, a cultivar, a certain stage of development, a disease state, a stress condition, or daily and seasonal environmental changes (215).

However, because of the diverse population of plant metabolites and their dynamic nature, the detection of the complete metabolome by means of a single and universal method is impossible. Covering the full spectrum of present metabolites indeed requires a platform of complementary analytical technologies (218,219). This implies that current metabolomics studies and strategies are elaborated according to specific research questions and objectives. In this regard, the applied experimental strategies are typically categorized as targeted analysis, metabolic profiling, or metabolic fingerprinting (220).

*TARGETED ANALYSIS* is regarded as a truly quantitative strategy in which the concentration levels are determined for one or a few known metabolites. This approach is associated with careful optimization of metabolite extraction, separation, and detection in order to avoid any interference from the plant matrix. As such, low limits of detection and high throughput are achieved for the target metabolites (221). The extensive optimization, however, narrows the applicability of the analytical method for assessment of global metabolic changes and identification of novel metabolic markers. In addition, targeted analysis requires prior knowledge about the selected metabolites and highly purified standards for accurate quantitation (222).

*METABOLIC PROFILING* aims at the measurement of a large set of both known and unknown metabolites in a sample. This strategy primarily focuses on the analysis of a group of metabolites, which are either related through their metabolic pathway or a specific class of compounds (223). This particular strategy offers medium throughput, is semi-quantitative, and provides no identification for the majority of signals (222).

*METABOLIC FINGERPRINTING* is largely implemented to define metabolite signatures or patterns, which are characteristic for the metabolic state of the plant. Herewith, identification or precise quantitation of the different metabolites in the plant samples is not addressed. As such, this approach mainly focuses on collecting and analyzing data from crude metabolite mixtures to rapidly classify samples. Subsequently, pattern recognition analysis may be performed to identify features that are specific for a fingerprint (224).

## 6.2 GENERAL WORKFLOW FOR METABOLOMIC ANALYSIS

A metabolomic (untargeted) analysis typically comprises four experimental stages; i.e. preparation of the sample, data acquisition by analytical methods, data mining using chemometric methods, and compound identification (Figure 1.19) (213,225). The obtained results may constitute the basis for an indispensable and meaningful biochemical interpretation (213). Description of the various steps primarily applies to the fingerprinting based metabolomics approach.

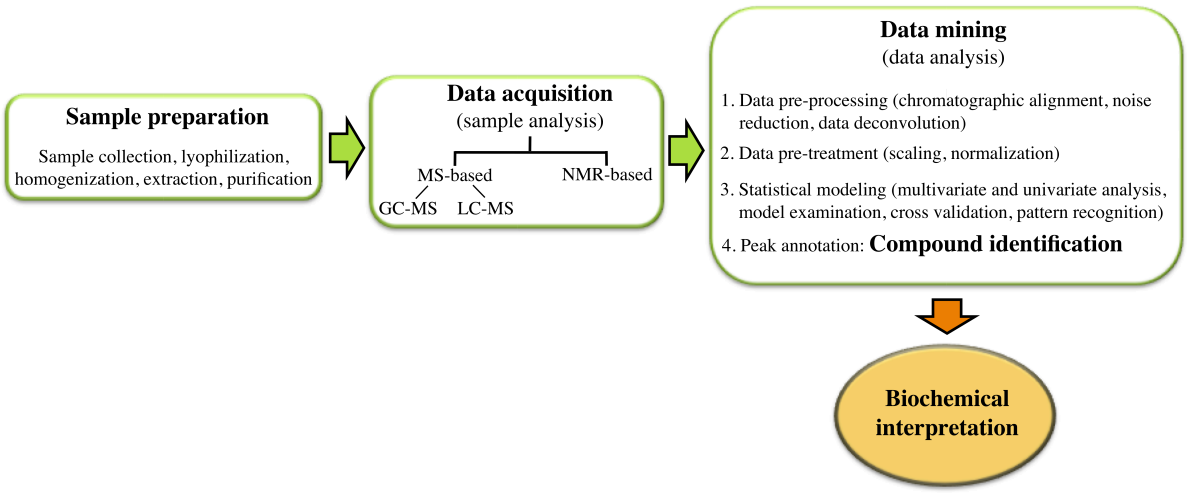



**FIGURE 1.19.** Typical workflow for metabolomic studies, including sample preparation, data acquisition, data mining, and identification. These steps may lead to biochemical interpretations (213).

*SAMPLE PREPARATION* should be as non-selective and comprehensive as possible. Herewith, care must be taken to avoid the introduction of any unwanted variability. In this regard, metabolite degradation (thermal, oxidative or enzymatically) and sample contamination represent the major causes of these variations. Some of the common extraction strategies include solvent extraction, supercritical fluid extraction, sonication and solid-phase extraction. However, none of these techniques has the ability of recovering all classes of compounds with high reproducibility and robustness (213).

*DATA ACQUISITION* requires advanced analytical techniques of which $^{1}$H nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry are the most intensively used for metabolomics. NMR spectroscopy provides direct identification and quantification of a range of abundant metabolites and is typically more robust and reproducible than hyphenated LC-MS based techniques. However, because of the superior sensitivity and widespread availability, mass spectrometry has emerged as the key technology for holistic metabolic experiments (226).

*DATA MINING* is regarded as the process during which the acquired metabolomics data are converted into valuable and meaningful information. Manual data handling is, however, no longer feasible with these highly complicated and multidimensional datasets. Therefore, specialized bioinformatics and data mining tools have been introduced for automated processing of the raw data. These tools comprise algorithms for data pre-processing (chromatographic alignment, noise

reduction, data deconvolution), pre-treatment (scaling, normalization), and statistical interpretation (pattern recognition, dimensional reduction, modeling) (224,227,228). Extensive data analysis may elucidate metabolites or metabolite patterns that are relevant for specific biological phenomena such as, for example, crop quality (227).

*IDENTIFICATION* and structural identification of biologically interesting metabolites is one of the key challenges in metabolomic studies. Nevertheless, mass spectrometry may encompass the potential for metabolite identification by providing information about *m*/*z*-ratios, relative isotope abundances, and fragmentation patterns. In addition, compound databases and mass spectral libraries containing experimentally acquired chromatographic and mass spectrometric data may be used as well for metabolite structural elucidation and identification (229).

## 6.3 APPLICATIONS OF METABOLOMICS IN PLANT RESEARCH

Plant metabolomics is a valuable tool for fundamental science, but has a potentially broad field of applications as well. Research areas where metabolomics are applied include amongst others the interpretation of metabolic pathways and networks, biomarker discovery that can assist in the identification of novel molecular targets, genotyping, gene function elucidation, plant breeding and crop quality assessment, discovery of metabolites that are involved in environmental adaptations, abiotic and abiotic stress responses, and host-pathogen interactions (227). Hence, even with its current limitations, plant metabolomics is an informative tool that is revolutionizing plant biology (213,227).

# 7. CONCEPTUAL FRAMEWORK OF THIS STUDY

The regulating role of phytohormones has been recognized to enfold an interesting fundament in studying diverse physiological and developmental plant processes. In this perspective, knowledge on the specific actions and functioning of these secondary metabolites may hold valuable implications for both horticultural and agricultural plant production. Indeed, targeted control of the endogenous phytohormone concentration levels by genetic modification, external applications, or agronomic measures may as such assist in receiving amongst others better plant growth, flowering, product quality, resistance against pathogens, stress tolerance, and plant architecture. Being able to monitor these aspects of plant production may evidently hold major economic benefits. One of these aspects, i.e. nutritional quality, was the subject of our study.

Improvement of tomato fruit quality has indeed become an important issue for growers, as such reflecting the increased interest of consumers in the nutritional aspects of tomato fruit. This finding has prompted research aiming at the identification of means, which enclose the ability to enhance the accumulation of health-promoting substances in tomato fruit. Although diverse agronomic practices have been claimed significant value within this context, less conclusive or even conflicting data have been reported as well. An improved knowledge about the underlying mechanisms of tomato fruit metabolism would be of particular interest for a better understanding of the effects, expressed upon imposed agronomic measures. Therefore, this study aimed at deepening the knowledge about tomato fruit carotenoid metabolism by investigating the possible regulatory role of phytohormones. Within this particular aim, the establishment of a metabolomic framework was considered critically important because of the complex crosstalk along hormone-signaling pathways. Moreover, this metabolomic framework was supposed of having potential to study various other aspects of plant physiology and development, and this with respect to tomato as well as other plant species. Hereby, it should be noted that in this study a rather broad interpretation of the term hormone was used, thereby covering both compounds with recognized physiological activity as well as inactive conjugates and derivatives.

The specific goals of this study were:

- ✓ To develop an analytical method for the accurate and specific determination of the major carotenoids in tomato fruit (**Chapter II**);
- ✓ To develop an analytical method for the metabolic survey of tomato fruit phytohormones and related metabolites (**Chapter III**);
- ✓ To define the phytohormonal profiles of developing tomato fruits (**Chapter IV**);
- ✓ To elucidate the regulating role of phytohormones towards carotenoid metabolism in tomato fruit, thereby using the established metabolomic framework (**Chapter V**);
- ✓ To evaluate the potential of altered nutrient solution salinity to improve tomato fruit carotenoid content, thereby using the established metabolomic framework (**Chapter VI**).

Finally, **Chapter VII** summarizes and discusses the eliciting findings of the different research chapters. Furthermore, some conclusions are formulated, which provoke various challenging future research topics.

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# CHAPTER II

## HIGH-RESOLUTION ORBITRAP MASS SPECTROMETRY FOR THE ANALYSIS OF CAROTENOIDS IN TOMATO FRUIT: VALIDATION AND COMPARATIVE EVALUATION TOWARDS UV-VIS AND TANDEM MASS SPECTROMETRY

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## ABSTRACT

In this study, a generic extraction protocol and full-scan high-resolution Orbitrap-MS detection method were developed, enabling the metabolomic screening for carotenoids in tomato fruit tissue. To this end, the carotenoids lutein, zeaxanthin, $\alpha$-carotene, $\beta$-carotene and lycopene (representing both xanthofylls and carotenes) were considered. The extraction procedure was optimized by means of a D-optimal design and consisted of a liquid-liquid extraction with methanol/methyl-tert-butylether (1:1, v/v). The considered compounds were detected by a single-stage Exactive$^{TM}$ Orbitrap mass spectrometer, operating at a mass resolution of 100,000 full width at half maximum. The validation study demonstrated excellent performance in terms of linearity ($R^2$ > 0.99), repeatability (CV ≤ 10.6%), within-laboratory reproducibility (CV ≤ 12.2%), and mean corrected recovery (ranging from 85 to 106%). Additionally, a comparative evaluation towards well-established detection techniques, i.e. MS/MS and UV-VIS PDA, indicated superior performance of high-resolution Orbitrap-MS with regard to specificity/selectivity and sensitivity (with LODs ranging from 8.0 to 304.0 ng $g^{-1}$ dry weight). As a result, it may be concluded that high-resolution Orbitrap-MS is a suited alternative for UV-VIS or MS/MS in analyzing carotenoids and may offer significant value in carotenoid research because of the metabolomic screening possibilities.

# 1. INTRODUCTION

Carotenoids are important secondary metabolites, which are mainly synthesized by plants, algae and certain types of bacteria and fungi (1). Up to now, more than 700 different carotenoid structures have been isolated and identified from various natural sources. As a consequence, this group of natural pigments is characterized by a wide distribution, structural diversity and numerous biological and physiological functions (2).

In epidemiological and clinical studies, associations were found between the intake of the concerned dietary compounds and the prevalence of chronic-degenerative diseases, including cancers, cardiovascular disorders and age-related macular degeneration (3). These findings were mainly attributed to a number of biological functions that have been ascribed to carotenoids, i.e. provitamin A activity, immune-response modulation, antioxidant effects and induction of gap-junction communication (4). Because of the outlined biological functions and associated beneficial health effects, carotenoids are intensively studied to further clarify and better understand their specific role and actions. Additionally, a further deepening of the knowledge about the metabolism and partitioning of carotenoids in plants is required, in particular when trying to increase carotenoid levels in fruits and vegetables by modifying environmental factors, cultivation techniques, or genetic manipulation (5).

Within this context, metabolomic profiling techniques are highly relevant since they can reveal a comprehensive view on the relative levels of hundreds to thousand metabolites, present in the plant material of interest (6). With regard to the metabolomic profiling aims, full-scan mass spectrometry appears to be most designated for such kind of approaches because of the associated specific properties (7-10). Full-scan mass analysis offers indeed the possibility to simultaneously analyze a virtually unlimited number of compounds. Furthermore, the retrospective post-acquisition evaluation of data allows screening for analytes that are not a priori selected (11). In this context, Fourier Transform Orbitrap mass spectrometric is extremely suited since the applied technology provides precise mass accuracy (mass deviations < 2 ppm, mass resolving power up to 100,000 FWHM), resulting in high selectivity and sensitivity for analysis of samples with complex matrix co-extracts (12).

Although the majority of these detected metabolites are in general not yet structurally characterized or even completely unknown, valuable information about the overall metabolome differences and similarities between samples under study (i.e. between well-defined treatments) is provided (13,14). As such, metabolomic profiling techniques have the potential to reveal the significance of a certain metabolite with regard to a specific process, i.e. the importance of a certain carotenoid towards observed beneficial health effects or specifically applied growing conditions. As a consequence, a metabolomic approach of carotenoids is believed to be significantly valuable in various studies and practical applications, and is considered to be strongly complimentary with genetic and functional genomic approaches (6).

Therefore, during this study, a full-scan high-resolution Fourier Transform Orbitrap-MS detection method was developed for the metabolomic profiling of carotenoids in tomato fruit. It is indeed stated that tomato (*Solanum lycopersicum* L.) is an excellent model for fleshy fruit research and, moreover, of great importance because of the high nutritional value (15). For example, tomatoes and tomato-based foods account for more than 85% of all the dietary sources of lycopene (16). The development and validation of the analytical method was based on five carotenoids, which represented both carotenes (lycopene, $\alpha$-carotene and $\beta$-carotene) and xanthofylls (lutein and zeaxanthin). In addition, the analytical performance of the developed method was comprehensively evaluated by means of a comparative study with consideration of well-established carotenoid detection techniques (15-23). Firstly, a PDA detection method was considered, whereby identification of the carotenoids was based on the UV-VIS absorption spectrum and the previously determined retention times of the authentic markers. Secondly, an MS/MS analytical method was considered, whereby information about the retention time, molecular mass and functional groups was used for carotenoid identification. By comparative evaluation of the proposed analytical methods, information could be provided about the suitability of a certain technique within a defined experimental framework and about its limitations and major considerations.

# 2. MATERIALS AND METHODS

## 2.1 CHEMICALS AND REAGENTS

The carotenoid standard *all-trans*-lycopene was purchased from PhytoLab GmbH & Co. KG (Vestenbergsgreuth, Germany), *all-trans*-β-carotene was from Sigma-Aldrich Co. (St. Louis, MO, USA), *all-trans*-α-carotene was from Wako Chemicals GmbH (Neuss, Germany), *trans*-lutein was from Extrasynthese (Genay, France), and *all-trans*-zeaxanthin was from TRC Inc. (Eching, Germany). The internal standard β-apo-8'-carotenal was obtained from Sigma Aldrich (St. Louis, MO, USA). Reagents were of analytical grade when used for extraction purposes and of LC-MS grade for LC-MS or LC-UV-VIS applications. They were respectively purchased from VWR International (Merck, Darmstadt, Germany) and Fisher Scientific (Loughborough, UK). Magnesium carbonate ($MgCO_3$) and butylated hydroxytoluene (BHT) were from Sigma Aldrich (St. Louis, MO, USA) and SAFC Supply Solutions (St. Louis, MO, USA), respectively, whereas ammonium acetate, formic acid and acetic acid were from VWR International (Merck, Darmstadt, Germany). Polyamide filter units (0.45 μm pore size), tested for clean-up of the extracts, were obtained from Chromafil® (Macherey-Nagel, Düren, Germany).

## 2.2 PREPARATION OF STOCK SOLUTIONS

Preparation of stock solutions was adapted from the method of Karppi *et al*. (2008) (24). In brief, stock solutions of lycopene (1 mg $mL^{-1}$), α-carotene (1 mg $mL^{-1}$), β-carotene (1 mg $mL^{-1}$) and β-apo-8'-carotenal (1 mg $mL^{-1}$) were prepared in a solution containing acetonitrile/methanol/chloroform (18:7.5:74.5, v/v/v) and 0.01% (w/v) BHT, whereas stock solutions of zeaxanthin (1 mg $mL^{-1}$) and lutein (1 mg $mL^{-1}$) were prepared in ethanol/acetonitrile/methanol/chloroform (70:18:7.5:4.5, v/v/v/v) and 0.01% (w/v) BHT. To prevent degradation, solutions were stored in amber glass bottles at -20 °C.

## 2.3 PLANT MATERIAL

Tomato plants (*Solanum lycopersicum* L. cv Moneymaker) were grown in a 60 $m^2$ greenhouse compartment of the Institute for Agricultural and Fisheries Research (ILVO, Melle, Belgium) and were subjected to normal cultural practices (17). Depending on the intended use – method

optimization or validation - tomato fruits were harvested when in their red-ripe or green-colored developmental stage, respectively. For each set of fruits, the consecutive cutting, lyophilization, grinding and sieving of the pooled fruits resulted in a homogenous powder, which allowed representative sampling. In order to avoid carotenoid degradation during sample homogenization, tomato fruit material was shielded from light as much as possible.

## 2.4 EXTRACTION OF CAROTENOIDS FROM TOMATO FRUIT TISSUE

For optimization of the extraction procedure, a design of experiments (i.e. a D-optimal design) was used. This technique represents a fractional factorial design and allows to efficiently identify variables, which significantly affect the extraction efficiency (25,26). During construction of the design, the optimality criterion (G-efficiency) and the exchange algorithm are used (27). By application of experimental designs, the number of experimental runs is strongly reduced compared to e.g. full factorial designs, which is reflected by both practical feasibility and economic benefits. Furthermore, a D-optimal design was found particularly suitable during the optimization of the extraction procedure because with this kind of design non-linear models can be applied. In addition, other optimal design strategies can be used to optimize individual parameters or the ability to distinguish between models, but these were not the objective of the current work (28).

With regard to the optimization of the extraction procedure, eight variables (reported in Table 2.1) were examined for their relevance towards the extraction of carotenoids from tomato fruit. These variables were selected based on literature (29,30) and included both qualitative and quantitative factor types. For development of the design, i.e. the scenario that describes the experiments to conduct, the software program Modde 5.0 (Umetrics, Cheshire, UK) was used. Based on the specifications (i.e. number and type of variables), the application of Modde 5.0 resulted in a number of experimental design proposals. Selection of the most suited scenario of experiments was selected based on the G-efficiency. The selected design included 28 experiments (25 design runs and 3 center points), with each of the experiments consisting of a specific combination of settings for the eight variables.

**TABLE 2.1** Selected variables and specified value ranges, which were included in the D-optimal design and investigated with regard to their effect on extraction efficiency.

| Quantitative variables | Lower value | Upper value |
|---|---|---|
| Mass tomato fruit tissue (mg dry weight) | 25 | 250 |
| Addition of $MgCO_3$ to tomato tissue (%, w/w[a]) | 0 | 2 |
| Volume of extraction solvent (%, w[a]/v) | 5 | 10 |
| Addition of BHT to extraction solvent (%, w/v) | 0 | 0.1 |
| Number of extraction phases | 1 | 2 |
| Enrichment by evaporation | no | yes |
| **Qualitative variables** | **Specifications** | |
| Way of purification | Centrifugation<br>Centrifugation and filtration (0.45 μm filter) | |
| Type of extraction solvent | Ethanol/hexane (4:3, v/v)<br>Methanol/methyl-tert-butylether (1:1, v/v)<br>Hexane/acetone/ethanol (1:1:2, v/v) | |

[a] weight of tomato fruit tissue (on a dry weight basis)

After implementation of the prescribed scenario, the main effect of each variable was determined and statistically evaluated (P-value < 0.05). Significant variables were built into the extraction protocol whereby the value resulting in the highest response was considered. For non-critical variables the least time consuming or least expensive options were selected. The described work methodology resulted in a relatively simple extraction protocol. First, 25 mg of homogenized tomato fruit material (dry weight) was weighed and 0.5 mg $MgCO_3$ was added in order to neutralize trace levels of organic acids (31). Subsequently, 500 μL extraction solvent consisting of methanol/tert-butyl methyl ether (1:1, v/v) was added to the plant material. The extraction solvent was supplemented with 0.4 ng $\mu L^{-1}$ β-apo-8'-carotenal internal standard to compensate for possible fluctuations during implementation of the extraction protocol. The sample was vigorously shaken until all plant material was homogenized into the extraction solvent and then rotated (1,200 x g) for 15 min. Subsequently, solid tomato fruit particles were separated by centrifugation (5 min at 11,250 x g) after which about 400 μL of extract was collected. The remaining plant residue was re-extracted with a second volume of 500 μL extraction solvent, shaken and again rotated for 15 min. Next, the sample was centrifuged and about 500 μL extract was collected. Then, extracts were pooled and 500 μL ultra-pure water was added to separate the polar and non-polar phase. Finally, the upper and colored layer was collected and transferred to an HPLC-vial.

## 2.5 ANALYSIS OF CAROTENOIDS BY HPLC-ORBITRAP-MS

Chromatographic separation of the carotenoids was achieved on an Accela U-HPLC system (Thermo Fisher Scientific, San José, USA), equipped with an Acclaim C30 column (3.0 μm, 150 mm x 4.6 mm internal diameter) (Thermo Fisher Scientific, Breda, The Netherlands). Since the selected column belongs to the HPLC-type, the low-pressure pumping mode was applied. The elution gradient was carried out with a quaternary solvent system of ultra-pure water (solvent A), methanol containing 0.04 g $L^{-1}$ ammonium acetate (solvent B), acetonitrile (solvent C) and methanol/ethyl acetate (1:1, v/v) (solvent D). The applied linear gradient profile started with 0.5% A, 84% B, 13.5% C and 2% D for 4 min. Then, in 3.50 min, the proportion of solvent B was decreased to 68.5% and solvent D was increased to 17.5%. Subsequently, in 7.50 min, the proportion of solvent B was further decreased to 51% whereas solvent D was further increased to 35%. This mobile phase condition was kept for 9 min and was then changed to 34.5% B, 5% C and 60% D in 8 min. Next, in 7.90 min the proportions of solvent B, C and D were changed to 9.5%, 0% and 90%, respectively. Subsequently, in 0.10 min, the composition of the mobile phase was changed to 100% D and was kept there for 9.90 min. Finally, the mobile phase was returned to initial conditions in 0.10 min and the column was re-equilibrated for 5 min. The flow rate was kept constant at 1 mL $min^{-1}$ and the column oven temperature was set at 22 °C. A 10 μL aliquot of each sample was injected for analysis.

High-resolution mass spectrometric analysis was performed on an Exactive™ single-stage Orbitrap mass spectrometer (Thermo Fisher Scientific, San José, USA), equipped with an atmospheric pressure chemical ionization probe (APCI), operating in positive ionization mode. Ionization source working parameters were optimized and involved sheath gas pressure (35 au, arbitrary units), auxiliary gas pressure (5 au), sweep gas pressure (1 au), vaporizer temperature (275 °C), capillary temperature (175 °C), discharge current (5 μA), capillary voltage (57.50 V), tube lens voltage (90 V), and skimmer voltage (18 V). An *m/z* scan range of 100 - 650 was selected and the resolution was set at 100,000 full width at half maximum (FWHM) at 1 Hz (1 cycle per second). The automatic gain control (AGC) target was set at the high dynamic range (3 x $e^6$ ions) and the maximum injection time was 50 ms. The option of "all ion fragmentation" using the High Energy Collisional Dissociation (HCD) cell was only used to investigate the confirmation potential of generated fragments, and was turned off during actual analyses.

Initial instrument calibration was achieved by infusing calibration mixtures (Thermo Fisher Scientific, San Jose, USA) for each ion polarity mode. The positive calibration mixture included caffeine, Met-Arg-Phe-Ala acetate salt (MRFA) and Ultramark® 1621, while the negative calibration solution comprised sodium dodecyl sulfate, sodium taurocholate and Ultramark® 1621. These compounds were dissolved in a mixture of acetonitrile, water and methanol, and both mixtures were infused using a Chemyx Fusion 100 syringe pump (Thermo Fisher Scientific, San Jose, USA). Instrument control and data processing were carried out by Xcalibur 2.1 software (Thermo Fisher Scientific, San José, USA).

## 2.6 ANALYSIS OF CAROTENOIDS BY HPLC-PDA-MS/MS

Prior to PDA and MS/MS analysis, chromatographic separation of the targeted carotenoids was carried out by means of an Accela UHPLC-system (Thermo Fisher Scientific, San José, USA), equipped with an Acclaim C30 column (3.0 μm, 150 mm x 4.6 mm internal diameter) (Thermo Fisher Scientific, Breda, The Netherlands). The developed HPLC-method was similar to the abovementioned method (Section 2.5). However, some minor adjustments with respect to the applied gradient profile had to be implemented because of small operating differences between the considered pumping systems.

Detection of the carotenoids by means of UV-VIS detection was carried out using a PDA-detector (Thermo Fisher Scientific, San José, USA). The UV-VIS spectra were obtained between 400 and 500 nm and the chromatograms were processed at the absorption maxima of the various carotenoids, i.e. 445 nm for lutein, 475 nm for zeaxanthin, 446 nm for β-carotene, 473 nm for lycopene, and 458 nm for both β-apo-8'-carotenal and α-carotene. Absorption maxima were defined experimentally and are in accordance with values found in the literature (18,21,23).

MS/MS analysis was performed on a TSQ Vantage triple-quadrupole mass spectrometer (Thermo Finnigan, San José, USA), equipped with an APCI-interface, operating in positive ionization mode. The mass spectrometer was connected in series to the PDA detector. General instrumental parameters are reported in Table 2.2. Remaining parameter values, to be optimized for each analyte separately, and *m*/*z*-values for precursor and product ions are reported in Table 2.3.

**TABLE 2.2** Instrumental parameter values, used for APCI-ionization of carotenoids by MS/MS.

| Instrumental parameter | Values for MS/MS |
|---|---|
| Sheath gas pressure | 40 au |
| Auxiliary gas pressure | 5 au |
| Sweep gas pressure | 2 au |
| Vaporizer temperature | 370 °C |
| Capillary temperature | 250 °C |
| Discharge current | 5 μA |
| Argon collision cell gas pressure | 1.5 mTorr |

au: arbitrary units

**TABLE 2.3** Precursor and product ion *m/z*-values and instrumental parameter values for MS/MS analysis, optimized for the targeted carotenoids and internal standard. All four product ions were considered for quantification and identification.

| Analyte | Precursor ion (*m/z*)[a] | Product ion (*m/z*) | Collision energy (eV) | S-lens voltage (V) |
|---|---|---|---|---|
| β-apo-8'-carotenal | 417 | 91 | 45 | 116 |
| | 417 | 95 | 26 | 116 |
| | 417 | 105 | 36 | 116 |
| | 417 | 119 | 32 | 116 |
| Lutein | 551 | 91 | 60 | 164 |
| | 551 | 105 | 49 | 164 |
| | 551 | 119 | 34 | 164 |
| | 551 | 145 | 31 | 164 |
| Zeaxanthin | 569 | 91 | 60 | 164 |
| | 569 | 105 | 52 | 164 |
| | 569 | 107 | 31 | 164 |
| | 569 | 119 | 42 | 164 |
| Lycopene, α-carotene, | 537 | 119 | 45 | 139 |
| β-carotene | 537 | 123 | 34 | 139 |
| | 537 | 133 | 32 | 139 |
| | 537 | 195 | 44 | 139 |

[a] For the various carotenoids and the internal standard, the protonated molecule (i.e. $[M+H]^+$) was observed as the most abundant precursor ion. Lutein was an exception since the most abundant ion concerned the dehydrated protonated molecule, i.e. $[M-H_2O+H^+]^+$.

## 2.7 QUALITY ASSURANCE

Prior to sample analysis, a standard mixture of the targeted carotenoids was injected to check the operational conditions of the respective analytical devices. To every sample 8 ng $mg^{-1}$ internal standard β-apo-8'-carotenal was added to the tomato plant material, prior to extraction. With regard to the identification of the carotenoids, identification parameters were selected according to the used detection technique. In the case of MS/MS analysis, carotenoids were identified based on the retention time relative to that of the internal standard and the ion ratios of the selected transitions. For UV-VIS PDA detection, both the relative retention time and the UV-VIS spectrum were used for identification of the targeted carotenoids. When Orbitrap-MS was employed, identification of the carotenoids was based on the relative retention time, the accurate mass (*m*/*z*-value) of the generated ion, and the $^{13}C/^{12}C$ relative isotopic ratio according to the criteria described in CD 2002/657/EC (32).

After identification, the concentration of the detected carotenoids was calculated by fitting the area ratio into six-point calibration curves, established by enrichment of green tomato fruit material (dry weight) with the internal standard at 8 ng $mg^{-1}$ and the five carotenoids in the ranges of 8 to 60 ng $mg^{-1}$ for lutein and zeaxanthin, 2 to 40 ng $mg^{-1}$ for lycopene and α-carotene, and 4 to 40 ng $mg^{-1}$ for β-carotene. Area ratios were determined by integration of the area of an analyte under the specific chromatogram in reference to the integrated area of the internal standard. Maximum allowed mass deviations, used during processing of the data, were compound-specific and depended on the detection capability, signal intensity and occurrence of matrix interferences (33). For lutein, zeaxantin and β-apo-8'-carotenal the mass extraction window was set at 3.5 ppm, while for lycopene, α-carotene, and β-carotene 3.0 ppm was found suited.

## 2.8 VALIDATION STUDY

Due to the absence of specific directives for the analysis of carotenoids in food matrices, the CD 2002/657 EC (32) was used as a guideline for validation of the optimized extraction procedure and the developed detection methods. With regard to the required blank reference material, a preliminary study was conducted in which tomato fruits, representing different developmental stages, were analyzed for their carotenoid content.

It was found that no actual blank reference material could be generated since for each of the considered developmental stages several of the targeted carotenoids were present at detectable concentrations (Figure 2.1). However, since for most carotenoids the lowest concentration levels could be associated with the earliest stage of fruit development (i.e. green-colored tomatoes) this kind of plant material was used for validation. For validation of the various performance criteria, samples of plant material were enriched with specific amounts of analytical standards and internal standard. The addition of the standards and internal standard was realized through the extraction solvent, which was supplemented with needed volumes of (internal) standard working solutions. In order to enable an optimal comparison of the instruments' analytical performances, it was opted to analyze all of the obtained extracts by each of the considered analytical instruments.

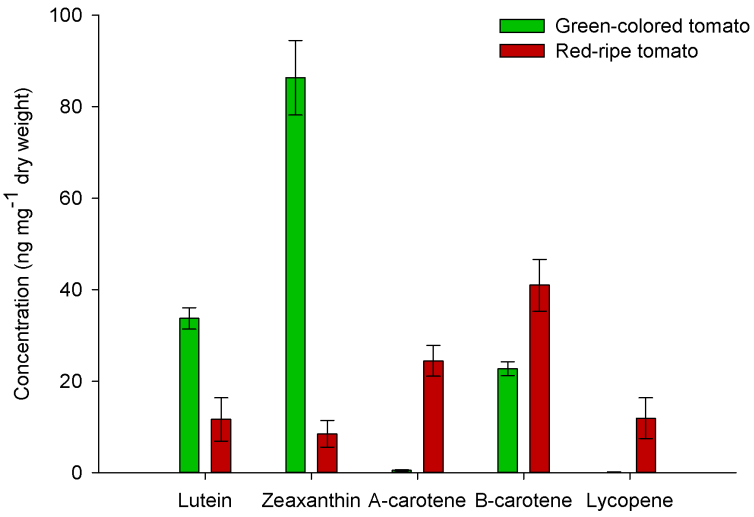


**FIGURE 2.1** Average concentration levels (ng $mg^{-1}$ dry weight) for green-colored and red-ripe tomato fruits. Variation is indicated by means of the standard deviation (n = 3, technical repetitions of pooled tomato fruit material).

# 3. RESULTS AND DISCUSSION

## 3.1. OPTIMIZATION OF THE EXTRACTION PROCEDURE FOR CAROTENOIDS

A D-optimal design was employed to statistically evaluate the effect of eight variables (or factors) (Table 2.1) with regard to the extraction efficiency. The selected design was characterized by a G-efficiency of 96%. This parameter is the default criterion, used by Modde 5.0 when selecting the optimal design and is based on the determinant of the information matrix [X'X] (27). Experiments were implemented in a random order and were executed by extracting red-ripe tomato fruit material. At this stage of fruit development, all of the targeted carotenoids were present at detectable concentration levels (Figure 2.1), which rendered addition of carotenoids to the considered plant material prior to extraction redundant. The effect of each factor was statistically evaluated by means of a coefficient plot, visualizing the by Modde 5.0 applied partial least squares (PLS) regression analysis. For this, the absolute peak areas of the individual carotenoids were taken into account. Significant positive effects were observed for the type of extraction solvent (i.e. methanol/tert-butyl methyl ether, 1:1, v/v), the implementation of a secondary extraction phase, the clean-up without usage of a filter-unit, and the omission of extract evaporation. Furthermore, based on the coefficient plot it was concluded that a limited amount of tomato plant material (25 mg), a large volume of extraction solvent (500 μL) and the addition of $MgCO_3$ tended to positively affect extraction efficiency, however, not significantly. After determining the significance of the considered factors, the application of response surface modelling enables a detailed optimization of the significant factors using quadratic and cubic models. However, since the factors, categorized as significantly affecting the extraction efficiency, were all of the qualitative type, such a profound approach was not performed.

The extraction method, as described in detail in section 2.4, has a number of significant advantages in comparison with well-established liquid-liquid extraction methodologies (29,34-38) that are applied for the extraction of carotenoids from fruits and vegetables. The in our study optimized low amount of starting plant material has interesting consequences towards the sampling process, the number of samples, and the sample material (e.g. early-stage tomato fruits). Furthermore, the low volumes of extraction solvent allow for a quick and efficient

completion of the various extraction steps, i.e. mixture homogenization, filtration, re-extraction, etc. As a consequence, samples are for a shorter period of time exposed to light, high temperatures, and oxygen, which are causes of carotenoid degradation (39,40). In addition, no evaporation of the extract was found to be necessary, which is again advantageous for reduced carotenoid degradation and increased recovery (29,34).

## 3.2. HPLC OPTIMIZATION

During optimization of the HPLC-method, the chromatographic resolution of the xanthophyll isomers and peak shape of all targeted carotenoids were considered as the main criteria of evaluation. It should hereby be noted that the chromatographic resolution was evaluated based on the chromatograms of the protonated xanthophyll peaks ($[M+H^+]^+$). Indeed, in the chromatograms in which the dehydrated and protonated structures ($[M+H^+-H_2O]^+$) were displayed, no zeaxanthin could be detected and no resolution problem was at issue. Chromatographic resolution ($R_s$) was determined using the half-height method, i.e. $R_s = (2\ (t_2 - t_1))/\ (1.7\ (w0.5_1 + w0.5_2))$ with $t_1$ and $t_2$ the retention times of the successive peaks, and $w0.5_1$ and $w0.5_2$ the width of the peaks at their half maximum. Peak shape ($A_s$) was calculated as $A_s = b/a$ with b being the width of the back half of the peak and a being the width of the front half of the peak (41). The initial composition of the mobile phase was based on Epler *et al.* (1993) (42) and consisted of ultra-pure water, acetonitrile and ethyl acetate. Use of methanol as an additional solvent significantly improved peak shape and chromatographic resolution of especially lutein and zeaxanthin ($A_s$ increased with 16.3 and 17.4%, respectively; $R_s$ increased from 0.67 to 1.04), which is supported by the study of Craft *et al.* (1992) (43). Furthermore, a number of organic modifiers, including ammonium acetate (0.4 g $L^{-1}$ and 0.04 g $L^{-1}$) (29), formic acid (1 g $L^{-1}$) (44) and acetic acid (0.5 g $L^{-1}$) (45), were tested with regard to their influence on recovery, peak shape and chromatographic resolution. It was observed that addition of 0.04 g $L^{-1}$ ammonium acetate significantly improved these variables for various carotenoids. More specifically, peak shape was improved with 81.8% for β-carotene, 50.0% for lutein, and 30.0% for α-carotene. For the other carotenoids, only small effects (< 5%) on peak shape were observed. The percentage increase in recovery was calculated for each of the targeted carotenoids by using the integrated peak areas and amounted for lutein 231.5%, for zeaxanthin 355.1%, for α-carotene 2.8%, for β-carotene 28.4%, and for lycopene 35.9%. Moreover, addition of this organic modifier also improved chromatographic resolution between lutein and zeaxanthin,

which was increased from 1.04 to 1.41. Finally, the column oven temperature was optimized by focusing on the chromatographic resolution of lutein and zeaxanthin over a 20 – 30 °C temperature range. It was concluded that a set column oven temperature of 22 °C enables the chromatographic separation of the targeted xanthophylls ($R_s$ = 1.94).

## 3.3. OPTIMIZATION OF HR-ORBITRAP-MS

Prior to determining the optimal MS conditions, each carotenoid standard and internal standard (10 ng $\mu L^{-1}$) was directly infused into the APCI source to assign accurate masses to the generated ions. For zeaxanthin, $\alpha$-carotene, $\beta$-carotene, lycopene and $\beta$-apo-8'-carotenal, the protonated molecule (i.e. $[M+H^+]^+$) was observed as the most abundant ion. For lutein, the protonated and dehydrated molecule (i.e. $[M-H_2O+H^+]^+$) was found most abundant. These findings are in accordance with the study of Hao *et al.* (2005) (31), in which the specific mechanisms of formation are described. For each analyte, the theoretical mass of the associated ion was calculated by Xcalibur 2.1 software. Mass deviations, expressed in parts per million (ppm), were defined as $10^6$ x ((measured mass – theoretical mass) / theoretical mass] and were below 2 ppm (Table 2.4).

**TABLE 2.4** Measured accurate masses of the targeted carotenoids by HR-Orbitrap-MS, with indication of the mass deviation (in ppm) with respect to the calculated theoretical mass.

| Analyte | Measured mass (m/z) | Theoretical mass (m/z) | Mass deviation (ppm) |
|---|---|---|---|
| Lutein | 551.42382 | 551.42474 | -1.67 |
| Zeaxanthin | 569.43456 | 569.43531 | -1.32 |
| $\alpha$-carotene | 537.44488 | 537.44548 | -1.11 |
| $\beta$-carotene | 537.44474 | 537.44548 | -1.38 |
| Lycopene | 537.44486 | 537.44548 | -1.15 |
| $\beta$-apo-8'-carotenal | 417.31492 | 417.31519 | -0.65 |

Optimal APCI working conditions, described in section 2.5, were determined based on the carotenoids' peak areas and signal-to-noise (S/N) ratios by analysis of red-ripe tomato fruit samples. The selection of APCI was justified by the study of Rivera *et al.* (2011) (30) in which APCI was demonstrated to be a more powerful technique for carotenoid ionization than ESI (electrospray ioniziation) or APPI (atmospheric pressure photoionization).

Optimization of the instrument's working parameters (i.e. AGC setting, mass resolution and mass extraction window) was realized by analysis of red-ripe tomato fruit samples. The AGC setting is a significant parameter towards spectral resolution by controlling the number of ions that enters the trap (33). The optimal AGC setting for carotenoid analysis was found to be the high dynamic range ($3 \times 10^6$ ions), which allowed assuming maximal spectral stability from scan to scan. Additionally, based on the improved S/N ratios and the verified low mass deviations (< 1 ppm) significant effects due to space charging were excluded. Optimization of the mass spectrometric resolution is often described as searching for a compromise between specificity/selectivity and sensitivity (33,34). An increased mass resolution is indeed associated with a higher mass accuracy and therefore positively affects specificity/selectivity by excluding matrix interferences. However, high-resolution mass scans result in increased scan time, decreasing the number of chromatographic data points over a peak. Insufficient data points can dramatically affect the repeatability of the measurement method and can increase LOD-values (46). A mass spectrometric resolution of 100,000 FWHM was found optimal since highest S/N ratios and peak areas were obtained with this parameter setting. The associated cycle time, including both the pre-scan (decisive for the AGC) and analytical scan, was 1 Hz. Additionally, since for each of the targeted carotenoids a minimum of 30 data points per peak was observed, the optimized mass resolution permitted precise and reproducible peak area integration and consequential adequate quantification performance. Furthermore, the selected mass extraction window was considered to be a crucial parameter with respect to specificity/selectivity. The optimal value (in ppm) is compound-specific and depends on the detection capability, signal intensity and occurrence of matrix interferences (i.e. S/N ratios) (47). Mass extraction windows were optimized for each carotenoid individually and were all below 5 ppm.

In case of HRMS applications, CD 2002/657/EC (32) states that the mere presence of the generated ions (in particular $[M+H^+]^+$ or $[M-H_2O+H^+]^+$ ion) with their respective *m/z*-values and specified retention times is insufficient for unambiguous identification and confirmation of the selected analytes since at least two diagnostic ions are required. Therefore, the potential of the monoisotopic pattern (i.e. the $^{13}C$ ion) and characteristic fragments with regard to additional confirmation of a compound's identity was investigated. With regard to the use of fragments for additional confirmation, the mass spectrometer offers the possibility of all ion fragmentation (i.e.

without precursor ion selection). Since no precursor ions are selected, a whole range of ion fragments is generated, of which some may be characteristic for the considered compound and selected as diagnostic ions (i.e. product ions). To this end, the HCD-cell collision energy was optimized by analysis of carotenoid standards at different collision energy settings, ranging from 30 to 50 eV. For all carotenoids, it was observed that an applied collision energy of 40 eV was sufficient to ensure about 90 to 95% fragmentation of the considered precursor ion. In Figure 2.2, these additional identification parameters were considered for lutein and β-carotene, representing both xanthofylls and carotenes.

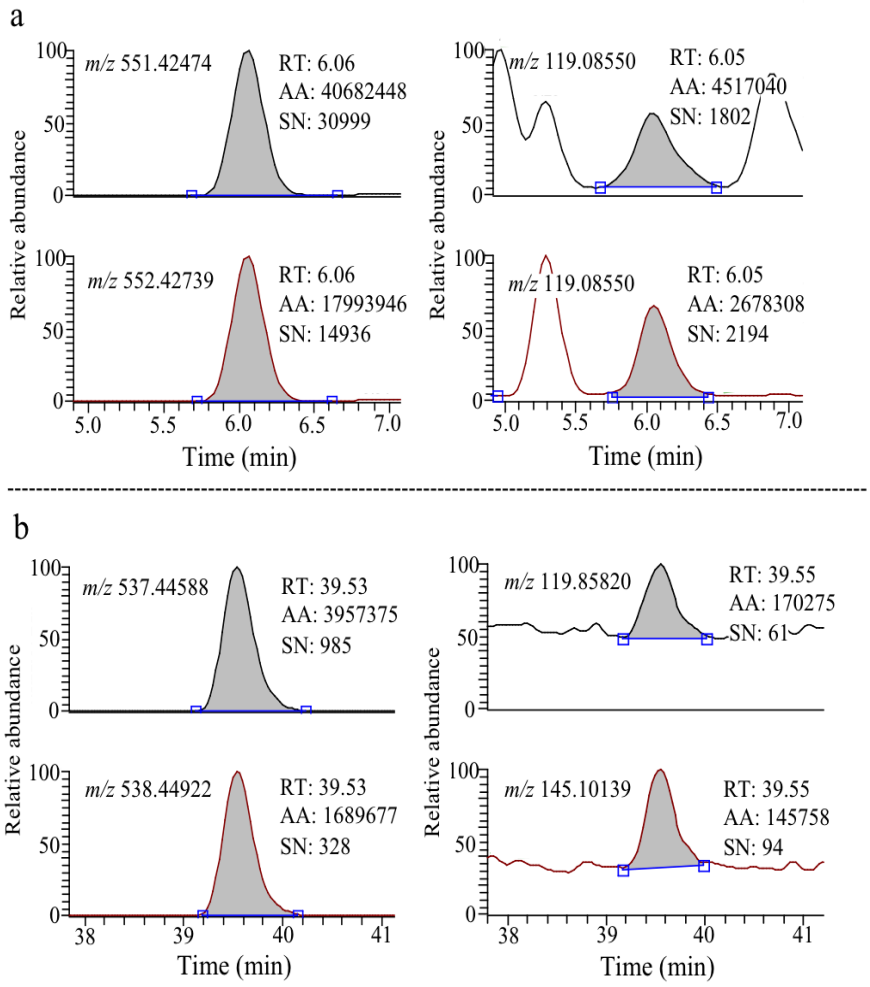


**FIGURE 2.2 a)** Chromatograms for lutein, obtained by full-scan HR-Orbitrap-MS, representing the $[M\text{-}H_2O+H^+]^+$ ion and corresponding $^{13}C$ isotopic ion (left). Chromatograms for two lutein fragments, obtained by HR-Orbitrap-MS with application of HCD fragmentation (right). **b)** Chromatograms for β-carotene, obtained by full-scan HR-Orbitrap-MS, representing the $[M+H^+]^+$ ion and corresponding $^{13}C$ isotopic ion (left). Chromatograms for two β-carotene fragments, obtained by HR-Orbitrap-MS with application of HCD fragmentation (right).

The respective fragments were selected based on both their relative abundance and correspondence with the observed *m/z*-values upon TSQ-MS/MS fragmentation. The usage of the $^{13}C$ isotope as a diagnostic ion was regarded as more appropriate since for the fragments significant lower S/N ratios and peak areas were observed, reflecting worse specificity/selectivity and sensitivity (Figure 2.2). The obtained spectra from this type of generic fragmentation may encounter more potential isobaric interferences among low *m/z* fragments, which can limit the accuracy of the peak area compared to MS/MS spectra, characterized by precursor selected fragmentation (48). Therefore, it was concluded that the $^{13}C$ isotopic ion had the highest associated identification potential, which is in line with expectations since the considered analytes are characterized by a relatively high molecular weight and a vast amount of carbon atoms, leading to a relative abundance of the $^{13}C$ isotopic ion of about 45%. The high identification potential of the monoisotopic pattern was extendable to all of the targeted carotenoids. With regard to the obtained fragmentation profiles, it was observed that the characteristic fragments, determined during MS/MS optimization, were also generated by HCD fragmentation. Furthermore, these profiles demonstrated the presence of several other fragments of which some may have more potential as diagnostic ion because of their higher abundances. For example, in the case of β-carotene, fragments with *m/z*-values of 119.08522, 123.11643, 133.110075 and 195.1151 were observed in the obtained mass spectrum (data not presented), corresponding to the values obtained when fragmenting with TSQ-MS/MS (Table 2.3). In addition, the fragments with *m/z* values of 95.08589 and 105.07023 were noted to display higher abundances and were identified as $C_7H_{11}^+$ (-3.7 ppm) and $C_8H_9^+$ (-3.4 ppm), respectively. Based on the associated chromatograms of these fragments (data not presented) and the corresponding S/N ratios, both product ions had some potential as diagnostic ion. However, the $^{13}C$ isotopic ion was still preferred as diagnostic ion. Although it was thus concluded that all ion fragmentation by HCD was of limited value only in confirming an analyte's identity, it may be of importance in the structural elucidation of "unknowns" (i.e. unidentified peaks) in future metabolomic profiling experiments (48,49).

## 3.4. OPTIMIZATION OF UV-VIS-MS/MS

Optimization of the UV-VIS detection method mainly included the determination of carotenoids' absorption maxima: 445 nm for lutein, 475 nm for zeaxanthin, 446 nm for β-carotene, 473 nm for lycopene, and 458 nm for both β-apo-8'-carotenal and α-carotene.

Acquisition parameters for MS/MS detection were optimized by direct infusion of standard working solution of the individual analytes (10 ng $\mu L^{-1}$) into the APCI-source. The MS/MS fragmentation conditions were investigated and collision energies and S-lens voltages were optimized for each individual compound and/or transition (Table 2.3). APCI working conditions were optimized, as described in Section 3.3. Quantification was based on the summed integrated peak areas of the 4 most abundant product ions.

## 3.5. VALIDATION STUDY

A brief, preliminary study demonstrated that several of the targeted carotenoids were present at detectable levels in both green-colored and red-ripe tomato fruit material (Figure 2.1). Based on these determined concentration levels, it was opted to use green-colored tomato fruits for validation. Average endogenous concentrations of the carotenoids, detected in the green-colored tomato fruit tissue, were calculated on the basis of 6 'blank' samples (i.e. samples with endogenously present carotenoids only) and were taken into account in the determination of the various performance characteristics. To verify whether the suggested work methodology (described in section 2.8) was feasible, a brief study was conducted to determine the stability of the targeted carotenoids in extract for up to seven days at -20 °C. For this purpose, a six-point calibration curve was established in tomato fruit matrix. The study demonstrated satisfying stability (relative standard deviations below 13.3%) of the targeted carotenoids in the tomato fruit extracts for the required period and justified the outlined work methodology.

### 3.5.1. SPECIFICITY/SELECTIVITY

In accordance with CD 2002/657/EC, analytes were identified on the basis of their relative retention time, i.e. the ratio of the chromatographic retention time of the analyte to that of the internal standard (32). Additionally, in the case of mass spectrometric detection, a system of identification points is used, whereby a minimum of 4 identification points (IPs) is required.

The developed HPLC-methods were nearly identical for the various detection techniques and yielded individual relative retention times (n = 6) with standard deviations lower than 0.02 min and coefficients of variation smaller than 0.58%. This falls well within the stated tolerance level of 2.5% for liquid chromatography (32). In the case of high-resolution MS, the 4 IPs were achieved by means of two diagnostic ions (2 IP per ion), i.e. the $[M+H^+]^+$ or $[M-H_2O+H^+]$ ion and the corresponding $^{13}C$ isotopic ion. The latter is, however, only suitable as a diagnostic ion when the calculated relative ion intensity complies with CD 2002/657/EC requirements: for theoretically determined relative intensities of > 20 to 50%, > 10 to 20% and ≤ 10%, the maximum permitted tolerances were, respectively, ±25%, ±30%, and ±50%. It is indeed demonstrated by Lehner *et al.* (2011) (50) and Stoev *et al.* (2012) (51) that isotope abundances provide increased confidence of identification, as employing high resolution enables observation of fine isotopic structures. For MS/MS analysis, the required number of identification points was achieved by means of 1 precursor ion (1 IP per ion) and at least 2 product ions (1.5 IP per ion). As a result, based on the relative retention time and the system of identification points, both mass spectrometric techniques are able to realize an unambiguous identification of the targeted carotenoids. In the case of UV-VIS detection, identification is solely based on relative retention time and absorption spectrum. As a consequence, such an elaborated system of identification points is not addressed. The specificity/selectivity is therefore often inferior compared to mass spectrometric detection techniques, as has been indicated by Fang *et al.* (2003) and Frenich *et al.* (2005) (52,53). Since in the plant material many of the targeted compounds were endogenously present, an actual blank sample was not available. As a consequence, specificity/selectivity could not be evaluated for all targeted carotenoids by comparing the chromatograms from 'blank' and enriched samples. For the carotenoids, which were present in the considered tomato fruit material, specificity/selectivity was exclusively evaluated on the basis of the chromatograms from 6 'blank' samples. For all analytical techniques, the carotenoids lutein, zeaxanthin and β-carotene were present in the blank material (Figure 2.3, Figure 2.4, Figure 2.5).

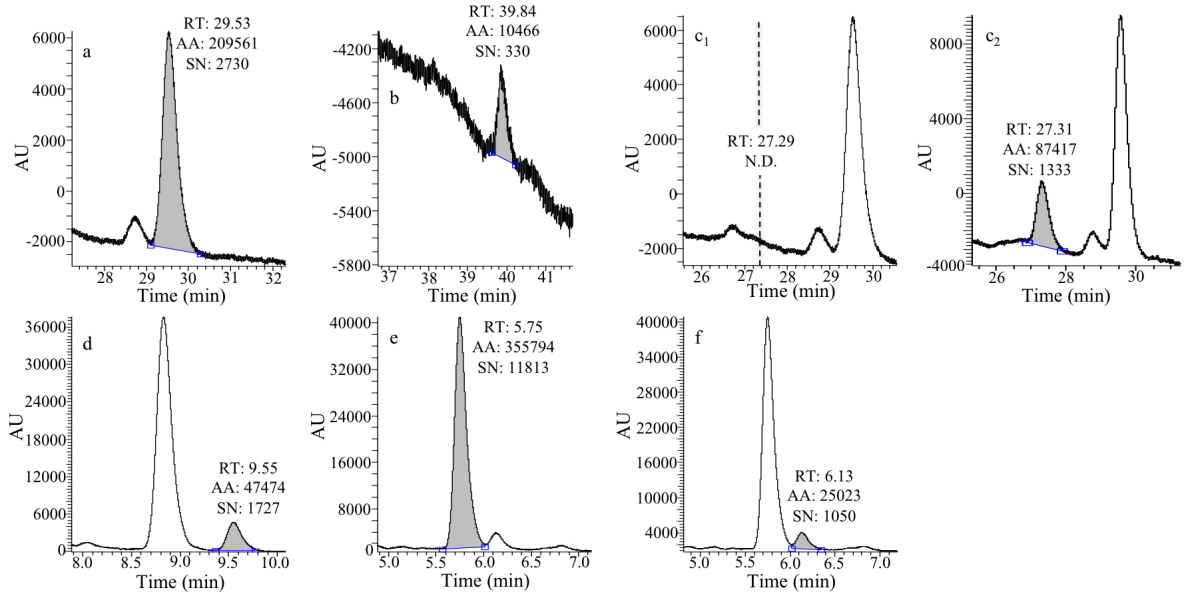


**FIGURE 2.3** Chromatograms of the various carotenoids, obtained by UV-VIS-PDA analysis. The extract under consideration was derived from green-colored tomato fruit material, which was solely enriched with the internal standard β-apo-8'-carotenal (8 ng $mg^{-1}$ dry weight). Only in the case of chromatogram $c_2$, the extracted tomato fruit material was enriched with α-carotene at a concentration level of 8 ng $mg^{-1}$ dry weight. Chromatograms were processed at the respective wavelength absorption maxima of the targeted analytes, i.e. β-carotene (a), lycopene (b), α-carotene ($c_1$ and $c_2$; blank and enriched sample, respectively), β-apo-8'-carotenal (d), lutein (e), and zeaxanthin (f).

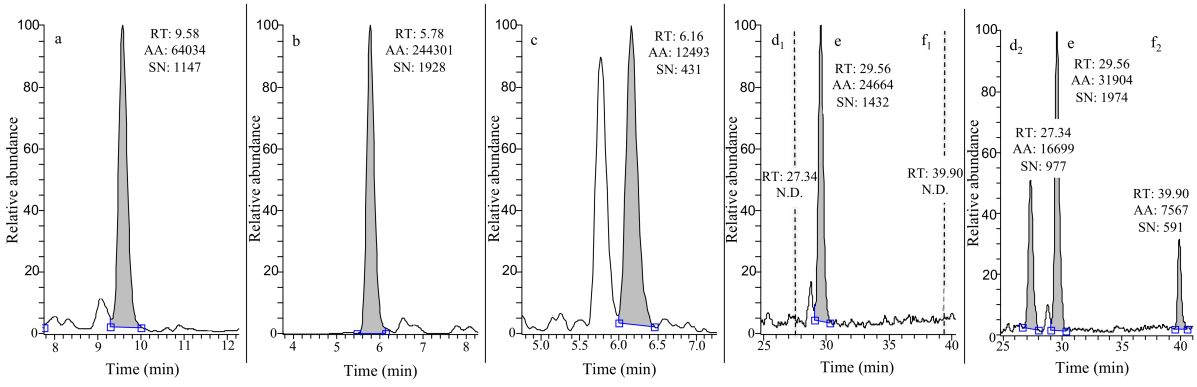


**FIGURE 2.4** Chromatograms of the various carotenoids, obtained by TSQ-MS/MS analysis. The extract under consideration was derived from green-colored tomato fruit material, which was solely enriched with the internal standard β-apo-8'-carotenal (8 ng $mg^{-1}$). Only in the case of chromatograms $d_2$ and $f_2$, the extracted tomato fruit material was enriched with α-carotene and lycopene, both at a concentration level of 8 ng $mg^{-1}$ dry weight. The figure represents the TIC-signal, including the 4 product ions. The targeted analytes concerned β-apo-8'-carotenal (a), lutein (b), zeaxanthin (c), α-carotene ($d_1$ and $d_2$; blank and enriched samples, respectively), β-carotene (e), and lycopene ($f_1$ and $f_2$; blank and enriched samples, respectively).

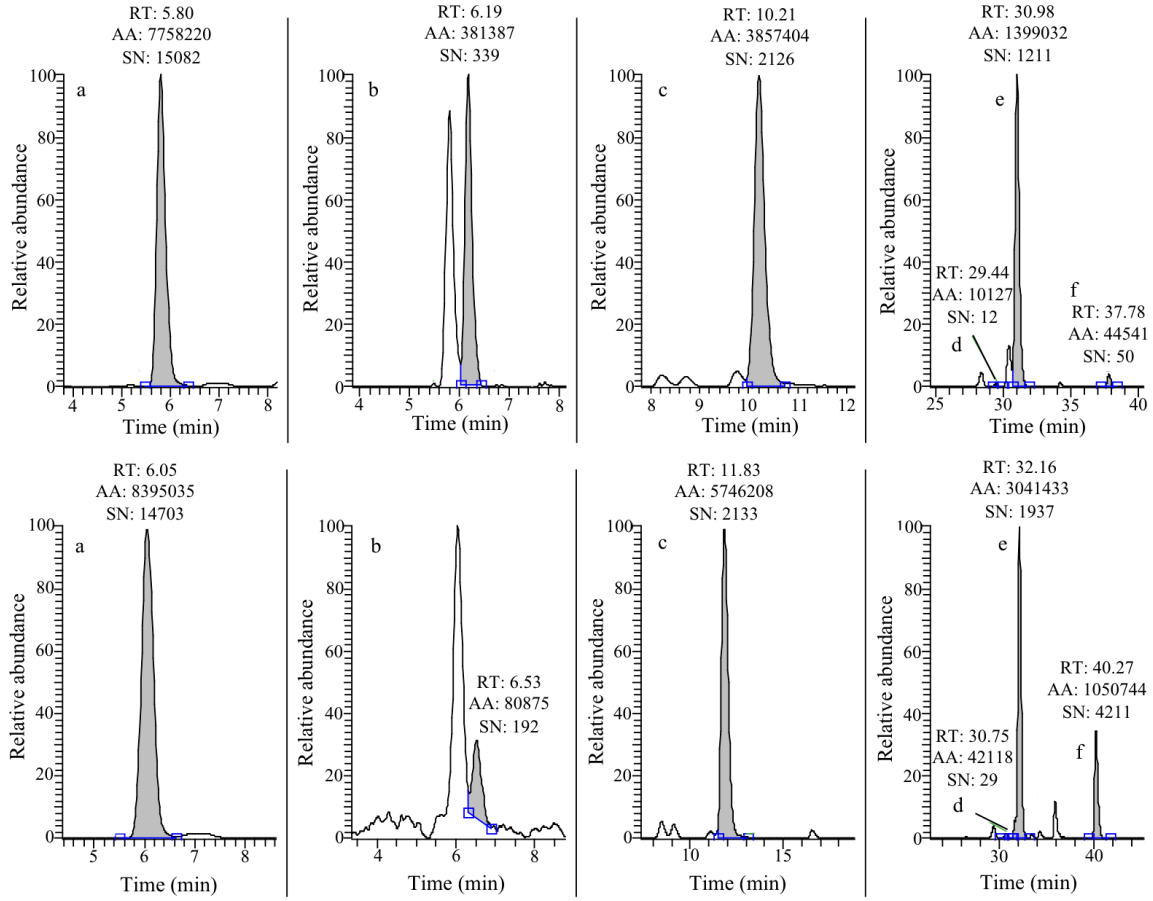


**FIGURE 2.5** Chromatograms of the various carotenoids, obtained by HR-Orbitrap-MS analysis. The mass extraction window was maximally set at 3.5 ppm. The extracts under consideration were derived from green-colored tomato fruit material (upper part) and red-ripe tomato fruit material (below), which were solely enriched with the internal standard β-apo-8'-carotenal (8 ng $mg^{-1}$ dry weight). The targeted analytes concerned β-apo-8'-carotenal (a), lutein (b), zeaxanthin (c), α-carotene (d), β-carotene (e), and lycopene (f).

The chromatograms for these carotenoids indicate that no other matrix substances significantly interfered since S/N ratios were for each of the considered detection techniques at least 3. The same is valid for lycopene and α-carotene in the case of Orbitrap-MS, and for lycopene in the case of UV-VIS PDA detection. For the other carotenoids, which were not detected by a certain analytical technique, it is demonstrated that the chromatograms of the enriched extracts displayed a significant increase in peak area intensity at the specific retention times of the considered compounds, taking a S/N ratio of at least 3 into account. In addition, no other matrix substances interfered significantly at these retention times. In general, the developed methods proved to be specific/selective for lutein, zeaxanthin, α-carotene, β-carotene, lycopene and β-

apo-8'-carotenal. However, when comparing the chromatograms of the various detection techniques, it was noted that the specificity/selectivity for Orbitrap-MS tended to exceed that of UV-VIS and MS/MS. Indeed, less matrix interferences were observed upon Orbitrap-MS, especially with regard to the carotenes. Additionally, the extract of a red-ripe tomato fruit was analyzed by Orbitrap-MS and evaluated with regard to specificity/selectivity. As demonstrated by Figure 2.5, good specificity/selectivity was obtained for lutein, $\alpha$-carotene, $\beta$-carotene and lycopene. In the case of zeaxanthin, however, more matrix interferences were noticeable although S/N ratio was still > 3. Based on the chromatograms from both green-colored and red-ripe tomato fruits, representing the major possible modifications with regard to the composition of the considered plant matrix, it may be concluded that the developed high-resolution Orbitrap-MS method has the ability to ensure good specificity/selectivity, irrespective of the matrix components present.

### 3.5.2. PRECISION

To evaluate the precision of the developed methods, repeatability and within-laboratory reproducibility were determined, whereby the coefficients of variation (CV) were calculated. The outcome of these calculations was evaluated by means of the Horwitz equation (32). To study the repeatability, six replicates of samples were analyzed and this at three fortification levels. Fortification levels (Table 2.5) were based on the carotenoid concentrations, found in both green-colored and red-ripe tomato fruits (Figure 2.1). The within-laboratory reproducibility was evaluated with two series of six replicates of samples, and this again at three fortification levels. These series were analyzed on different days by different operators. Calculated CV values, presented in Table 2.5, indicated good repeatability and within-laboratory reproducibility for each of the considered detection techniques and for all carotenoids. The obtained CV values were similar to the values found in the majority of the consulted papers concerning carotenoid analysis (54-57). However, in the study of Lyan *et al.* (2001) and Marinova and Ribarova (2007) (58,59) better precision was achieved during carotenoid analysis, which possibly can be attributed in part to the difference in matrix.

**TABLE 2.5** Performance criteria for the various detection techniques (i.e. Orbitrap-MS, UV-VIS PDA and MS/MS), evaluated during the validation study (n = 6). LOD and LOQ are expressed on a dry weight basis.

| Analyte | Detection | LOD (ng $g^{-1}$) | LOQ (ng $g^{-1}$) | Nominal conc. (ng $\mu L^{-1}$) | Repeatab. CV (%) | Within-lab. reprod. CV (%) | Recovery (%) |
|---|---|---|---|---|---|---|---|
| Lutein | UV-VIS | 224 | 774 | 1.25 | 12.6 | 12.7 | 87.4 |
| | | | | 2.50 | 11.5 | 14.4 | 98.2 |
| | | | | 3.75 | 5.3 | 14.5 | 95.6 |
| | MS/MS | 64 | 208 | 1.25 | 2.9 | 12.0 | 91.6 |
| | | | | 2.50 | 8.0 | 13.0 | 99.8 |
| | | | | 3.75 | 9.7 | 11.7 | 94.6 |
| | Orbitrap | 8 | 40 | 1.25 | 4.7 | 12.2 | 91.5 |
| | | | | 2.50 | 7.4 | 10.9 | 99.1 |
| | | | | 3.75 | 4.9 | 7.4 | 89.4 |
| Zeaxanthin | UV-VIS | 5184 | 17288 | 1.25 | 12.4 | 14.0 | 95.5 |
| | | | | 2.50 | 10.5 | 8.3 | 98.7 |
| | | | | 3.75 | 7.9 | 13.4 | 83.7 |
| | MS/MS | 1376 | 4576 | 1.25 | 7.8 | 13.8 | 102.1 |
| | | | | 2.50 | 2.8 | 5.9 | 102.3 |
| | | | | 3.75 | 8.3 | 12.7 | 97.8 |
| | Orbitrap | 304 | 1024 | 1.25 | 8.1 | 10.0 | 96.3 |
| | | | | 2.50 | 9.9 | 10.3 | 90.4 |
| | | | | 3.75 | 10.6 | 11.1 | 91.7 |
| $\alpha$-carotene | UV-VIS | 352 | 1184 | 0.50 | 5.9 | 7.4 | 94.7 |
| | | | | 1.00 | 5.8 | 7.3 | 97.1 |
| | | | | 1.50 | 8.2 | 8.3 | 91.6 |
| | MS/MS | 48 | 160 | 0.50 | 4.1 | 9.6 | 101.5 |
| | | | | 1.00 | 7.4 | 9.0 | 93.6 |
| | | | | 1.50 | 4.0 | 5.5 | 97.8 |
| | Orbitrap | 8 | 16 | 0.50 | 6.7 | 11.1 | 94.9 |
| | | | | 1.00 | 6.4 | 9.3 | 84.9 |
| | | | | 1.50 | 4.1 | 9.2 | 91.5 |
| $\beta$-carotene | UV-VIS | 600 | 2008 | 1.00 | 3.6 | 12.6 | 98.2 |
| | | | | 2.00 | 7.8 | 9.9 | 84.8 |
| | | | | 3.00 | 8.9 | 11.1 | 87.0 |
| | MS/MS | 32 | 96 | 1.00 | 11.7 | 9.7 | 96.0 |
| | | | | 2.00 | 7.2 | 14.5 | 92.9 |
| | | | | 3.00 | 12.4 | 13.2 | 95.1 |
| | Orbitrap | 8 | 24 | 1.00 | 4.7 | 7.6 | 106.2 |
| | | | | 2.00 | 7.1 | 13.3 | 96.9 |
| | | | | 3.00 | 8.5 | 10.0 | 101.3 |
| Lycopene | UV-VIS | 248 | 816 | 0.50 | 5.5 | 9.6 | 101.0 |
| | | | | 1.00 | 4.9 | 7.9 | 93.0 |
| | | | | 1.50 | 2.4 | 7.9 | 88.6 |
| | MS/MS | 64 | 208 | 0.50 | 9.8 | 10.7 | 97.6 |
| | | | | 1.00 | 9.4 | 12.1 | 81.6 |
| | | | | 1.50 | 7.2 | 10.1 | 97.8 |
| | Orbitrap | 16 | 40 | 0.50 | 2.4 | 9.9 | 104.0 |
| | | | | 1.00 | 3.2 | 3.4 | 92.4 |
| | | | | 1.50 | 7.0 | 8.4 | 89.8 |

### 3.5.3. MEAN CORRECTED RECOVERY

Since no certified reference material was available, trueness was determined as the mean corrected recovery by using fortified tomato fruit samples. To this end, three fortification levels were considered with six replicates for each level. These fortification levels were based on the endogenous concentrations of the carotenoids in green-colored and red-ripe tomato fruit tissue. For each sample and carotenoid, the area ratio determined was diminished with an average area ratio, which reflected the endogenous carotenoid level and was calculated based on the 6 'blank' samples that were used for evaluation of specificity/selectivity. Next, based on the net area ratio, recovered carotenoid concentrations were calculated and compared to expected concentrations. Mean corrected recoveries for each of the considered detection techniques are reported in Table 2.5 and were satisfactory according to CD 2002/657/EC (32). The obtained recovery values were similar to values reported in the literature (60-62).

### 3.3.4. LINEARITY

The linearity of the developed methods was evaluated by preparing six-point calibration curves in matrix for the various carotenoids. The blank samples were fortified to reach extract concentrations (exclusively the endogenous carotenoid concentrations), ranging from 1.0 to 7.5 ng $\mu L^{-1}$ for lutein and zeaxanthine, 0.25 to 5.0 ng $\mu L^{-1}$ for $\alpha$-carotene and lycopene, and 0.5 to 5.0 ng $\mu L^{-1}$ for $\beta$-carotene. Determination coefficients ($R^2$), obtained for these compounds, were all > 0.99, which suggested good linearity.

### 3.3.5. LIMIT OF DETECTION AND LIMIT OF QUANTIFICATION

Since no blank samples were available, the limit of detection (LOD, S/N ≥ 3) and the limit of quantification (LOQ, S/N ≥ 10) for each of the carotenoids were theoretically calculated based on six-point calibration curves, relating concentrations and S/N ratios. The values for these performance characteristics are reported in Table 2.5. It may be concluded that Orbitrap-MS is more sensitive for carotenoid analysis than UV-VIS or MS/MS. Based on the literature (31,63-66), it may be concluded that the here established Orbitrap-MS analytical method is extremely suited for sensitive analysis of carotenoids since the obtained LOD and LOQ values for this detection technique are among the lowest in the field of carotenoid analysis. It should be noted that large differences were observed in LOD and LOQ values between lutein and zeaxanthin. These

differences are likely to originate from the specific in-source ion fragmentation, which arises from the structural difference between the considered xanthophylls. Because of the presence of a 4', 5'-double bond, lutein possesses an allylic hydroxyl group, which allows the elimination of water after protonation (68,69). The resulting base peak at *m/z* 551 corresponds to the $[M+H-H_2O]^+$ ion, which is stabilized by mesomeric effects (68,69). Zeaxanthin will not as easily fragment to $[M+H-H_2O]^+$ fragments, because the double bonds are not able to better stabilize a positive charge (70). It is assumed that less matrix interferences are present in the extract with an *m/z*-value of 551. In addition, differences in ionization efficiency may also contribute to the observed LOD and LOQ differences between lutein and zeaxanthin. With regard to the well-established analytical methods (i.e. UV-VIS and MS/MS), the literature (31,63-66) indicates in general equivalent sensitivity as obtained in this study.

### 3.6. METABOLOMIC PROFILING

Although the analytical method was developed and validated by focusing on five carotenoids only, a metabolomic profiling of the tomato fruit was premised. An efficient screening of the full scan data for carotenoid compounds was executed by using the software program ToxID 2.1.2 (Thermo Fisher Scientific, San José, USA). For this purpose, a ToxID database was constructed by implementing the molecular formula of 355 relevant carotenoids (71), which corresponded to 137 different chemical formulas. Since no information about the retention time of these compounds was available, identification was solely based on the presence of two diagnostic ions: the $(M+H^+]^+$ ion and the corresponding $^{13}C$ isotopic ion. An isotopic ion was found suitable as diagnostic ion when the calculated relative ion intensities met the CD 2002/657/EC (32) requirements. However, unequivocal identification cannot always be guaranteed because of the presence of isomers, which may lead to the detection of multiple chromatographic peaks. Taking these findings into account, the metabolomic approach enables a first screening for relevant compounds and is indicative for the possible presence or absence of a certain compound, characterized by a certain elemental composition and molecular weight. Main ToxID settings included a minimum peak intensity of 1000 and a maximum mass deviation of 5 ppm. The application of ToxID was able to detect 59 and 97 peaks (Table 2.6 and Table 2.7) in the extract of a random green and red tomato, respectively. Since only one red and one green tomato fruit sample were considered, this ToxID application had the sole purpose of demonstrating the profiling capabilities.

# 4. CONCLUSIONS

Within this research, a generic extraction protocol and full-scan high-resolution Orbitrap-MS detection method were successfully developed and validated for five carotenoids (lutein, zeaxanthin, $\alpha$-carotene, $\beta$-carotene and lycopene) in tomato fruit tissue. The extraction protocol was efficiently optimized by means of a D-optimal design and was characterized by a low solvent usage and a small needed amount of plant material. The detection method used a single-stage Exactive™ Orbitrap mass spectrometer, operating at a mass resolution of 100,000 FWHM. The validation study demonstrated good performance for the various criteria under investigation, whereas the comparative study with well-established detection techniques (i.e. MS/MS and UV-VIS PDA) proved superior achievements with regard to selectivity/specificity and sensitivity. Although the method was developed for only five carotenoids, representing both carotenes and xanthofylls, the full-scan principle of the Orbitrap-MS allows screening all known carotenoids and to search for unknown compounds, which are closely involved in carotenoid metabolism. As such, the developed method enables a metabolomic screening approach, which is of significant importance for a better understanding of the carotenoids' metabolism, partitioning in plants, actions and role.

**TABLE 2.6** Carotenoids and related compounds, detected in the extract of a red-ripe tomato fruit. The results were obtained by usage of the software program ToxID, for which a minimum peak intensity of 1000 and a maximum mass deviation of 5 ppm were taken into account. The possible compound names are indicated by a number, specified in Straub and Pfander (1987) (71) and defined in Table 2.8.

| Elemental composition | RT (min) | Peak intensity $[M+H]^+$ | Peak intensity $[^{13}C$ isotope + $H]^+$ | Isotope ratio (%) | Possible compound names |
|---|---|---|---|---|---|
| $C_{22}H_{30}O$ | 3.63 | 6.19 $e^5$ | 1.55 $e^5$ | 25.04 | 263 |
| | 4.18 | 11.2 $e^5$ | 2.47 $e^5$ | 22.05 | |
| $C_{25}H_{34}O$ | 3.28 | 1.16 $e^5$ | 3.35 $e^4$ | 28.88 | 262 |
| | 4.68 | 1.46 $e^5$ | 3.78 $e^4$ | 25.89 | |
| | 5.25 | 4.23 $e^5$ | 9.90 $e^4$ | 23.40 | |
| | 6.38 | 3.21 $e^5$ | 7.55 $e^4$ | 23.52 | |
| $C_{25}H_{34}O_2$ | 3.28 | 1.16 $e^5$ | 3.35 $e^4$ | 28.88 | 262, 263 |
| $C_{27}H_{36}O_2$ | 3.72 | 1.66 $e^5$ | 4.22 $e^4$ | 25.36 | 257, 260 |
| $C_{30}H_{40}O$ | 8.45 | 4.58 $e^5$ | 1.31 $e^5$ | 28.60 | 248, 254 |
| | 11.80 | 6.18 $e^6$ | 1.94 $e^6$ | 31.39 | |
| | 16.42 | 7.25 $e^5$ | 1.96 $e^5$ | 27.03 | |
| $C_{30}H_{40}O_2$ | 5.63 | 2.49 $e^5$ | 7.51 $e^4$ | 30.16 | 249, 250, 251, 264 |
| $C_{30}H_{46}$ | 4.18 | 2.44 $e^5$ | 6.28 $e^4$ | 25.73 | 264 |
| $C_{32}H_{48}$ | 13.24 | 9.13 $e^5$ | 3.07 $e^5$ | 33.63 | 264 |
| | 14.04 | 3.81 $e^5$ | 1.16 $e^5$ | 30.45 | |
| | 7.20 | 5.06 $e^6$ | 1.61 $e^6$ | 31.82 | |
| | 41.45 | 1.01 $e^6$ | 3.05 $e^5$ | 30.20 | |
| | 42.31 | 2.42 $e^6$ | 7.27 $e^5$ | 30.04 | |
| | 44.56 | 1.40 $e^7$ | 4.46 $e^6$ | 31.90 | |
| | 49.85 | 1.16 $e^6$ | 3.14 $e^5$ | 27.07 | |
| $C_{32}H_{42}O$ | 20.20 | 7.62 $e^5$ | 1.77 $e^5$ | 23.23 | 242 |
| $C_{40}H_{46}$ | 26.61 | 5.55 $e^5$ | 1.74 $e^5$ | 30.99 | 12, 13 |
| | 27.71 | 1.58 $e^3$ | 6.26 $e^2$ | 39.62 | |
| $C_{40}H_{50}$ | 26.17 | 9.94 $e^4$ | 2.79 $e^4$ | 28.07 | 35 |
| $C_{40}H_{54}$ | 18.50 | 3.05 $e^5$ | 1.12 $e^5$ | 36.72 | 2, 7, 18, 36, 37 |
| | 33.62 | 7.29 $e^4$ | 2.09 $e^4$ | 28.67 | |
| $C_{40}H_{54}O$ | 6.06 | 4.50 $e^6$ | 1.85 $e^6$ | 41.11 | 38, 41, 44, 50, 53, 143, 148, |
| | 20.16 | 4.77 $e^5$ | 1.98 $e^5$ | 41.51 | 149, 151 |
| $C_{40}H_{54}O_2$ | 4.53 | 4.33 $e^5$ | 1.66 $e^5$ | 38.33 | 66, 72, 84, 117, 143, 153, |
| | 5.29 | 2.63 $e^5$ | 1.15 $e^5$ | 43.73 | 156, 158, 161, 162, 163, 165, 194 |
| $C_{40}H_{56}$ | 39.55 | 1.02 $e^7$ | 4.22 $e^6$ | 41.37 | 3, 4, 5, 11, 19, 20 |
| | 35.30 | 3.99 $e^5$ | 1.71 $e^5$ | 42.86 | |
| | 31.53 | 5.25 $e^6$ | 2.12 $e^6$ | 40.38 | |
| | 28.97 | 2.99 $e^5$ | 1.19 $e^5$ | 39.80 | |
| $C_{40}H_{56}O$ | 7.80 | 3.30 $e^5$ | 1.23 $e^5$ | 37.27 | 37, 39, 40, 42, 45, 46, 47, 49, |
| | 13.19 | 1.72 $e^5$ | 5.71 $e^4$ | 33.20 | 55, 62, 63, 114, 115, 116, |
| | 16.66 | 2.87 $e^5$ | 1.35 $e^5$ | 47.04 | 125, 126, 273 |
| | 18.03 | 1.39 $e^5$ | 5.31 $e^4$ | 38.20 | |
| | 19.14 | 2.05 $e^5$ | 9.77 $e^4$ | 47.66 | |
| | 23.41 | 2.89 $e^6$ | 1.21 $e^6$ | 41.87 | |
| | 27.18 | 1.08 $e^5$ | 4.58 $e^4$ | 42.41 | |
| | 27.83 | 1.79 $e^5$ | 6.42 $e^4$ | 35.87 | |
| | 31.95 | 1.82 $e^6$ | 7.68 $e^5$ | 42.20 | |
| | 33.62 | 2.73 $e^5$ | 1.10 $e^5$ | 40.29 | |
| | 38.34 | 2.57 $e^5$ | 1.02 $e^5$ | 39.69 | |

| | | | | | |
|---|---|---|---|---|---|
| $C_{40}H_{56}O_2$ | 6.05 | 1.57 $e^5$ | 5.58 $e^4$ | 35.54 | 63, 64, 67, 69, 70, 71, 73, 75, |
| | 6.56 | 6.09 $e^4$ | 2.94 $e^4$ | 48.28 | 76, 78, 79, 83, 116, 117, 127, |
| | 14.97 | 3.49 $e^5$ | 1.33 $e^5$ | 38.11 | 128, 133, 136, 139, 144, 157, |
| | 16.50 | 8.37 $e^4$ | 3.58 $e^4$ | 42.77 | 160, 194, 213, 214 |
| $C_{40}H_{56}O_3$ | 4.53 | 5.67 $e^5$ | 2.42 $e^5$ | 42.68 | 84, 86, 88, 89, 119, 120, 124, |
| | 5.29 | 1.02 $e^5$ | 4.14 $e^4$ | 40.59 | 129, 130, 134, 170, 215 |
| $C_{40}H_{56}O_4$ | 3.23 | 7.25 $e^4$ | 2.81 $e^4$ | 38.76 | 93, 94, 122, 123, 131, 132, |
| | 3.73 | 5.93 $e^5$ | 2.52 $e^5$ | 42.50 | 135, 138, 174, 188, 191, 197, |
| | 4.32 | 4.07 $e^5$ | 1.70 $e^5$ | 41.77 | 205, 206, 216 |
| $C_{40}H_{58}$ | 5.05 | 4.90 $e^4$ | 1.98 $e^4$ | 40.41 | 12, 21, 22, 37 |
| | 27.94 | 9.06 $e^4$ | 2.87 $e^4$ | 31.68 | |
| $C_{40}H_{58}O$ | 16.24 | 1.58 $e^5$ | 6.70 $e^4$ | 42.41 | 46, 48, 56, 57, 115 |
| $C_{40}H_{58}O_2$ | 9.09 | 2.14 $e^5$ | 7.84 $e^4$ | 36.64 | 75, 78, 80 |
| | 13.19 | 9.68 $e^5$ | 3.81 $e^5$ | 39.36 | |
| | 14.49 | 4.28 $e^4$ | 1.76 $e^4$ | 41.12 | |
| | 16.62 | 5.78 $e^4$ | 2.53 $e^4$ | 43.77 | |
| $C_{40}H_{60}O$ | 5.08 | 3.26 $e^5$ | 1.41 $e^5$ | 43.25 | 57, 58, 115 |
| | 16.26 | 3.57 $e^5$ | 1.52 $e^5$ | 42.58 | |
| | 17.43 | 5.02 $e^5$ | 1.88 $e^5$ | 37.45 | |
| | 19.99 | 4.60 $e^5$ | 1.85 $e^5$ | 40.22 | |
| $C_{40}H_{62}$ | 6.36 | 2.47 $e^6$ | 9.92 $e^5$ | 40.16 | 27, 28, 29, 30 |
| | 11.33 | 6.42 $e^5$ | 2.80 $e^5$ | 43.61 | |
| | 16.09 | 5.99 $e^6$ | 2.48 $e^6$ | 41.40 | |
| | 26.10 | 2.36 $e^6$ | 9.22 $e^5$ | 39.07 | |
| | 27.61 | 3.82 $e^6$ | 1.61 $e^6$ | 42.15 | |
| $C_{40}H_{62}O$ | 4.03 | 6.09 $e^5$ | 2.58 $e^5$ | 42.36 | 43 |
| | 5.40 | 7.61 $e^5$ | 3.09 $e^5$ | 40.60 | |
| | 6.52 | 3.63 $e^5$ | 1.39 $e^5$ | 38.29 | |
| | 8.19 | 5.16 $e^5$ | 2.07 $e^5$ | 40.11 | |
| $C_{40}H_{62}O_2$ | 6.20 | 1.68 $e^6$ | 7.52 $e^5$ | 44.76 | 117 |
| | 31.35 | 9.47 $e^4$ | 4.10 $e^4$ | 43.29 | |
| $C_{40}H_{64}$ | 14.43 | 3.98 $e^6$ | 1.68 $e^6$ | 42.21 | 31, 32 |
| | 15.24 | 1.18 $e^5$ | 4.87 $e^5$ | 41.27 | |
| | 26.03 | 8.14 $e^6$ | 3.42 $e^6$ | 42.01 | |
| | 35.72 | 9.70 $e^4$ | 3.68 $e^4$ | 37.94 | |
| | 36.18 | 1.69 $e^5$ | 6.26 $e^4$ | 37.04 | |
| | 38.21 | 2.27 $e^6$ | 9.12 $e^5$ | 40.18 | |
| | 38.62 | 3.81 $e^6$ | 1.62 $e^6$ | 42.52 | |
| | 40.36 | 1.43 $e^5$ | 6.21 $e^4$ | 43.43 | |
| | 41.08 | 2.49 $e^6$ | 1.02 $e^6$ | 40.96 | |
| | 41.54 | 2.74 $e^6$ | 1.10 $e^6$ | 40.15 | |
| | 45.26 | 2.89 $e^5$ | 1.24 $e^5$ | 42.91 | |
| | 45.99 | 2.42 $e^5$ | 8.49 $e^4$ | 35.08 | |
| $C_{40}H_{64}O_2$ | 4.03 | 2.02 $e^5$ | 8.23 $e^4$ | 40.07 | 79, 82 |
| | 6.50 | 1.67 $e^5$ | 6.32 $e^4$ | 37.84 | |
| | 8.18 | 6.36 $e^5$ | 2.51 $e^5$ | 39.47 | |
| $C_{41}H_{58}O$ | 4.53 | 4.32 $e^5$ | 1.66 $e^5$ | 38.43 | 96, 97 |
| | 5.29 | 2.63 $e^5$ | 1.07 $e^5$ | 40.68 | |
| | 15.09 | 1.23 $e^5$ | 5.22 $e^4$ | 42.44 | |
| | 22.36 | 2.19 $e^5$ | 8.13 $e^4$ | 37.12 | |
| $C_{41}H_{60}O_2$ | 18.12 | 3.14 $e^5$ | 1.28 $e^5$ | 40.76 | 106, 147, 182 |
| $C_{42}H_{64}O_2$ | 29.63 | 1.69 $e^5$ | 7.27 $e^4$ | 43.02 | 109, 110, 111 |

**TABLE 2.7** Carotenoids and related compounds, detected in the extract of a green-colored tomato fruit. The results were obtained by usage of the software program ToxID, at which a minimum peak intensity of 1000 and a maximum mass deviation of 5 ppm were taken into account. The possible compound names are indicated by a number as defined in Straub and Pfander (1987) (71) and specified in Table 2.8.

| Elemental composition | RT (min) | Peak intensity $[M+H]^+$ | Peak intensity $[^{13}C$ isotope + $H]^+$ | Isotope ratio | Possible compound names |
|---|---|---|---|---|---|
| $C_{27}H_{40}O_3$ | 3.19 | 5.56 $e^4$ | 9.92 $e^3$ | 17.84 | 255 |
| $C_{30}H_{40}O$ | 8.66 | 2.49 $e^5$ | 6.74 $e^4$ | 27.07 | 248, 254 |
| | 9.35 | 2.31 $e^5$ | 5.78 $e^4$ | 25.02 | |
| | 11.29 | 5.23 $e^5$ | 1.19 $e^5$ | 22.75 | |
| | 11.89 | 6.65 $e^5$ | 2.07 $e^6$ | 31.13 | |
| $C_{30}H_{40}O_2$ | 3.00 | 5.84 $e^4$ | 1.18 $e^4$ | 20.20 | 249, 250, 251, 264 |
| | 3.80 | 6.30 $e^4$ | 1.25 $e^4$ | 19.84 | |
| | 5.78 | 1.75 $e^5$ | 4.88 $e^4$ | 27.89 | |
| $C_{30}H_{48}$ | 3.92 | 6.21 $e^4$ | 1.73 $e^4$ | 27.85 | 264 |
| | 4.45 | 7.76 $e^4$ | 2.33 $e^4$ | 30.02 | |
| | 13.27 | 1.58 $e^6$ | 5.34 $e^5$ | 33.80 | |
| | 34.71 | 2.11 $e^5$ | 7.08 $e^4$ | 33.55 | |
| | 35.82 | 2.54 $e^5$ | 8.88 $e^4$ | 34.96 | |
| | 39.25 | 2.43 $e^5$ | 9.48 $e^4$ | 39.01 | |
| | 41.51 | 2.23 $e^5$ | 8.38 $e^4$ | 37.57 | |
| | 44.59 | 4.80 $e^5$ | 2.10 $e^5$ | 43.75 | |
| | 49.79 | 2.62 $e^5$ | 9.53 $e^4$ | 36.37 | |
| $C_{32}H_{48}O_2$ | 29.67 | 8.72 $e^4$ | 2.32 $e^4$ | 26.61 | 241 |
| $C_{40}H_{52}$ | 6.30 | 2.88 $e^5$ | 1.15 $e^5$ | 39.93 | 1, 6, 15, 17 |
| $C_{40}H_{52}O$ | 4.95 | 2.46 $e^3$ | 8.91 $e^2$ | 36.22 | 147, 150 |
| | 6.33 | 1.19 $e^5$ | 3.04 $e^4$ | 25.55 | |
| $C_{40}H_{52}O_2$ | 3.29 | 2.57 $e^5$ | 1.04 $e^5$ | 40.47 | 65, 152, 156, 192, 193, 194, |
| | 3.75 | 3.16 $e^5$ | 1.37 $e^5$ | 43.35 | 211 |
| | 5.02 | 3.65 $e^4$ | 9.92 $e^3$ | 27.17 | |
| $C_{40}H_{54}O$ | 5.52 | 1.29 $e^5$ | 4.62 $e^4$ | 35.81 | 38, 41, 44, 50, 53, 143, |
| | 6.29 | 1.66 $e^7$ | 7.14 $e^6$ | 43.01 | 148, 149, 151 |
| | 7.57 | 3.19 $e^5$ | 1.19 $e^5$ | 37.30 | |
| | 9.29 | 4.14 $e^5$ | 1.66 $e^5$ | 40.09 | |
| | 10.03 | 2.56 $e^5$ | 9.05 $e^4$ | 35.35 | |
| $C_{40}H_{54}O_2$ | 3.83 | 4.81 $e^3$ | 1.65 $e^3$ | 34.30 | 66, 72, 84, 117, 143, 153, |
| | 4.68 | 1.33 $e^5$ | 6.15 $e^4$ | 46.24 | 156, 158, 161, 162, 163, |
| | 6.35 | 1.73 $e^5$ | 4.51 $e^5$ | 26.07 | 165, 194 |
| $C_{40}H_{54}O_3$ | 3.29 | 7.31 $e^5$ | 3.04 $e^5$ | 41.59 | 118, 128, 167, 169, 171, |
| | 3.75 | 1.06 $e^6$ | 4.22 $e^5$ | 39.81 | 177, 197 |
| | 4.49 | 5.54 $e^4$ | 2.20 $e^4$ | 39.71 | |
| | 5.19 | 1.36 $e^5$ | 5.10 $e^4$ | 37.50 | |
| $C_{40}H_{56}$ | 28.98 | 1.06 $e^5$ | 3.23 $e^4$ | 30.47 | 3, 4, 5, 11, 19, 20 |
| | 31.28 | 3.35 $e^5$ | 1.20 $e^5$ | 35.82 | |
| | 31.57 | 2.78 $e^6$ | 1.15 $e^6$ | 41.37 | |
| $C_{40}H_{56}O$ | 20.34 | 2.29 $e^3$ | 6.69 $e^2$ | 29.21 | 37, 39, 40, 42, 45, 46, 47, |
| | 23.48 | 2.33 $e^5$ | 8.56 $e^4$ | 36.74 | 49, 55, 62, 63, 114, 115, 116, 125, 126, 273 |
| $C_{40}H_{56}O_2$ | 6.29 | 7.69 $e^4$ | 2.49 $e^4$ | 32.38 | 63, 64, 67, 69, 70, 71, 73, |
| | 6.82 | 2.70 $e^5$ | 1.03 $e^5$ | 38.15 | 75, 76, 78, 79, 83, 116, |
| | 15.04 | 6.69 $e^5$ | 2.76 $e^5$ | 41.26 | 117, 127, 128, 133, 136, |
| | 21.66 | 3.89 $e^5$ | 1.45 $e^5$ | 37.28 | 139, 144, 157, 160, 194, 213, 214 |

| | | | | | |
|---|---|---|---|---|---|
| $C_{40}H_{56}O_3$ | 4.69 | 2.13 $e^5$ | 9.06 $e^4$ | 42.54 | 84, 86, 88, 89, 119, 120, |
| | 4.95 | 7.35 $e^5$ | 2.94 $e^5$ | 40.00 | 124, 129, 130, 134, 170, |
| | 5.82 | 5.34 $e^4$ | 1.57 $e^4$ | 29.40 | 215 |
| | 6.38 | 1.34 $e^5$ | 3.80 $e^4$ | 28.36 | |
| $C_{40}H_{56}O_4$ | 3.29 | 1.33 $e^6$ | 5.50 $e^5$ | 41.35 | 93, 94, 122, 123, 131, 132, |
| | 3.82 | 5.76 $e^6$ | 2.46 $e^6$ | 42.71 | 135, 138, 174, 188, 191, |
| | 4.49 | 7.48 $e^5$ | 2.81 $e^5$ | 37.57 | 197, 205, 206, 216 |
| | 5.23 | 9.15 $e^5$ | 3.59 $e^5$ | 39.23 | |
| $C_{40}H_{64}$ | 14.26 | 3.81 $e^5$ | 1.56 $e^5$ | 40.68 | 31, 32 |
| | 38.27 | 3.81 $e^5$ | 1.57 $e^5$ | 41.10 | |
| | 38.68 | 1.05 $e^5$ | 3.62 $e^5$ | 40.80 | |
| | 41.11 | 5.00 $e^5$ | 3.35 $e^5$ | 43.00 | |
| | 41.58 | 7.79 $e^4$ | 2.04 $e^4$ | 34.48 | |
| $C_{40}H_{68}O_6$ | 5.62 | 6.91 $e^4$ | 3.09 $e^4$ | 44.71 | 57 |

**TABLE 2.8** Possible compound names, corresponding to the numbering (N°) of carotenoids in Straub and Pfander (1987) (71).

| N° | Possible compound name | N° | Possible compound name |
|---|---|---|---|
| 1 | 3,4,3',4'-Bisdehydro-β-carotene | 115 | α-carotene epoxide |
| 2 | 3,4-Dehydro-β-carotene | 116 | Phytoene 1,2-oxide |
| 3 | β-carotene | 117 | Cryptoxanthin epoxide |
| 4 | η-carotene | 118 | Diadinoxanthin |
| 5 | α-carotene | 119 | Antheraxanthin |
| 6 | β-isorenieratene | 120 | Taraxanthin |
| 7 | Torulene | 122 | Neoxanthin |
| 11 | δ-carotene | 123 | Trollixanthin |
| 12 | α-zeacarotene | 124 | Vaucheriaxanthin |
| 13 | Isorenieratene | 125 | Mutatochrome |
| 15 | Chlorobactene | 126 | Flavochrome |
| 17 | Bisdehydrolycopene | 127 | Cryptoflavin |
| 18 | 3,4-Dehydrolycopene | 128 | Rubichrome |
| 19 | Lycopene | 129 | Mutatoxanthin |
| 20 | 1,2-Dihydro-3,4-dehydrolycopene | 130 | Flavoxanthin |
| 21 | 1,2-Dihydrolycopene | 131 | Neochrome |
| 22 | Neurosporene | 132 | Trollichrome |
| 27 | 1,2,7,8,11,12-Hexahydrolycopene | 133 | β-carotene 5,6,5',6'-diepoxide |
| 28 | 1,2,1',2'-Tetrahydroneurosporene | 134 | Cryptoxanthin diepoxide |
| 29 | 7,8,1',2',7',8'-Hexahydrolycopene | 135 | Violaxanthin |
| 30 | Phytofluene | 136 | Luteochrome |
| 31 | 1,2-Dihydrophytofluene | 138 | Luteoxanthin |
| 32 | Phytoene | 139 | Aurochrome |
| 35 | Anhydroeschscholtzxanthin | 143 | 13-*cis*-Lycopen-20-al |
| 36 | Isocarotene | 144 | 13-*cis*-Rhodopin-20-al |
| 37 | 7,7'-Dihydro-β-carotene | 147 | 3,4,3',4'-Tetrahydrospirilloxanthin-20-al |
| 38 | Anhydrolutein | 148 | Echinone |
| 39 | β-Cryptoxanthin | 149 | Phoenicopterone |
| 40 | Isocryptoxanthin | 150 | 4-Ketotorulene |
| 41 | Crocoxanthin | 151 | 4-Keto-γ-carotene |
| 42 | α-Cryptoxanthin | 152 | 3-Oxoechinenone |
| 44 | Celaxanthin | 153 | Hydroxyechinenone |
| 45 | Rubixanthin | 156 | 3,4-Diketo-α-carotene |
| 46 | Gazaniaxanthin | 157 | Cryptocapsin |
| 47 | Myxobactin | 158 | Deoxyflexixanthin |
| 48 | 1',2'-Dihydro-1'-hydroxy-γ-carotene | 160 | 1',2'-Dihydro-1'hydroxy-4-keto-γ-carotene |
| 49 | Aleuriaxanthin | 161 | 1',2'-Dihydro-2'-hydroxy-3',4'-dehydro-4-keto-γ-carotene |
| 50 | 3',4'-Dihydro-18'-hydroxy-γ-carotene | 162 | 2'-Dehydroplectaniaxanthin |
| 53 | OH-Chlorobactene | 163 | Rubixanthone |

| | |
|---|---|
| 55 | 3,4-Dehydrorhodopin |
| 56 | Rhodopin |
| 57 | Rhodopin β-D-glucoside |
| 58 | Chloroxanthin |
| 62 | Lycoxanthin |
| 63 | Anhydrowarmingol |
| 64 | 3,4-Dihydroxy-β-carotene |
| 65 | Alloxanthin |
| 66 | Diatoxanthin |
| 67 | Zeaxanthin |
| 69 | Caloxanthin |
| 70 | Nostoxanthin |
| 71 | Isozeaxanthin |
| 72 | Monadoxanthin |
| 73 | Lutein |
| 75 | Saproxanthin |
| 76 | Plectaniaxanthin |
| 78 | Tunaxanthin |
| 79 | 3,3'-Dihydroisorenieratene |
| 80 | Rhodopinol |
| 82 | 1,1'-Dihydroxy-1,2,1',2'-tetrahydro-ζ-carotene |
| 83 | Lycophyll |
| 84 | Eschscholtzxanthin |
| 86 | Deepoxyneoxanthin |
| 88 | Loroxanthin |
| 89 | Pyrenoxanthin |
| 93 | Crustaxanthin |
| 94 | Heteroxanthin |
| 96 | Oscillol 2,2'-di(O-methyl-methylpentoside) |
| 97 | Anhydrorhodovibrin |
| 106 | Rhodovibrin |
| 109 | 3,4-Dihydrospirilloxanthin |
| 110 | 3,4,3',4'-Tetrahydrospirrilloxanthin |
| 111 | 3,4,7,8-Tetrahydrospirrilloxanthin |
| 114 | β-Carotene 5,6-epoxide |
| 165 | 4-Keto-3'hydroxylycopene |
| 167 | Adonixanthin |
| 169 | α-Doradexanthin |
| 170 | Capsanthin |
| 171 | Flexixanthin |
| 174 | Siphonaxanthin |
| 177 | 2'-Dihydrophillisiaxanthin |
| 182 | Spheroidenone |
| 188 | Capsanthin monoepoxide |
| 191 | Capsochrome |
| 192 | Eschscholtzxanthone |
| 193 | Canthaxanthin |
| 194 | Diketopirardixanthin |
| 197 | Capsanthinone |
| 205 | Capsorubin |
| 206 | 7,8,7',8'-Tetrahydrocapsorubin |
| 211 | Torularhodin |
| 213 | Semi-β-carotenone |
| 214 | Semi-α-carotenone |
| 215 | Triphasiaxanthin |
| 216 | β-Carotenone |
| 241 | 15-Hydroxy-7',8'-dihydroreticulaxanthin |
| 242 | Apo-6'-carotenal |
| 248 | β-Apo-8'-carotenal |
| 249 | β-Citraurin |
| 250 | β-Apo-8'-carotenoic acid |
| 251 | Methyl β-apo-8'-carotenoate |
| 254 | Apo-8'-lycopenal |
| 255 | Methyl 1-hexosyl-1,2-dihydro-3,4-didehydroapo-8'-lycopenoate |
| 257 | 3-Hydro-β-apo-10'-carotenal |
| 260 | β-Apo-10'-carotenoic acid |
| 262 | β-Apo-12'-carotenal |
| 263 | Apo-12'-violaxanthal |
| 264 | Carotenonaldehyde |
| 273 | Peridinin |

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# CHAPTER III

## ULTRA-HIGH PERFORMANCE LIQUID CHROMATOGRAPHY COUPLED TO HIGH-RESOLUTION ORBITRAP MASS SPECTROMETRY FOR METABOLOMIC PROFILING OF THE ENDOGENOUS PHYTOHORMONAL STATUS OF THE TOMATO PLANT

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**Adapted from:**

Van Meulebroek L., Vanden Bussche J., Steppe K., Vanhaecke L. (2012). *Journal of Chromatography A* 1260:67-68

## ABSTRACT

Phytohormones are key signaling biomolecules and are of particular interest because of their regulating role in numerous physiological and developmental plant processes. Since the plant response to a given stimulus results amongst others from the complex interaction between phytohormones, there is a mounting interest for multiple phytohormone analysis. Therefore, with the primary aim of profiling the hormonal status of the tomato plant, a generic extraction protocol and an UHPLC-Orbitrap-MS analysis were developed and validated for both tomato fruit and leaf tissue. To this end, eight phytohormones were considered, i.e. gibberellic acid, indol-3-acetic acid, abscisic acid, jasmonic acid, salicylic acid, zeatin, $N^6$-benzyladenine and epibrassinolide, representing the major hormonal classes. The sample pre-treatment involved liquid extraction with a buffer of methanol, ultra-pure water and formic acid (75:20:5, v/v/v), after which the extract was purified by means of an Amicon® Ultra centrifugal unit. Subsequently, analytes were chromatographically separated on a sub-2 μm particles Nucleodur Gravity $C_{18}$ column and detected by an Exactive™ high-resolution mass spectrometer. Validation of the analytical method demonstrated that linearity (≥ 0.99), precision (CV ≤ 15%) and mean corrected recovery (between 80% and 110%) performed well for the majority of the eight targeted phytohormones. In addition, the generic nature of the extraction protocol and the full scan approach of the Orbitrap mass spectrometer allowed metabolomic profiling of the hormonal status of the tomato plant.

# 1. INTRODUCTION

As a minor component of the metabolome, phytohormones are of significant value because of their regulating actions towards various vital plant processes (1-3). Herewith, an effectuated plant response generally results from the considerable crosstalk among phytohormone-signaling pathways and is the primary reason for the arisen interest in multiple phytohormone analysis (4-6). The development of a method for the simultaneous analysis of phytohormones from all classes is thus of critical importance, but remains a challenging task. After all, phytohormones are typically present and active in plant tissues at very low concentrations (ng $g^{-1}$ fresh weight), in a background of a wide range of more abundant primary and secondary metabolites (3,7). Therefore, during the sample preparation phase, the targeted phytohormones have to be extracted efficiently without co-extraction of too much interfering substances from the considered plant tissue (4,7). Diverse solvents such as methanol, water/methanol mixture, dichloromethane, and neutral or acid pH buffers were found to be suited for this and are frequently used (7,8). Subsequent to the actual extraction, purification of the resulting extract is generally inevitable and diverse purification strategies including liquid-liquid extraction, ion exchange, solid phase extraction, solid phase micro extraction and vapor phase extraction have been reported (2,8-10). These techniques, however, may require significant amounts of solvent, time, and labor (8). For the quantitative measurement of phytohormones in plant extracts, traditionally, enzyme- or radioimmunoassays (ELISA, RIA), which are described as sensitive and selective, have been implemented. However, when applied to low purity extracts, quantification can be misleading due to the cross-reactivity of the antibodies and their inhibition or activation by interfering substances (11).

In recent years, (tandem) mass spectrometry became increasingly important for the profiling and quantification of phytohormones, mainly because of the high sensitivity, selectivity, accuracy and reproducibility of this approach (4,10). Gas chromatography coupled to mass spectrometry is a well-recognized technique for phytohormone analysis, which is reflected by numerous studies (12-16). However, prior to analysis, this approach typically requires derivatization to enhance the volatility and stability of the phytohormones (3). In addition, as certain phytohormones are thermally labile, the high temperatures in the gas chromatographic system could induce thermal disintegration (4,11). High-performance liquid chromatography has emerged as an effective

alternative that is essentially free of the limitations outlined above (5). Especially the application of multiple reaction monitoring (MRM) and the corresponding selectivity should allow to simultaneously analyze a wide range of phytohormones (3). However, it was only recently achieved by Pan *et al*. (2010) (10) to analyze phytohormones from the seven major classes within one run using HPLC-MS/MS. To the best of our knowledge, the study of Pan *et al*. (2010) (10) is the most comprehensive approach towards the concept of metabolomic profiling of phytohormones in plants, but still encounters some limitations. There is no possibility for post-acquisition re-interrogation of data, when for example a new type of phytohormone would be discovered; only a limited number of compounds can be measured per run and it is impossible to screen for unidentified, unknown compounds (17). Because of these limitations there is currently a trend towards full scan MS experiments, which are considered to be crucially important to bridge the gap between the still more targeted analyses and the broader metabolomic profiling approach (18).

Within this research a generic extraction procedure and an UHPLC-Fourier Transform Orbitrap-MS detection method were developed for the metabolomic profiling of phytohormones in different tomato plant tissues. The generic character of the envisioned extraction procedure implied a fully new approach since previously developed methods (e.g. 4,6,11) were specifically aimed towards a limited number of compounds and had as a consequence to be very selective. The extraction method was therefore kept as simple as possible without the co-extraction of too much interfering substances. The developed chromatographic separation method was, to the best of our knowledge, the first to apply UHPLC, resulting in a significant decrease in run time and reduction of solvent usage. Additionally, this research is also the first to utilize Orbitrap-MS for the analysis of phytohormones, which is indispensable with respect to the metabolomic profiling of the hormonal plant status. Although the development and validation of the analytical procedures were based on eight phytohormones only, coming from the major hormonal classes, the generic nature of the extraction procedure and the full scan approach of the Orbitrap mass spectrometer allow to screen for all known phytohormones and for unknown compounds with possible hormonal activity in plant tissues. This approach is believed to be most valuable in the further elucidation of hormone-signaling pathways in regulating vital plant processes.

# 2. MATERIALS AND METHODS

## 2.1 CHEMICALS AND REAGENTS

The phytohormone standards (±)-*cis, trans*-abscisic acid (ABA), gibberellic acid (GA3), indol-3-acetic acid (IAA), jasmonic acid (JA), epibrassinolide (BL), *trans*-zeatin (Z) and $N^6$-benzyladenine (BA) were all purchased from Sigma-Aldrich Co. (St. Louis, USA), while salicylic acid (SA) was from Fisher Scientific (Loughborough, UK). The deuterium-labeled internal standards $d_5$-indol-3-acetic acid ($d_5$-IAA), $d_5$-zeatin ($d_5$-Z), $d_7$-$N^6$-benzyladenine ($d_7$-BA) and $d_6$-abscisic acid ($d_6$-ABA) were obtained from OlChemIm (Olomouc, Czech Republic). For each standard and internal standard a stock solution was prepared in methanol at a concentration of 1 mg $mL^{-1}$. Reagents were of analytical grade when used for extraction purposes and of LC-MS grade for UHPLC-MS applications. They were respectively purchased from VWR International (Merck, Darmstadt, Germany) and Fisher Scientific (Loughborough, UK). Ultra-pure water (0.055 $\mu$S $cm^{-1}$) was obtained by usage of a purified-water system (VWR International, Merck, Darmstadt, Germany).

## 2.2 PLANT MATERIAL AND SAMPLE PREPARATION

Tomato plants (*Solanum lycopersicum* L. cv. Moneymaker) were grown in a 60 $m^2$ greenhouse compartment of the Institute for Agricultural and Fisheries Research (ILVO, Melle, Belgium) and were subjected to normal cultural practices (19). For the development and validation of the generic extraction procedure and the detection method, two different plant tissues were considered: tomato leaf and tomato fruit tissue. The collected tomato leaves were free from any damage and were immediately stored in liquid nitrogen to prevent enzymatic and thermal degradation of the phytohormones. Prior to the extraction, homogenization of the leaf material was realized by removal of the biggest veins and by grinding with liquid nitrogen, using pestle and mortar. The selected tomato fruits were almost fully-grown and were completely green, which coincided with the stage of fruit development whereby the concentration of phytohormones is most abundant for the majority of the hormonal classes (20). After harvest, fruits were immediately put into cool boxes and transported to the laboratory. Subsequently, the consecutive cutting, lyophilization, grinding and sieving of each harvested tomato fruit resulted in a homogenous powder, allowing representative sampling.

## 2.3 EXTRACTION AND CLEAN-UP

Although the generic extraction procedure was developed for the tomato leaf and tomato fruit tissue separately, the most optimal procedure was found to be almost identical for both tissue types. First, 100 mg of homogenized plant material was weighed in a 1.5 mL plastic recipient and 1 mL of cold extraction buffer (-20 °C) was added. This buffer consisted of methanol, ultra-pure water and formic acid (75:20:5, v/v/v) and was supplemented with the deuterium-labeled internal standards (2000 pg $\mu L^{-1}$ $d_5$-IAA, 100 pg $\mu L^{-1}$ $d_5$-Z, 100 pg $\mu L^{-1}$ $d_6$-ABA and 10 pg $\mu L^{-1}$ $d_7$-BA). Subsequently, the sample was vigorously vortexed until all plant material was homogenized into the extraction solvent, after which the sample was subjected to cold extraction (-25 °C) for 12h. After extraction, plant particles were separated by centrifugation (5 min at 14,000 rpm and 4 °C) and 500 µl of the supernatant was transferred to a 30 kDa Amicon® Ultra centrifugal filter unit (Merck Millipore Corporation, Massachusetts, USA), which was centrifuged for 10 min at 14,000 rpm and 4 °C. Then, the purified extract was reduced under vacuum at 35 °C by means of a Gyrovap centrifugal evaporator (Howe, Banbury, UK) to a final volume, which depended of the considered type of tomato tissue. The extract, obtained from tomato fruit tissue was reduced to half, while the extract from tomato leaf tissue was reduced to a fourth of the original volume. The obtained extract was transferred to an HPLC-vial and 10 µL was injected.

## 2.4 REVERSED-PHASE ULTRA-HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

The UHPLC system consisted of a Thermo Fisher Scientific (San José, USA) Accela UHPLC pumping system, coupled to an Accela Autosampler and Degasser. Chromatographic separation of the phytohormones was achieved on a Nucleodur Gravity $C_{18}$ column (1.8 µm, 50 mm x 2.1 mm ID) (Macherey-Nagel, Düren, Germany) and by implementation of a gradient elution program. This gradient was carried out with a binary solvent system consisting of 0.1% formic acid in ultra-pure water (A) and methanol (B) at a constant flow rate of 300 $\mu L\ min^{-1}$. A linear gradient profile with the following proportions (v/v) of solvent A was applied: 0 to 1 min at 98%, 1 to 2.50 min from 98 to 60%, 2.50 to 4 min from 60 to 50%, 4 to 5 min from 50 to 20%, 5 to 7 min at 20%, 7 to 7.10 min from 20 to 0%, 7.10 to 8 min at 0%, 8 to 8.01 min from 0 to 98%, followed by 2 min of re-equilibration. Furthermore, the divert valve was used for the first 1.60 min to avoid caramel formation due to a build-up of sugars in the heated capillary. Column oven temperature was 30 °C.

## 2.5 HESI-ORBITRAP MASS SPECTROMETRY

Mass spectrometric analysis was carried out using a Exactive™ benchtop Orbitrap mass spectrometer (Thermo Fisher Scientific, San José, USA), which was equipped with a heated electrospray ionization source (HESI), operating in switching polarity mode. Ionization source working parameters were optimized and are reported in Table 3.1.

**TABLE 3.1** Instrumental parameters used for HESI(II)-ionization of phytohormones.

| Instrumental parameter | Value |
|---|---|
| Spray voltage | 4 kV |
| Sheath gas flow rate | 50 au |
| Auxiliary gas flow rate | 5 au |
| Sweep gas flow rate | 2 au |
| Capillary temperature | 220 °C |
| Heater temperature | 330 °C |
| Capillary voltage | 65 (-62.5) V |
| Tube lens voltage | 75 (-100) V |
| Skimmer voltage | 24 (-27) V |

au: arbitrary units

An *m/z* scan range from 100 to 800 was chosen and the resolution was set at 100,000 full width half maximum (FWHM) at 1 Hz (1 scan per sec). The automatic gain control (AGC) target was set at high dynamic range ($3 \times e^6$) and maximum injection time was 20 ms. The option of "all ions fragmentation" using the High Energy Collision Dissociation (HCD) cell was turned off and the fore vacuum, high vacuum and ultra-high vacuum were maintained around 2 mbar, from $1 \times e^{-5}$ to $3 \times e^{-5}$ mbar and below $8 \times e^{-10}$ mbar, respectively. Initial instrument calibration was achieved by infusing calibration mixtures (Thermo Fisher Scientific, San José, USA) for the positive and negative ion modes. The positive calibration mixture included caffeine, Met-Arg-Phe-Ala acetate salt (MRFA) and Ultramark® 1621, while the negative calibration solution comprised sodium dodecyl sulfate, sodium taurocholate and Ultramark® 1621. These compounds were dissolved in a mixture of acetonitrile, water and methanol, and both mixtures were infused using a Chemyx Fusion 100 syringe pump (Thermo Fisher Scientific, San José, USA). Instrument control and data processing were carried out by Xcalibur 2.1 software (Thermo Fisher, Scientific, San José, USA).

## 2.5 QUALITY ASSURANCE

Prior to sample analysis, a standard mixture of the targeted phytohormones was injected to check the operational conditions of the UHPLC-MS device. Phytohormones were identified based on both their retention time relative to that of the internal standard and the accurate mass of each compound (Table 3.2). To this end, a mixture of the internal standards $d_5$-IAA, $d_5$-Z, $d_6$-ABA and $d_7$-BA, at respective concentrations of 2000 pg $\mu L^{-1}$, 100 pg $\mu L^{-1}$, 100 pg $\mu L^{-1}$ and 10 pg $\mu L^{-1}$, was added to every sample, prior to extraction.

**TABLE 3.2** Accurate masses of the various phytohormones with indication of the ionization modus, internal standard used, retention time (RT) and mass deviation.

| Phytohormone | Ionization modus | Internal standard used | RT (min) | Accurate mass (*m/z*) | Mass deviation (ppm) |
|---|---|---|---|---|---|
| Zeatin (Z) | + | $d_5$-Z | 2.89 | 220.11845 | -0.84 |
| Gibberellic acid (GA3) | - | $d_6$-ABA | 4.06 | 345.13461 | 1.35 |
| Benzyladenine (BA) | + | $d_7$-BA | 4.08 | 226.10781 | -0.91 |
| Indol-3-acetic acid (IAA) | + | $d_5$-IAA | 4.35 | 176.06966 | -0.95 |
| Salicylic acid (SA) | - | $d_6$-ABA | 4.91 | 137.02357 | 0.25 |
| Abscisic acid (ABA) | - | $d_6$-ABA | 5.17 | 263.12885 | 1.06 |
| Jasmonic acid (JA) | - | $d_6$-ABA | 5.69 | 209.11794 | 0.72 |
| Epibrassinolide (BL) | + | $d_6$-ABA | 6.86 | 481.35120 | -1.17 |

After identification, the concentrations of the detected phytohormones were calculated by fitting their area ratios into eight-point calibration curves, set up in matrix. For both the tomato leaf tissue and the tomato fruit tissue, a specific calibration curve was set up by fortifying the corresponding matrix with a mixture of standards (GA3, ABA, IAA, JA, SA, Z, BA and BL) and internal standards ($d_5$-IAA, $d_5$-Z, $d_6$-ABA and $d_7$-BA). The considered concentration ranges for each type of plant tissue were based on expected phytohormone concentration levels and are described in sections 3.4.3 and 3.5.3.

## 2.6 METHOD VALIDATION

Due to the absence of specific guidelines for the analysis of phytohormones in plant tissue, the Commission Directive 2002/657/EC (24) was used as a guideline for the validation of the developed extraction and detection method for GA3, ABA, IAA, JA, SA, Z, BA and BL. Appropriate deuterium-labelled internal standards were selected, capable of anticipating fluctuations in signal intensity

upon extraction of phytohormones from tomato plant tissue. $d_5$-IAA, $d_5$-Z, $d_6$-ABA and $d_7$-BA were used as internal standard for the corresponding phytohormones, while for the other compounds $d_6$-ABA was found to be satisfactory.

When tomato fruit tissue was considered as initial plant material, an extensive validation protocol was implemented. In the case of tomato leaf tissue, a more limited (i.e. with a reduced number of replicates) protocol was considered, mainly to demonstrate the applicability of the developed method towards different types of tissue.

# 3. RESULTS AND DISCUSSION

## 3.1 OPTIMIZATION OF SAMPLE PREPARATION

Sample preparation is a crucial step towards the unambiguous detection and accurate quantification of phytohormones in plant tissue (10). After all, in response to mechanical wounding, which includes the removal of tissue from the plant, various defense and healing mechanisms are activated through hormonal control (10,21). Therefore, to limit hormonal changes, the gathered plant material was kept cold at all times and low temperatures were handled during sample preparation. For each type of plant tissue, both lyophilization and grinding with liquid nitrogen, using pestle and mortar, were tested as possible sample preparation methods and were evaluated based on the absolute peak areas, repeatability and linearity. For tomato leaf material, both linearity and repeatability performed better when the extraction protocol used grounded leaf tissue ($R^2$ > 0.997 and a maximum coefficient of variance (CV) of 24.4%) instead of lyophilized tissue ($R^2$ > 0.988 and a maximum CV of 45.2%). In addition, since a higher proportion of the targeted phytohormones were detected and higher peak areas were obtained, grinding with liquid nitrogen proved to be the most appropriate sample preparation method for tomato leaf tissue. For tomato fruit material, lyophilization was selected as the most appropriate sample preparation method, mainly because of practicality. After all, the differences between both preparation methods for the outlined performance criteria were limited. Linearity and repeatability were further improved for both types of tomato plant tissue and corresponding sample preparation methods when the development of the extraction and clean-up procedure advanced.

## 3.2 OPTIMIZATION OF EXTRACTION AND CLEAN-UP

The first step within the development of the generic extraction protocol concerned the selection of an appropriate solvent for the efficient extraction of phytohormones from tomato plant tissue. To this end, the extraction buffers, used in the protocols of Gianarelli *et al.* (2010) (22) and Pan *et al.* (2008) (5), were compared in terms of extraction efficiency. The extraction buffer as described by Gianarelli *et al.* (2010) (22) demonstrated higher extraction efficiencies since a higher proportion of the phytohormones, which were added to the plant material prior to extraction, were detected in the extract and higher peak areas and signal-to-noise ratios were obtained. The enrichment of the prepared plant material with phytohormone (internal) standards (120 ng $g^{-1}$ dry weight for each standard compound; 700 ng $g^{-1}$ dry weight for each internal standard) was realized by adding limited amounts of the standard solutions, which were prepared in the respective extraction buffers. In conclusion, the development of the generic extraction protocol used a solution of methanol, ultra-pure water and formic acid (75:20:5, v/v/v) as extraction buffer. Secondly, various amounts of tomato plant tissue (100, 200, 300, 400 and 500 mg dry weight tomato fruit tissue or fresh weight leaf material) and volumes of extraction buffer (1, 2 and 3 mL) were considered to determine an optimal ratio. Based on the resulting peak areas, repeatability and signal-to-noise ratios, it was concluded that 100 mg of tissue and 1 mL of extraction buffer was the optimal combination for good repeatability and efficient extraction of the targeted phytohormones.

Subsequently, an appropriate purification strategy was selected for the removal of plant particles, lipids, pigments and other interferences (23). The removal of these interferences aimed to increase the signal-to-noise ratios and corresponding limits of detection and quantification. To this end, various purification strategies, including centrifugation, solid-phase extraction and filtration, were evaluated. After extraction of the tomato plant tissue, which was enriched with the phytohormone standards, the suggested purification strategies were implemented to the obtained extracts in triplicate. Purification by centrifugation was realized by an eppendorf centrifuge for 10 min at 14,000 rpm and 4 °C after which the supernatant was collected. Solid-phase extraction (SPE) was tested with Sep-Pak® (Waters, Munich, Germany), Isolute® $C_{18}$ (EC) (Biotage, Eke, Belgium) and Bond Elut® (Agilent Technologies, Santa Clara, USA) cartridges whereby the protocol of Hou *et al.* (2008) (2) was applied and solely purification was intended. In brief, cartridges were

preconditioned with 3 mL ultra-pure water and 3 mL methanol, washed with 1 mL 20% methanol (containing 0.1% FA), and eluted with 1 mL 80% methanol. Filtration was evaluated by usage of 0.45 μm Chromafil® (Macherey Nagel, Düren, Germany) and 0.22 μm Millex® (Merck Millipore Corporation, Massachusetts, USA) syringe driven filter devices and 10 and 30 kDa Amicon® Ultra centrifugal filter units. Based upon the signal-to-noise ratios and absolute peak areas of the targeted phytohormones, the most appropriate method for purification of the extract was selected (Figure 3.1 and 3.2; data for tomato fruit is not presented). To this end, purified extracts were also enriched by partial evaporation of the extraction solvent. After all, it can be assumed that the potential of a certain purification strategy with regard to the outlined evaluation criteria only fully manifests when the extract is enriched. Filtration by Amicon® Ultra centrifugal filter units (30 kDa) proved to be most appropriate and was executed by means of an eppendorf centrifuge for 10 min at 14,000 rpm and 4 °C.

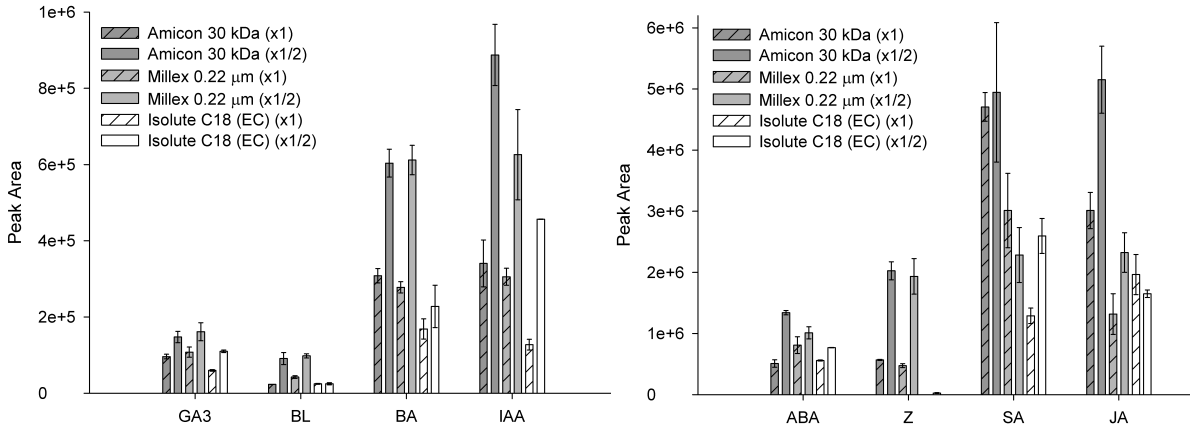


**FIGURE 3.1.** Relation between the considered purification strategy and the absolute peak areas of the targeted phytohormones, present in tomato leaf extract. For each type of purification strategy (i.e. syringe driven filtration, SPE and Amicon® ultra centrifugal filtration) the variant, which performed best for the outlined evaluation criteria, is presented. The variance is indicated by the standard deviation (n = 3) and fortified concentrations were 50 pg $\mu L^{-1}$ IAA, 5 pg $\mu L^{-1}$ GA3, 5 pg $\mu L^{-1}$ BL and 2 pg $\mu L^{-1}$ BA. The other phytohormones were naturally present in the considered tomato leaf material and were therefore not added. The degree of enrichment is expressed by means of the volume reduction and is indicated between brackets (x1 or x1/2).

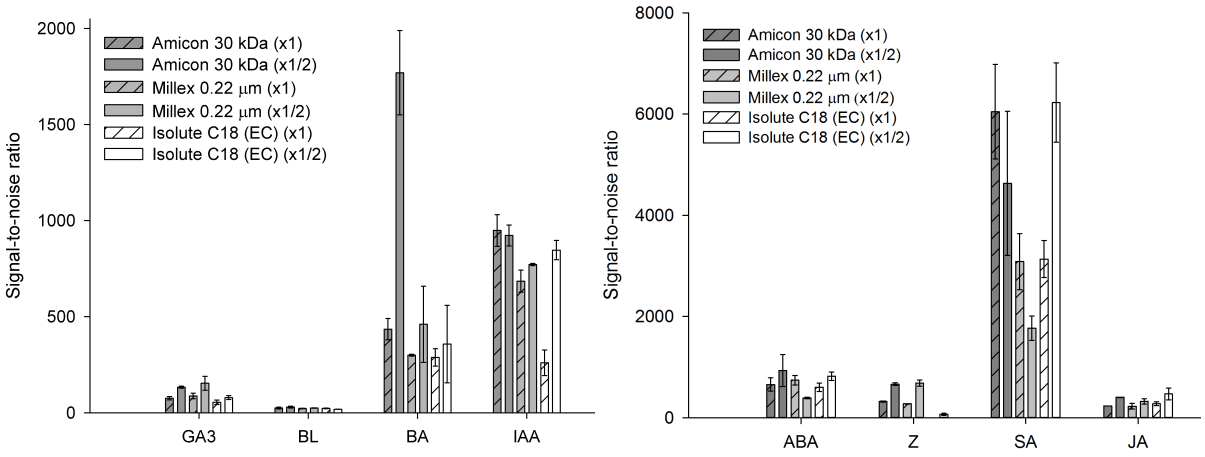


**FIGURE 3.2.** Relation between the considered purification strategy and the signal-to-noise ratios of the targeted phytohormones, present in tomato leaf extract. For each type of purification strategy (i.e. syringe driven filtration, SPE and Amicon® ultra centrifugal filtration) the variant, which performed best for the outlined evaluation criteria, is presented. The variance is indicated by the standard deviation (n = 3) and fortified concentrations were 50 pg $\mu L^{-1}$ IAA, 5 pg $\mu L^{-1}$ GA3, 5 pg $\mu L^{-1}$ BL and 2 pg $\mu L^{-1}$ BA. The other phytohormones were naturally present in the considered tomato leaf material and were therefore not added. The degree of enrichment is expressed by means of the volume reduction and is indicated between brackets (x1 or x1/2).

The final step within the development of the generic extraction protocol comprised the determination of the final extract volume by optimizing the enrichment factor. Reduction of the extract volume was achieved under vacuum by means of a Gyrovap centrifugal evaporator at 35 °C. Optimization of the enrichment factor was based on the signal-to-noise ratios and absolute peak areas and was found to be four for tomato leaf extract and two for tomato fruit extract. In order to clarify this, Figure 3.3 presents the relation between the outlined evaluation criteria and the considered extract volume reduction for fortified tomato leaf tissue (data for tomato fruit is not presented). It is demonstrated that a high enrichment factor (x4 or x6) generally resulted in an increase of the absolute peak areas, while the signal-to-noise ratios remained predominantly constant. For IAA, however, a declining peak area was observed when the evaporation continued, which is most likely due to thermal degradation. Since a metabolomic approach is pursued, the result of the evaporation process was evaluated across all phytohormonal classes. Therefore, the optimal enrichment factor for tomato leaf extract was concluded to be four. In general, higher peak areas were obtained while sensitivity, expressed as the signal-to-noise ratio, was maintained.

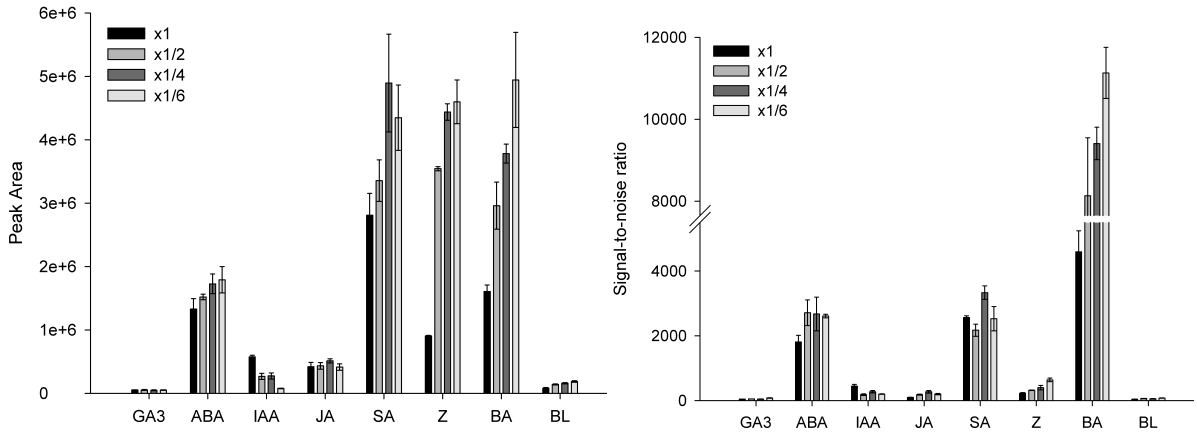


**FIGURE 3.3.** Relation between the considered volume reduction (x1, x1/2, x1/4 or x1/6) and the absolute peak areas and signal-to-noise ratios of the targeted phytohormones, present in tomato leaf extract. The variation is indicated by the standard deviation (n = 3). Fortified concentrations were 10 pg $\mu L^{-1}$ IAA, 5 pg $\mu L^{-1}$ GA3, 5 pg $\mu L^{-1}$ BL and 1 pg $\mu L^{-1}$ BA. Other phytohormones were naturally present and were therefore not added to the tomato leaf material.

## 3.3 UHPLC AND MS PARAMETERS

For the chromatographic separation of the targeted phytohormones, three different UHPLC-columns were tested, i.e. Nucleodur Pyramid $C_{18}$ (1.8 µm, 100 mm x 2.1 mm ID, Macherey-Nagel), Nucleodur Gravity C18 (1.8 µm, 50 mm x 2.1 mm ID, Macherey Nagel) and Acquity HSS $C_{18}$ (1.8 µm, 50 mm x 2.1 mm ID, Waters) column. Based on the baseline separation and the retention time of the first and last eluting analyte (and thus also total run time), the Nucleodur Gravity $C_{18}$ column was found to be the most appropriate column. Additional separation and optimal retention times were obtained by careful selection of the gradient program, which resulted in a total run time of 10 min. Since in other studies HPLC instead of UHPLC is used to accomplish chromatographic separation, longer run times can be expected in those studies. Indeed, the run time for HPLC-based analyses is frequently about 15 min (3,6,9,21), but even longer run times of ≥ 20 minutes have often been reported (4,5,11). Furthermore, since large amounts of sugar may be present in the tomato leaf and fruit extract, caramel formation could occur at the heated capillary of the HESI interface. Therefore, by means of an Alltech® 3300 Evaporative Light Scattering Diode (Grace, Illinois, USA) the retention times of the most abundant sugars, in particular sucrose, fructose and glucose, were determined by analysis of thirty tomato fruit extracts and it was deduced that the usage of the divert valve during the first 1.60 min was able to redirect the major part of the

present sugars to the waste instead of to the detector. Before determining the optimal MS conditions, phytohormone standards and deuterium-labelled standards were infused into the HESI. For each compound, the observed mass of the corresponding ion was compared to the theoretical mass, which was calculated by Xcalibur 2.1 software. The mass deviations, expressed in parts per million (ppm), were defined as $e^6$ x ((measured mass – theoretical mass) / theoretical mass) and found to be below 2 ppm (Table 3.2). In addition, the monoisotopic pattern (i.e. $^{13}C$ ion) was evaluated and confirmed as a second diagnostic ion for identification purposes. Indeed, calculated relative intensities (i.e. $^{13}C/^{12}C$ ion) were for each compound compliant with CD 2002/657/EC (24) requirements: for theoretical determined relative intensities of >20 to 50%, >10 to 20% and ≤10%, the maximum permitted tolerances are, respectively, ±25%, ±30% and ±50%. Unambiguous identification of the considered compounds was therefore based on the accurate masses of the $[M-H^+]^-$ or $[M+H^+]^+$ ions, the monoisotopic patterns, and the specific relative retention times. Instrumental MS parameters (Table 3.1) were optimized based on the peak intensities, peak areas and signal-to-noise ratios of the individual analytes. The most optimal AGC setting was found to be the high dynamic scan range (3 x $e^6$ ions) and was determined using the peak area, peak shape and signal-to-noise ratio for a spiked sample. The mean mass resolution was also determined by analysis of spiked tomato plant samples at 50,000 and 100,000 FWHM. Evaluation of the peak shape and area indicated that the best results were obtained with a mass resolution of 100,000 FWHM at 1 Hz. Additionally, the high resolution is able to strongly limit the contribution of false positives to the integrated peak area, detected for the compound of interest. On the other hand, the occurrence of false negatives is not expected to increase, unless a narrower mass window would be selected. However, because of the intended metabolomic approach a relatively broad mass window of 5 ppm was selected in order that no compounds will remain completely undetected (i.e. false negatives).

## 3.4 METHOD VALIDATION FOR TOMATO FRUIT TISSUE

### 3.4.1 SPECIFICITY

Since in the 'blank' samples, i.e. samples that were not enriched with phytohormone standards, many of the targeted phytohormones were present, an actual blank sample was not available. Average endogenous phytohormone concentrations (on fresh weight basis) were calculated on the basis of 18 'blank' samples and amounted 2.2 ng $g^{-1}$ GA3, 209.1 ng $g^{-1}$ ABA, 23.2 ng $g^{-1}$ IAA, 51.1 ng

$g^{-1}$ JA, 354.2 ng $g^{-1}$ SA, and 199.9 ng $g^{-1}$ Z. BA and BL were not detected in the used tomato fruit tissue. As a consequence, specificity (24) could not be evaluated for all targeted phytohormones by comparing the chromatograms from blank and enriched samples. For the phytohormones, which were present in the considered tomato fruits, specificity was exclusively based on the chromatograms from 18 'blank' samples. These chromatograms (Figure 3.4 A) indicated that no other matrix substances significantly interfered with these phytohormones since signal-to-noise ratios were at least 3. To evaluate the specificity of the other phytohormones, 18 tomato fruit samples were fortified with phytohormone standards to reach concentrations, expected to occur naturally in tomato fruits (15 pg $\mu L^{-1}$ GA3, 200 pg $\mu L^{-1}$ABA, 15 pg $\mu L^{-1}$ IAA, 20 pg $\mu L^{-1}$ JA, 200 pg $\mu L^{-1}$ SA, 150 pg $\mu L^{-1}$ Z, 10 pg $\mu L^{-1}$ BA and 10 pg $\mu L^{-1}$ BL). For each analyte spiked, the obtained chromatograms (Figure 3.4 B) displayed a significant increase in peak area intensity at the specific retention times of the considered compounds, taking a signal-to-noise ratio of at least 3 into account. In addition, no other matrix substances interfered at these retention times. As a result, the developed method proved to be specific for GA3, ABA, IAA, JA, SA, Z, BA and BL in the presence of matrix compounds.

### 3.4.2 SELECTIVITY

Analytes were identified on the basis of their relative retention time, which is the ratio of the retention time of the analyte to that of the corresponding internal standard. In addition, the accurate mass of the ions ($[M\text{-}H^+]^-$ or $[M+H^+]^+$) was taken into account when the chromatographic peak of interest had a signal-to-noise ratio of at least 3. A maximum mass deviation of 5 ppm was allowed within this study. Based on the outlined identification parameters, high selectivity (24) was guaranteed.

### 3.4.3 LINEARITY

The linearity of the developed method was evaluated by preparing eight-point calibration curves in matrix for the different phytohormones in triplicate. The 'blank' samples were fortified with concentrations, ranging from 2.5 to 125 pg $\mu L^{-1}$ for GA3, 2.5 to 150 pg $\mu L^{-1}$ for IAA, 5 to 150 pg $\mu L^{-1}$ for JA, 1.5 to 400 pg $\mu L^{-1}$ for Z, 1 to 75 pg $\mu L^{-1}$ for BA, 1 to 100 pg $\mu L^{-1}$ for BL and from 25 to 500 pg $\mu L^{-1}$ for both ABA and SA. Determination coefficients ($R^2$) obtained for these compounds were all > 0.99, which indicated good linearity within the considered concentration ranges.

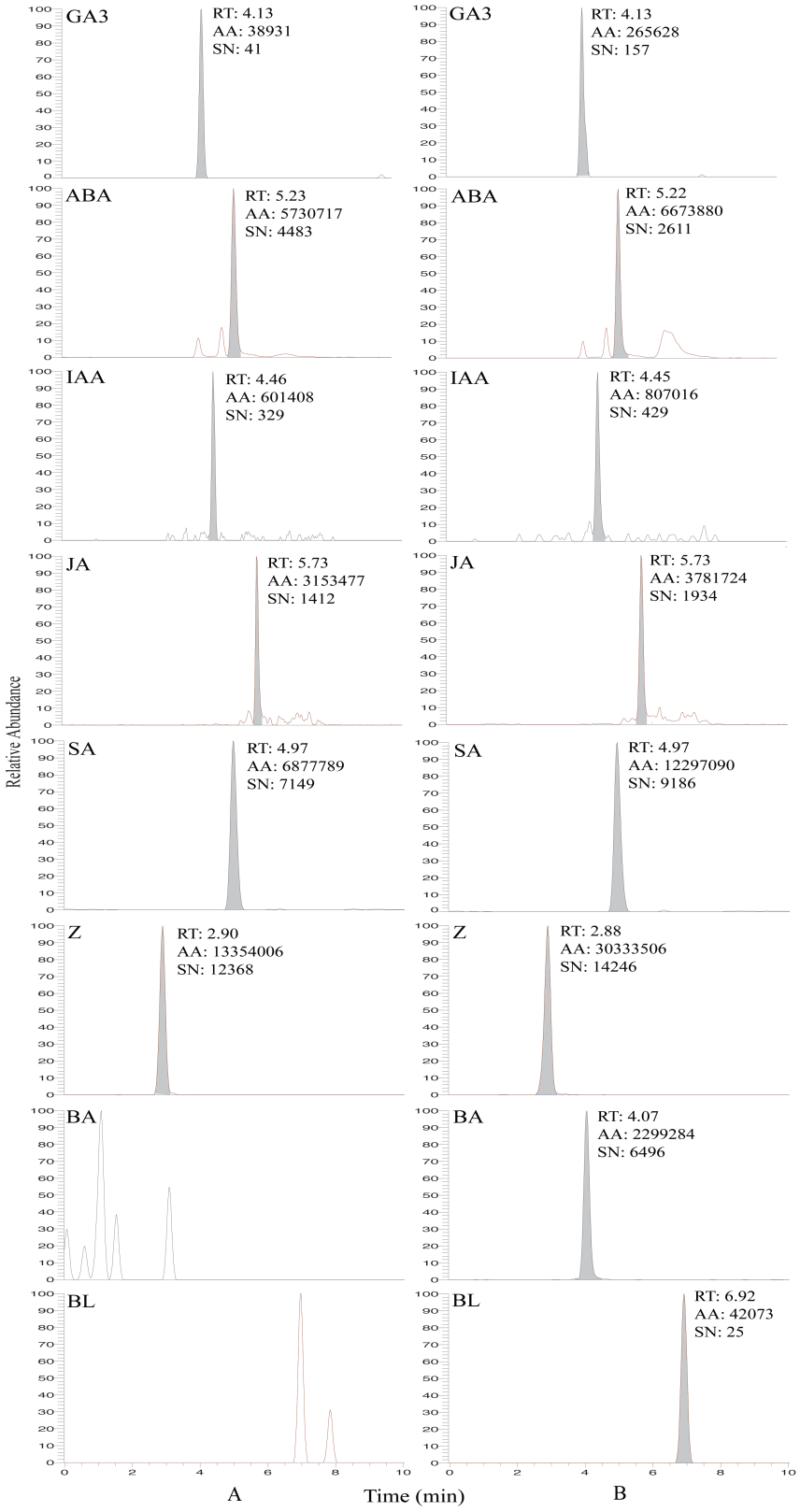


**FIGURE 3.4.** Chromatogram of a 'blank' tomato fruit sample, i.e. a sample that was not enriched with phytohormone standards (A), and chromatogram of a fortified (10 pg $\mu L^{-1}$ BA, 10 pg $\mu L^{-1}$ BL, 15 pg $\mu L^{-1}$ GA3, 15 pg $\mu L^{-1}$ IAA, 20 pg $\mu L^{-1}$ JA, 150 pg $\mu L^{-1}$ Z, 200 pg $\mu L^{-1}$ ABA and 200 pg $\mu L^{-1}$ SA) tomato fruit sample (B). The maximum mass deviation was 5 ppm.

### 3.4.4 PRECISION

To evaluate the precision of the developed method, repeatability and within-laboratory reproducibility were determined and evaluated by calculating the relative standard deviations (%RSD). To study the repeatability, three series of six replicates of samples were analysed, and this at three fortification levels. For the phytohormones, which were detected in the considered tomato fruit tissue (i.e. GA3, IAA, ABA, JA, SA and Z), the fortification levels were based on the determined endogenous concentrations. For BA and BL, which were not detected in the tomato fruit material, fortification levels were based on endogenous concentrations, frequently reported in literature to occur in plant material (11,25). The three fortification levels (nominal concentrations) for each of the targeted phytohormones are reported in Table 3.3 and were ½ time, 1 time and 2 times the considered endogenous concentration. Analyses were carried out by the same analyst under repeatable conditions. To evaluate the repeatability for the three fortification levels as such, additional calculations were made for GA3, IAA, ABA, JA, SA and Z, whereby for each sample the endogenous concentration (described under section 3.4.1) was subtracted from the calculated total concentration. RSD values are presented in Table 3.3 and were evaluated by comparison with criteria, calculated accordingly CD 2002/657/EC (24). Only for IAA, slightly worse repeatability was observed.

The within-laboratory reproducibility was evaluated with four series of six replicates of samples, and this again at three fortification levels, which were determined as previously described. These series were analysed on different days by different operators. Calculated RSD values (Table 3.3) are presented in Table 3.3 and indicate excellent within-laboratory reproducibility for the targeted phytohormones, accordingly CD 2002/657/EC (24).

### 3.4.5 LIMIT OF DETECTION AND LIMIT OF QUANTIFICATION

For the phytohormones, which were detected in the tomato fruits that were used for validation, the limits of detection (LOD, S/N ≥ 3) and quantification (LOQ, S/N ≥ 10) were theoretically calculated based on eight-point calibration curves, relating concentrations with signal-to-noise ratios. For BA and BL, the phytohormones that were not detected in the 'blank' samples, the LOD and LOQ were determined experimentally by enriching the tomato fruit tissue with concentrations near the theoretically calculated LOD and LOQ. The values for these performance characteristics

are reported in Table 3.3. In comparison with other studies, the developed method resulted in comparable (3,4,21,26) or better (6,11,22,27) LOD and LOQ values. Only in the study of Pan *et al*. (2008) (5) values outperformed the results, achieved in this study.

### 3.4.6 MEAN CORRECTED RECOVERY

Since no certified reference material was available, trueness was determined as the mean corrected recovery by using fortified tomato fruit samples. To this end, three fortification levels were considered with six replicates for each level. As previously described, fortification levels were based on the endogenous concentrations of the phytohormones in tomato fruit tissue. For each sample, calculated concentrations were adjusted with the determined endogenous concentration levels where necessary. Mean corrected recoveries are reported in Table 3.3 and were considered to be satisfactory according to CD 2002/657/EC (24).

**TABLE 3.3.** Limits of detection (LOD) and quantification (LOQ), mean corrected recovery and precision, determined for the eight targeted phytohormones in tomato fruit tissue. LOD and LOQ are expressed on a dry weight basis.

| Analyte | LOD (ng $g^{-1}$) | LOQ (ng $g^{-1}$) | Nominal concentration (pg $\mu L^{-1}$) | Recovery Mean ± SD (%) | Repeatability RSD (%) | Within-laboratory reproducibility RSD (%) |
|---|---|---|---|---|---|---|
| GA3 | 1.0 | 3.3 | 7.5 | 95.8 ± 5.5 | 9.9 | 12.6 |
| | | | 15 | 86.8 ± 5.7 | 7.2 | 7.9 |
| | | | 30 | 94.7 ± 3.4 | 4.4 | 8.4 |
| ABA | 2.5 | 8.3 | 100 | 86.4 ± 3.8 | 5.2 | 9.4 |
| | | | 200 | 97.5 ± 6.9 | 7.4 | 8.3 |
| | | | 400 | 96.8 ± 3.0 | 3.4 | 3.6 |
| IAA | 2.2 | 7.3 | 7.5 | 99.0 ± 29.0 | 22.2 | 22.2 |
| | | | 15 | 99.0 ± 14.6 | 17.0 | 19.6 |
| | | | 30 | 99.5 ± 6.7 | 12.6 | 16.9 |
| JA | 0.5 | 1.7 | 10 | 98.6 ± 6.0 | 7.1 | 7.3 |
| | | | 20 | 95.7 ± 2.4 | 5.9 | 9.9 |
| | | | 40 | 99.6 ± 5.2 | 7.2 | 7.2 |
| SA | 7.5 | 25.0 | 100 | 90.9 ± 5.2 | 6.9 | 7.5 |
| | | | 200 | 92.5 ± 7.0 | 7.4 | 8.2 |
| | | | 400 | 92.7 ± 1.4 | 4.8 | 5.0 |
| Z | 4.2 | 13.99 | 75 | 99.5 ± 9.4 | 9.7 | 12.6 |
| | | | 150 | 95.7 ± 2.5 | 8.3 | 8.3 |
| | | | 300 | 107.9 ± 2.9 | 6.7 | 8.4 |
| BL | 1.0 | 3.33 | 5 | 88.7 ± 5.6 | 9.9 | 9.9 |
| | | | 10 | 79.1 ± 3.8 | 7.2 | 7.2 |
| | | | 20 | 67.6 ± 3.2 | 4.4 | 5.6 |
| BA | 1.0 | 3.33 | 5 | 98.2 ± 6.0 | 6.4 | 6.5 |
| | | | 10 | 97.6 ± 5.5 | 5.7 | 7.0 |
| | | | 20 | 101.5 ± 4.9 | 6.4 | 6.7 |

## 3.5 METHOD VALIDATION FOR TOMATO LEAF TISSUE

### 3.5.1 SPECIFICITY

For the evaluation of the specificity only six 'blank' and six fortified tomato leaf samples were considered and fortification levels were adjusted to the considered matrix (i.e. tomato leaf tissue) and the corresponding, expected endogenous concentrations (10 pg $\mu L^{-1}$ for GA3, ABA, IAA, JA and BA, 20 pg $\mu L^{-1}$ for BL, 100 pg $\mu L^{-1}$ SA and 125 pg $\mu L^{-1}$ Z). The developed method was specific for the considered phytohormones in the presence of matrix compounds (Figure 3.5 A and B).

### 3.5.2 SELECTIVITY

High selectivity was guaranteed by the high mass accuracy (with a maximum mass deviation of 5 ppm) and the identification of compounds based on their relative retention times.

### 3.5.3 LINEARITY

The linearity of the developed method was evaluated by preparing an eight-point calibration curve for each compound in tomato leaf matrix. Considered ranges were as follows: from 2.5 to 150 pg $\mu L^{-1}$ for JA, ABA, BL and IAA, from 2.5 to 100 pg $\mu L^{-1}$ for GA3, from 15 to 250 pg $\mu L^{-1}$ for SA, from 15 to 350 pg $\mu L^{-1}$ for Z and from 1 to 150 pg $\mu L^{-1}$ for BA. Linearity performed well since correlation coefficients ($R^2$) were all > 0.99.

### 3.5.4 PRECISION

To evaluate precision both the repeatability and within-laboratory reproducibility were determined by calculating RSD values. For the repeatability three fortification levels were considered, with 6 replicates for each level (Table 3.4). Adjustments with the endogenous levels (96.2 ng $g^{-1}$ ABA, 99.6 ng $g^{-1}$ JA, 356.9 ng $g^{-1}$ SA and 268.1 ng $g^{-1}$ Z, on fresh weight basis) were made where necessary and repeatability was found to perform well for all phytohormones accordingly CD 2002/657/EC (24) (Table 3.4). Within-laboratory reproducibility was evaluated on the basis of two times six replicates, and this for three fortification levels, which were again based on the endogenous concentrations of the considered phytohormones in tomato leaf tissue. This validation parameter performed well since all RSD values were below the criteria, which were calculated according to CD 2002/657/EC (24) (Table 3.4).

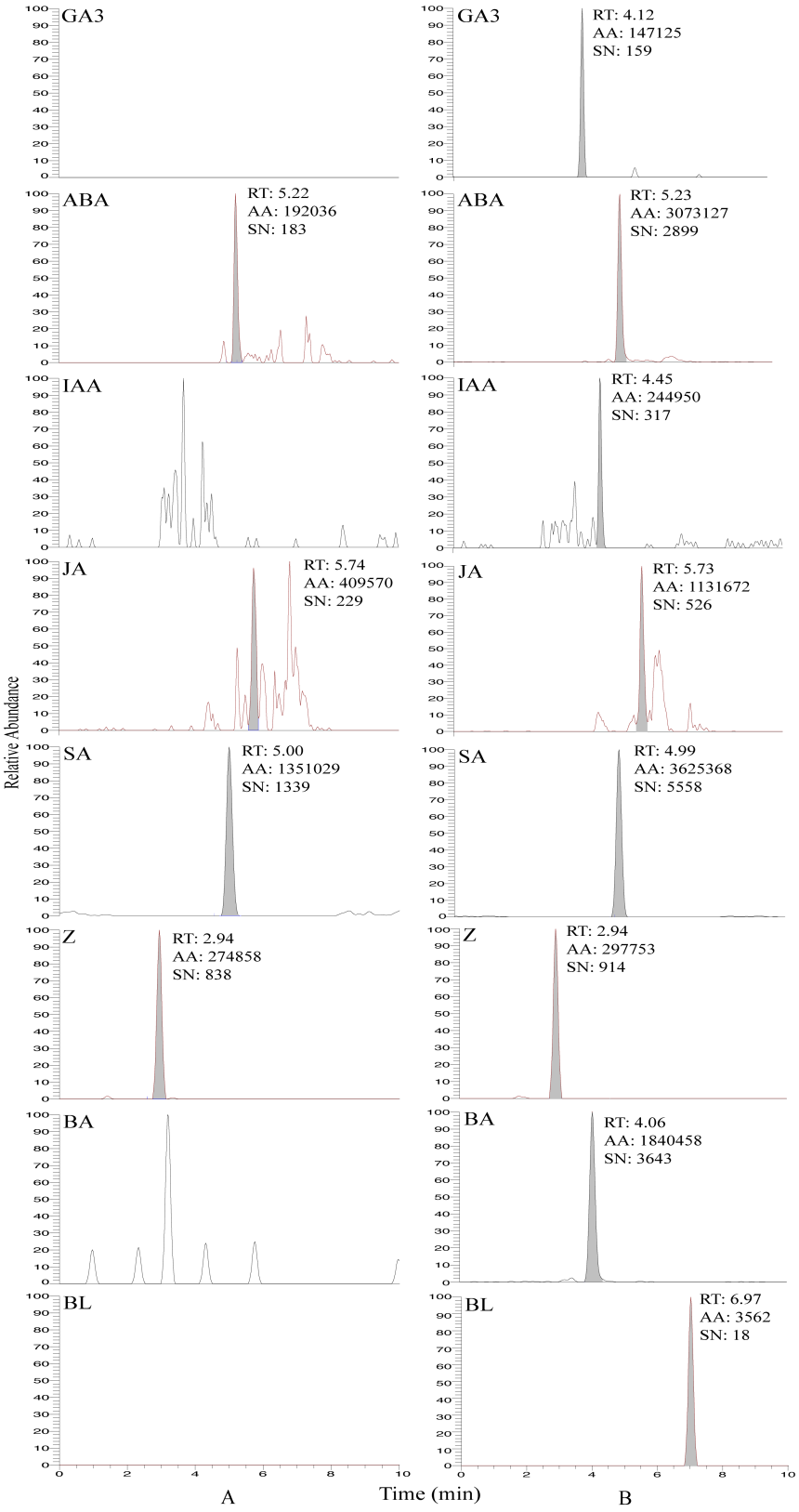


**FIGURE 3.5.** Chromatogram of a 'blank' tomato leaf sample, i.e. a sample that was not enriched with phytohormone standards (A), and chromatogram of a fortified (10 pg $\mu L^{-1}$ BA, 10 pg $\mu L^{-1}$ IAA, 10 pg $\mu L^{-1}$ GA3, 10 pg $\mu L^{-1}$ JA, 10 pg $\mu L^{-1}$ ABA, 20 pg $\mu L^{-1}$ BL, 100 pg $\mu L^{-1}$ SA, 125 pg $\mu L^{-1}$ Z) tomato leaf sample (B). The maximum mass deviation was 5 ppm.

### 3.5.5 LIMIT OF DETECTION AND LIMIT OF QUANTIFICATION

For the phytohormones, which were detected in the tomato leaf material that was used for validation, the limits of detection (LOD, S/N ≥ 3) and quantification (LOQ, S/N ≥ 10) were theoretically calculated based on eight-point calibration curves, whereby concentrations were related with signal-to-noise ratios. For the other phytohormones (i.e. GA3, IAA, BA, BL), which could not be detected in the 'blank' samples, the LOD and LOQ were determined experimentally by enriching the tomato leaf tissue with concentrations near the theoretically calculated LOD and LOQ. Values for these performance characteristics are reported in Table 3.4.

**TABLE 3.4** Limits of detection (LOD) and quantification (LOQ), mean corrected recovery and precision, determined for the eight targeted phytohormones in tomato leaf tissue. LOD and LOQ are expressed on a fresh weight basis.

| Analyte | LOD (ng $g^{-1}$) | LOQ (ng $g^{-1}$) | Nominal concentration (pg $\mu L^{-1}$) | Recovery Mean ± SD (%) | Repeatability RSD (%) | Within-laboratory reproducibility RSD (%) |
|---|---|---|---|---|---|---|
| GA3 | 10.0 | 33.3 | 5 | 81.1 ± 7.8 | 9.3 | 9.3 |
| | | | 10 | 81.7 ± 5.4 | 6.6 | 6.6 |
| | | | 20 | 80.3 ± 6.4 | 7.9 | 7.9 |
| ABA | 1.5 | 5.00 | 5 | 87.7 ± 7.5 | 7.8 | 7.8 |
| | | | 10 | 84.4 ± 6.1 | 6.9 | 6.9 |
| | | | 20 | 105.6 ± 4.6 | 4.5 | 8.7 |
| IAA | 10.0 | 33.3 | 5 | 92.1 ± 3.8 | 4.1 | 5.1 |
| | | | 10 | 87.7 ± 6.8 | 7.8 | 9.9 |
| | | | 20 | 99.6 ± 7.5 | 7.6 | 7.6 |
| JA | 1.1 | 3.7 | 5 | 97.8 ± 10.2 | 10.5 | 10.5 |
| | | | 10 | 104.2 ± 10.4 | 9.9 | 11.5 |
| | | | 20 | 109.3 ± 9.1 | 8.7 | 8.7 |
| SA | 0.9 | 3.0 | 50 | 105.2 ± 14.7 | 11.6 | 14.9 |
| | | | 100 | 101.4 ± 13.7 | 13.6 | 14.8 |
| | | | 200 | 94.1 ± 7.8 | 8.4 | 8.4 |
| Z | 1.6 | 5.3 | 62.5 | 89.2 ± 6.0 | 5.3 | 7.9 |
| | | | 125 | 90.1 ± 8.9 | 10.2 | 10.2 |
| | | | 250 | 102.5 ± 7.6 | 10.7 | 10.7 |
| BL | 2.5 | 8.3 | 10 | 81.1 ± 6.6 | 8.2 | 8.2 |
| | | | 20 | 81.5 ± 5.9 | 7.3 | 8.8 |
| | | | 40 | 89.8 ± 11.5 | 9.6 | 13.7 |
| BA | 1.0 | 3.3 | 5 | 99.4 ± 0.9 | 0.9 | 1.3 |
| | | | 10 | 99.1 ± 2.1 | 2.1 | 2.5 |
| | | | 20 | 102.7 ± 1.6 | 1.6 | 1.6 |

### 3.5.6 MEAN CORRECTED RECOVERY

Since no certified reference material was available for this type of plant tissue, trueness was determined as the mean corrected recovery using fortified tomato leaf samples. For this purpose, three fortification levels were considered with six replicates for each level. Mean corrected recoveries are reported in Table 3.4 and were considered to be satisfactory according to CD 2002/657/EC (24).

## 3.6 METABOLOMIC PROFILING OF THE ENDOGENOUS HORMONAL STATUS OF THE TOMATO PLANT

Although the analytical method was developed and validated by focussing on eight phytohormones only, a metabolomic profiling of the endogenous hormonal status of the tomato plant was premised. An efficient screening of the full scan data for compounds, relevant to the hormonal concept, was executed by using the software program ToxID 2.1.2 (Thermo Fisher Scientific, San José, USA). For this purpose, a database was constructed by implementing the molecular formula of 275 relevant compounds (found in the ChemIm Ltd Catalogue 2008-2010). Since no information about the retention time of these compounds is available, identification was mainly based on the elemental composition and related molecular weight (i.e. $[M+H^+]^+$ and $[M-H^+]^-$ ions). However, additional confirmation by the presence of a second diagnostic ion, including the isotopic ions ($^{13}C$, $^{37}Cl$, $^{34}S$ or $^{78}Br$), may assist in the identification of compounds. An isotopic ion ($^{13}C$, $^{37}Cl$, $^{34}S$ or $^{78}Br$) was only found suitable as a diagnostic ion when the corresponding $[M+H]^+$ or $[M-H]^-$ ion was also detected and the calculated relative ion intensities met the CD 2002/657/EC (24) requirements: for theoretical determined relative intensities of >20 to 50%, >10 to 20% and ≤10%, the maximum permitted tolerances were, respectively, ±25%, ±30% and ±50%. Additionally, the retention time of a detected compound could also provide information about the identity (both polarity and structure) of that compound when evaluated relative towards the retention times of the eight reference phytohormones, considered in this study and representing the major hormonal classes. With the consideration of these additional identification parameters, the identity of a compound can be determined with a high degree of certainty. However, an unequivocal identification cannot always be guaranteed. Taking these findings into account, the metabolomic approach enables a first screening for relevant compounds and is indicative for the presence or absence of certain compounds, characterized by a certain molecular weight and elemental composition.

Optionally, characteristic fragments or retention time (based on standards) could be considered to verify the compound's identity if there would still be any doubt. Main ToxID settings concerned a minimum peak intensity of 1000 and a maximum mass deviation of 5 ppm. The application of ToxID was able to demonstrate the possible presence of 78 and 63 relevant compounds in the extract of a random tomato leaf and a green tomato fruit, respectively (Table 3.5 and 3.6). As a consequence, the developed analytical method proved its potential in the metabolomic profiling of the endogenous hormonal status of (tomato) plants.

# 4. CONCLUSIONS

Within this research a generic extraction procedure and detection method were successfully developed and validated for eight phytohormones (GA3, ABA, IAA, JA, SA, Z, BA and BL) in both tomato fruit and leaf tissue. As a consequence, since the method could easily be applied to different types of plant tissue, the capability of the considered detection method to counteract background interferences, caused by a wide range of matrix compounds, was demonstrated. Although the analytical method was successfully developed and validated for eight phytohormones, representing the major hormonal classes, the full scan approach of the Orbitrap-MS resulted in data that includes information about hundreds of compounds. The obtained full scan data can be screened for relevant compounds in various ways, depending on the objectives of the concerned study. Firstly, with consideration of a defined number of known compounds, a rather targeted screening of the data is possible, which can be efficiently be realized by appropriate software programs such as ToxID. Therefore, in this study, a database comprising 275 relevant compounds was established. Secondly, an actual metabolomic profiling is also possible and can lead to the discovery of compounds that were previously not known to possess any hormonal activity or to fulfil a role in the hormonal regulation of certain plant processes. In conclusion, the developed methods are considered to be most valuable for the further elucidation of hormone-signalling pathways in the regulation of several vital processes in plants.

**TABLE 3.5** Phytohormones and related compounds, detected in the extract of a tomato leaf. The results were obtained by usage of the software program ToxID, at which a minimum peak intensity of 1000 and a maximum mass deviation of 5 ppm were taken into account. For correct identification of a compound, at least two diagnostic ions had to be present. An isotopic ion ($^{13}C$, $^{37}Cl$, $^{34}S$ or $^{78}Br$) was only suited as diagnostic ion when the corresponding $[M+H^+]^+$ or $[M-H^+]^-$ ion was also found and the calculated relative intensity (ion ratio) met the CD 2002/657/EC requirements.

| Compound name | Elemental composition | RT (min) | Positive ionization modus | | | Negative ionization modus | | |
|---|---|---|---|---|---|---|---|---|
| | | | Peak intensity $[M+H]^+$ | Peak intensity $[^{13}C$ isotope + $H]^+$ | Ion ratio (%) | Peak intensity $[M-H]^-$ | Peak intensity $[^{13}C$ isotope - $H]^-$ | Ion ratio (%) |
| Adenosine | $C_{10}H_{13}N_5O_4$ | 3.19 | 1.63 $e^4$ | 1.54 $e^3$ | 9.44 | | | |
| $N^6$-benzyladenine | $C_{12}H_{11}N_5$ | 3.45 | 2.64 $e^4$ | | | 2.97 $e^4$ | | |
| $N^6$-benzyladenine-3-glucoside | $C_{18}H_{21}N_5O_5$ | 3.08 | | | | 5.68 $e^4$ | 8.94 $e^3$ | 15.74 |
| $N^6$-benzyladenine-7-glucoside | $C_{18}H_{21}N_5O_5$ | 3.08 | | | | 5.68 $e^4$ | 8.94 $e^3$ | 15.74 |
| $N^6$-benzyladenine-9-glucoside | $C_{18}H_{21}N_5O_5$ | 3.08 | | | | 5.68 $e^4$ | 8.94 $e^3$ | 15.74 |
| N-(2-chloro-4-pyridyl-)-N'-phenylurea | $C_{10}H_{12}ClN_3O$ | 2.80 | 1.05 $e^3$ | | | 2.01 $e^2$ | | |
| $N^6$-cyclopentyladenosine-5'-monophosphate sodium salt | $C_{15}H_{20}N_5Na_2O_7PH_2O$ | 2.99 | 1.50 $e^4$ | | | 2.27 $e^3$ | | |
| Dihydrozeatin-O-glucoside riboside | $C_{21}H_{33}N_5O_{10}$ | 3.15 | 1.45 $e^4$ | | | 9.63 $e^3$ | 2.33 $e^3$ | 24.20 |
| $N^6$-isopentenyladenine | $C_{10}H_{13}N_5$ | 4.38 | 7.29 $e^3$ | | | 2.53 $e^4$ | | |
| 2-Methylthio-$N^6$-isopentenyladenosine | $C_{16}H_{23}N_5O_4S$ | 3.67 | 4.33 $e^3$ | 6.62 $e^2$ | 15.29 | | | |
| $N^6$-isopentenyladenine-3-glucuronide amide | $C_{16}H_{22}N_6O_5$ | 4.52 | 5.28 $e^3$ | | | 5.40 $e^2$ | | |
| $N^6$-isopentenyladenine-7-glucoside | $C_{16}H_{23}N_5O_5$ | 3.40 | 1.05 $e^5$ | 1.35 $e^4$ | 12.86 | | | |
| $N^6$-isopentenyladenine-9-glucoside | $C_{16}H_{23}N_5O_5$ | 3.40 | 1.05 $e^5$ | 1.35 $e^4$ | 12.86 | | | |
| $N^6$-isopentenyladenosine-5'-monophosphate sodium salt | $C_{15}H_{20}N_5Na_2O_7PH_2O$ | 2.99 | 1.50 $e^4$ | | | 2.27 $e^3$ | | |
| Meta-topolin riboside | $C_{17}H_{19}N_5O_5$ | 4.18 | 1.32 $e^5$ | 2.13 $e^4$ | 16.14 | | | |
| Ortho-topolin riboside | $C_{17}H_{19}N_5O_5$ | 4.18 | 1.32 $e^5$ | 2.13 $e^4$ | 16.14 | | | |
| Para-topolin riboside | $C_{17}H_{19}N_5O_5$ | 4.18 | 1.32 $e^5$ | 2.13 $e^4$ | 16.14 | | | |
| Ortho-methoxytopolin riboside | $C_{18}H_{21}N_5O_5$ | 6.22 | | | | 1.97 $e^5$ | 3.14 $e^4$ | 15.94 |
| Trans-zeatin | $C_{10}H_{13}N_5O$ | 2.93 | | | | 1.18 $e^5$ | 1.08 $e^4$ | 9.15 |
| Cis-zeatin | $C_{10}H_{13}N_5O$ | 2.93 | | | | 1.18 $e^5$ | 1.08 $e^4$ | 9.15 |
| Trans-zeatin riboside | $C_{15}H_{21}N_5O_5$ | 2.73 | | | | 8.67 $e^4$ | 1.30 $e^4$ | 14.99 |
| Zeatin mixed isomers (E/Z) | $C_{10}H_{13}N_5O$ | 2.93 | | | | 1.18 $e^5$ | 1.08 $e^4$ | 9.15 |
| Zeatin riboside mixed isomers (E/Z) | $C_{15}H_{21}N_5O_5$ | 2.73 | | | | 8.67 $e^4$ | 1.30 $e^4$ | 14.99 |
| Trans-zeatin-7-glucoside | $C_{16}H_{23}N_5O_6$ | 3.10 | | | | 1.91 $e^5$ | 2.85 $e^4$ | 14.92 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Trans-zeatin-9-glucoside | $C_{16}H_{23}N_5O_6$ | 3.10 | | | | 1.91 $e^5$ | 2.85 $e^4$ | 14.92 |
| Trans-zeatin-9-glucuronide | $C_{16}H_{21}N_5O_7$ | 3.99 | | | | 1.28 $e^4$ | 2.26 $e^3$ | 17.66 |
| Trans-zeatin-O-glucoside | $C_{16}H_{23}N_5O_6$ | 3.10 | | | | 1.91 $e^5$ | 2.85 $e^4$ | 14.92 |
| Trans-zeatin-O-glucuronide | $C_{16}H_{21}N_5O_7$ | 3.99 | | | | 1.28 $e^4$ | 2.26 $e^3$ | 17.66 |
| Trans-zeatin-O-glucoside riboside | $C_{21}H_{23}N_5O_6$ | 3.10 | | | | 1.91 $e^5$ | 2.85 $e^4$ | 14.92 |
| Cis-zeatin riboside | $C_{15}H_{21}N_5O_5$ | 2.96 | 8.64 $e^4$ | | | 1.79 $e^5$ | | |
| Cis-zeatin-9-glucoside | $C_{16}H_{23}N_5O_6$ | 3.10 | | | | 1.91 $e^5$ | 2.85 $e^4$ | 14.92 |
| Cis-zeatin-O-glucoside | $C_{16}H_{23}N_5O_6$ | 3.10 | | | | 1.91 $e^5$ | 2.85 $e^4$ | 14.92 |
| 1-Acetylindole-3-carboxaldehyde | $C_{11}H_9NO_2$ | 2.89 | 8.06 $e^6$ | 9.61 $e^5$ | 11.92 | 4.89 $e^3$ | | |
| 5-Benzyloxyindol-3-acetic acid | $C_{10}H_9NO$ | 4.06 | 8.43 $e^5$ | | | 2.82 $e^5$ | | |
| 4-Hydroxyindole | $C_8H_7NO$ | 4.05 | | | | 1.80 $e^4$ | 1.24 $e^3$ | 6.89 |
| 5-Hydroxyindole | $C_8H_7NO$ | 4.05 | | | | 1.80 $e^4$ | 1.24 $e^3$ | 6.89 |
| 5-Hydroxyindole-2-carboxylic acid | $C_9H_7NO_3$ | 3.16 | 1.04 $e^4$ | 9.21 $e^3$ | 8.86 | | | |
| 5-Hydroxyindole-2-carboxylic acid ethyl ester | $C_{11}H_{11}NO_3$ | 4.16 | 1.50 $e^5$ | 1.33 $e^4$ | 8.87 | | | |
| Indole | $C_8H_7N$ | 4.05 | | | | 1.80 $e^4$ | 1.24 $e^3$ | 6.89 |
| Indole-3-acetone | $C_{11}H_{11}NO$ | 5.68 | 2.32 $e^5$ | 2.38 $e^4$ | 10.26 | | | |
| Indole-3-acetyl-L-alanine | $C_{13}H_{14}N_2O_3$ | 4.22 | | | | 1.91 $e^6$ | 2.47 $e^5$ | 12.93 |
| Indole-3-acetyl-L-alanine methyl ester | $C_{14}H_{16}N_2O_3$ | 7.17 | 7.02 $e^4$ | 1.24 $e^4$ | 17.66 | | | |
| Indole-3-acetyl-L-aspartic acid | $C_{14}H_{14}N_2O_5$ | 4.22 | 5.60 $e^5$ | 7.02 $e^4$ | 12.54 | 3.10 $e^4$ | 3.78 $e^3$ | 12.19 |
| Indole-3-acetyl-L-glutamic acid dimethyl ester | $C_{17}H_{20}N_2O_5$ | 6.47 | | | | 3.04 $e^4$ | 5.19 $e^3$ | 17.07 |
| Indole-3-acetyl-L-isoleucine | $C_{16}H_{20}N_2O_3$ | 7.29 | 1.26 $e^7$ | 1.70 $e^6$ | 13.49 | | | |
| Indole-3-acrylic acid | $C_{11}H_9NO_2$ | 2.89 | 1.11 $e^7$ | 8.89 $e^5$ | 8.01 | 8.81 $e^3$ | | |
| Indole-3-butyric acid methyl ester | $C_{13}H_{15}NO_2$ | 3.08 | 1.78 $e^4$ | 1.95 $e^3$ | 10.96 | | | |
| Indole-2-carboxylic acid | $C_9H_7NO_2$ | 4.04 | 4.45 $e^4$ | | | 1.67 $e^3$ | | |
| Indole-3-carboxylic acid | $C_9H_7NO_2$ | 4.04 | 4.45 $e^4$ | | | 1.67 $e^3$ | | |
| DL-indole-3-lactic acid | $C_{11}H_{11}NO_3$ | 4.16 | 1.50 $e^5$ | 1.33 $e^4$ | 8.87 | | | |
| DL-indole-3-lactic acid methyl ester | $C_{12}H_{13}NO_3$ | 3.67 | 8.44 $e^4$ | 1.02 $e^4$ | 12.09 | | | |
| 5-Methoxyindole-3-acetic acid | $C_{11}H_{11}NO_3$ | 4.16 | 8.89 $e^4$ | 8.86 $e^3$ | 9.97 | | | |
| 5-Methoxyindole-2-carboxylic acid ethyl ester | $C_{12}H_{13}NO_3$ | 3.67 | 6.53 $e^4$ | 5.87 $e^3$ | 8.99 | | | |
| 5-Methoxy-2-methylindole-3-acetic acid | $C_{12}H_{13}NO_3$ | 3.67 | 6.53 $e^4$ | 5.87 $e^3$ | 8.99 | | | |
| Gibberellin A12 aldehyde | $C_{20}H_{28}O_3$ | 6.56 | 3.00 $e^5$ | 6.31 $e^4$ | | | | |
| Abscisic acid | $C_{15}H_{20}O_4$ | 5.26 | | | | 4.75 $e^4$ | 6.56 $e^3$ | 13.81 |
| R-abscisic acid | $C_{15}H_{20}O_4$ | 5.26 | | | | 4.75 $e^4$ | 6.56 $e^3$ | 13.81 |
| S-abscisic acid | $C_{15}H_{20}O_4$ | 5.26 | | | | 4.75 $e^4$ | 6.56 $e^3$ | 13.81 |
| Trans trans-abscisic acid | $C_{15}H_{20}O_4$ | 5.26 | | | | 4.75 $e^4$ | 6.56 $e^3$ | 13.81 |
| Trans trans-abscisic acid methyl ester | $C_{16}H_{22}O_4$ | 7.36 | 5.03 $e^6$ | 8.45 $e^5$ | 16.80 | | | |
| S-trans trans-abscisic acid | $C_{15}H_{20}O_4$ | 5.26 | | | | 4.75 $e^4$ | 6.56 $e^3$ | 13.81 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| S-trans trans-abscisic acid methyl ester | $C_{16}H_{22}O_4$ | 7.36 | 5.03 $e^6$ | 8.45 $e^5$ | 16.80 | | | |
| Abscisic acid methyl ester | $C_{16}H_{22}O_4$ | 7.36 | 5.03 $e^6$ | 8.45 $e^5$ | 16.80 | | | |
| S-abscisic acid methyl ester | $C_{16}H_{22}O_4$ | 7.36 | 5.03 $e^6$ | 8.45 $e^5$ | 16.80 | | | |
| Jasmonic acid | $C_{12}H_{18}O_3$ | 5.79 | 1.14 $e^5$ | 1.23 $e^4$ | 10.79 | | | |
| Jasmonic acid methyl ester | $C_{13}H_{20}O_3$ | 6.24 | | | | 1.28 $e^6$ | 1.56 $e^5$ | 12.19 |
| 9,10-Dihydrojasmonic acid | $C_{12}H_{20}O_3$ | 6.36 | | | | 2.75 $e^5$ | 2.85 $e^4$ | 10.36 |
| 3-Oxo-2-(2-(Z)-pentenyl)cyclopentane-1-butyric acid | $C_{14}H_{22}O_3$ | 6.36 | | | | 8.25 $e^4$ | 1.11 $e^4$ | 13.45 |
| 3-Oxo-2-(2-(Z)-pentenyl)cyclopentane-1-hexanoic acid | $C_{16}H_{26}O_3$ | 6.82 | | | | 3.03 $e^6$ | 4.95 $e^5$ | 16.34 |
| 3-Oxo-2-(2-(Z)-pentenyl)cyclopentane-1-octanoic acid | $C_{18}H_{30}O_3$ | 6.26 | 1.28 $e^6$ | 2.16 $e^5$ | 17.28 | | | |
| Cis-12-oxo-phytodienoic acid | $C_{18}H_{28}O_3$ | 6.14 | 5.59 $e^6$ | 1.07 $e^6$ | 19.14 | | | |
| Dinor-12-oxo-phytodienoic acid | $C_{16}H_{24}O_3$ | 6.80 | 8.14 $e^5$ | 1.29 $e^5$ | 15.48 | 2.04 $e^6$ | 3.70 $e^5$ | 18.14 |
| Cucurbic acid | $C_{12}H_{20}O_3$ | 6.44 | 1.70 $e^5$ | 2.53 $e^4$ | 14.88 | | | |
| Tuberonic acid | $C_{12}H_{18}O_4$ | 3.93 | 1.47 $e^5$ | 1.75 $e^4$ | 11.90 | 1.93 $e^5$ | 2.11 $e^4$ | 10.93 |
| Tuberonic acid methyl ester | $C_{13}H_{20}O_4$ | 6.31 | | | | 9.33 $e^5$ | 1.14 $e^5$ | 12.22 |
| 6-Deoxo-28-norcastasterone | $C_{27}H_{48}O_4$ | 6.16 | 2.65 $e^4$ | 8.47 $e^3$ | 31.96 | | | |
| Ponasterone A | $C_{27}H_{44}O_6$ | 6.27 | | | | 4.28 $e^4$ | 1.07 $e^4$ | 25.00 |
| Salicylic acid | $C_7H_6O_3$ | 5.07 | | | | 7.38 $e^5$ | 4.91 $e^4$ | 6.65 |

**TABLE 3.6.** Phytohormones and related compounds, detected in the extract of a tomato fruit. The results were obtained by usage of the software program ToxID, at which a minimum peak intensity of 1000 and a maximum mass deviation of 5 ppm were taken into account. For correct identification of a compound, at least two diagnostic ions had to be present. An isotopic ion ($^{13}C$, $^{37}Cl$, $^{34}S$ or $^{78}Br$) was only suited as diagnostic ion when the corresponding $[M+H^+]^+$ or $[M-H^+]^-$ ion was also found and the calculated relative intensity (ion ratio) met the CD 2002/657/EC requirements.

| Compound name | Elemental Composition | RT (min) | Positive ionization modus | | | Negative ionization modus | | |
|---|---|---|---|---|---|---|---|---|
| | | | Peak intensity $[M+H]^+$ | Peak intensity $[^{13}C$ isotope + $H]^+$ | Ion ratio (%) | Peak intensity $[M-H]^-$ | Peak intensity $[^{13}C$ isotope - $H]^-$ | Ion ratio (%) |
| Adenine | $C_5H_5N_5$ | 3.15 | 4.30 $e^4$ | | | 4.68 $e^3$ | | |
| $N^6$-cyclopentyladenosine-5'-monophosphate sodium salt | $C_{15}H_{20}N_5Na_2O_7PH_2O$ | 2.88 | 5.66 $e^3$ | | | 1.00 $e^4$ | | |
| Dihydrozeatin hydrochloride | $C_{10}H_{15}N_5OHCl$ | 2.96 | 2.61 $e^3$ | 9.70 $e^{2*}$ | 37.16 | | | |
| $N^6$-isopentenyladenine-7-glucoside | $C_{16}H_{23}N_5O_5$ | 3.40 | 1.07 $e^5$ | 1.64 $e^4$ | 15.33 | 6.12 $e^3$ | | |
| $N^6$-isopentenyladenine-9-glucoside | $C_{16}H_{23}N_5O_5$ | 3.40 | 1.07 $e^5$ | 1.64 $e^4$ | 15.33 | 6.12 $e^3$ | | |
| $N^6$-isopentenyladenosine-5'-monophosphate sodium salt | $C_{15}H_{20}N_5Na_2O_7PH_2O$ | 2.88 | 5.66 $e^3$ | | | 1.00 $e^4$ | | |
| Kinetin hydrochloride | $C_{10}H_9N_5OHCl$ | 2.92 | 1.00 $e^5$ | | | 6.60 $e^4$ | | |
| Trans-zeatin riboside | $C_{15}H_{21}N_5O_5$ | 2.75 | | | | 6.70 $e^3$ | 7.39 $e^2$ | 11.03 |
| $N^6$-isopentenyladenosine-5'-monophosphate sodium salt | $C_{15}H_{20}N_5$ $Na_2O_7PH_2O$ | 2.88 | 5.66 $e^3$ | | | 1.00 $e^4$ | | |
| Trans-zeatin | $C_{10}H_{13}N_5O$ | 2.93 | 5.53 $e^5$ | 7.71 $e^4$ | 7.17 | | | |
| Cis-zeatin | $C_{10}H_{13}N_5O$ | 2.93 | 5.53 $e^5$ | 7.71 $e^4$ | 7.17 | | | |
| Zeatin riboside mixed isomers (E/Z) | $C_{15}H_{21}N_5O_5$ | 2.75 | | | | 6.70 $e^3$ | 7.39 $e^2$ | 11.03 |
| Trans-zeatin-7-glucoside | $C_{16}H_{23}N_5O_6$ | 3.12 | 7.05 $e^4$ | 8.82 $e^3$ | 12.51 | 5.20 $e^5$ | 7.76 $e^4$ | 14.92 |
| Trans-zeatin-9-glucoside | $C_{16}H_{23}N_5O_6$ | 3.12 | 7.05 $e^4$ | 8.82 $e^3$ | 12.51 | 5.20 $e^5$ | 7.76 $e^4$ | 14.92 |
| Trans-zeatin-O-glucoside | $C_{16}H_{23}N_5O_6$ | 3.12 | 7.05 $e^4$ | 8.82 $e^3$ | 12.51 | 5.20 $e^5$ | 7.76 $e^4$ | 14.92 |
| Trans-zeatin-O-acetyl | $C_{12}H_{15}N_5O_2$ | 3.70 | | | | 3.31 $e^3$ | 3.25 $e^2$ | 9.82 |
| 2-Methylthio-trans-zeatin | $C_{11}H_{15}N_5OS$ | 4.87 | 8.08 $e^3$ | | | 1.41 $e^3$ | | |
| Cis-zeatin-9-glucoside | $C_{16}H_{23}N_5O_6$ | 3.12 | 7.05 $e^4$ | | | 5.20 $e^5$ | 7.76 $e^4$ | 14.92 |
| Cis-zeatin-O-glucoside | $C_{16}H_{23}N_5O_6$ | 3.12 | 7.05 $e^4$ | | | 5.20 $e^5$ | 7.76 $e^4$ | 14.92 |
| 1-Acetylindole-3-carboxaldehyde | $C_{11}H_9NO_2$ | 2.93 | 5.26 $e^6$ | 6.04 $e^5$ | 11.48 | 1.83 $e^3$ | | |
| 5-Benzyloxyindole-3-acetic acid | $C_{17}H_{15}NO_3$ | 3.89 | 1.38 $e^4$ | | | 9.84 $e^3$ | | |
| 5-Hydroxyindole-2-carboxylic acid ethyl ester | $C_{11}H_{11}NO_3$ | 4.11 | 1.24 $e^5$ | 1.82 $e^4$ | 14.68 | | | |
| Indole-3-acetyl-L-alanine | $C_{13}H_{14}N_2O_3$ | 4.20 | | | | 6.12 $e^5$ | 7.65 $e^4$ | 12.50 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Indole-3-acetyl-L-aspartic acid | $C_{14}H_{14}N_2O_5$ | 4.21 | 2.77 $e^5$ | 4.02 $e^4$ | 14.51 | 9.86 $e^3$ | | |
| Indole-3-acetyl-L-aspartic acid dimethyl ester | $C_{16}H_{18}N_2O_5$ | 7.11 | 9.01 $e^3$ | 1.49 $e^3$ | 16.53 | | | |
| Indole-3-acetyl-L-glutamic acid dimethyl ester | $C_{17}H_{20}N_2O_5$ | 6.47 | 1.90 $e^5$ | 3.22 $e^4$ | 16.95 | | | |
| Indole-3-acetyl-L-tryptophan | $C_{21}H_{19}N_3O_3$ | 4.23 | 1.77 $e^3$ | | | 6.67 $e^4$ | | |
| Indole-3-acetyl-L-valine | $C_{15}H_{18}N_2O_3$ | 3.50 | 1.06 $e^6$ | 1.28 $e^5$ | 12.08 | | | |
| Indole-3-acrylic acid | $C_{11}H_9NO_2$ | 2.93 | 5.26 $e^6$ | 6.04 $e^5$ | 11.48 | 1.83 $e^3$ | | |
| Indole-2-carboxylic acid | $C_9H_7NO_2$ | 3.94 | 1.90 $e^5$ | | | 3.34 $e^3$ | | |
| Indole-3-carboxylic acid | $C_9H_7NO_2$ | 3.94 | 1.90 $e^5$ | | | 3.34 $e^3$ | | |
| Indole-5-carboxylic acid | $C_9H_7NO_2$ | 3.94 | 1.90 $e^5$ | | | 3.34 $e^3$ | | |
| DL-indole-3-lactic acid | $C_{11}H_{11}NO_3$ | 4.11 | 1.24 $e^5$ | 1.82 $e^4$ | 14.68 | | | |
| 5-Methoxytryptamine | $C_{11}H_{14}N_2O$ | 4.11 | 1.24 $e^5$ | 1.82 $e^4$ | 14.68 | | | |
| 5-Methoxy-DL-tryptophan | $C_{12}H_{14}N_2O_3$ | 7.83 | 9.56 $e^3$ | | | 8.62 $e^4$ | 7.33 $e^3$ | 8.50 |
| Tryptophol | $C_{10}H_{11}NO$ | 4.04 | 6.86 $e^4$ | 5.40 $e^3$ | 7.87 | | | |
| Gibberellin A1 methyl ester | $C_{20}H_{26}O_6$ | 6.46 | | | | 2.36 $e^5$ | 3.90 $e^4$ | 16.53 |
| Gibberellin A9 methyl ester | $C_{20}H_{26}O_4$ | 5.91 | 1.33 $e^4$ | 2.27 $e^3$ | 17.07 | | | |
| Gibberellin A13 | $C_{20}H_{26}O_7$ | 2.93 | 2.37 $e^4$ | 5.57 $e^3$ | 23.50 | | | |
| Gibberellin A19 | $C_{20}H_{26}O_4$ | 6.46 | | | | 1.22 $e^5$ | 2.03 $e^4$ | 16.64 |
| Gibberellin A23 | $C_{20}H_{26}O_7$ | 2.93 | 2.37 $e^4$ | 5.57 $e^3$ | 23.50 | | | |
| Abscisic acid | $C_{15}H_{20}O_4$ | 5.24 | | | | 1.30 $e^5$ | 1.86 $e^4$ | 14.31 |
| R-abscisic acid | $C_{15}H_{20}O_4$ | 5.24 | | | | 1.30 $e^5$ | 1.86 $e^4$ | 14.31 |
| S-abscisic acid | $C_{15}H_{20}O_4$ | 5.24 | | | | 1.30 $e^5$ | 1.86 $e^4$ | 14.31 |
| Trans,trans-abscisic acid | $C_{15}H_{20}O_4$ | 5.24 | | | | 1.30 $e^5$ | 1.86 $e^4$ | 14.31 |
| Trans,trans-abscisic acid methyl ester | $C_{16}H_{22}O_4$ | 7.25 | | | | 4.90 $e^4$ | 8.87 $e^3$ | 18.10 |
| S-trans,trans-abscisic acid | $C_{15}H_{20}O_4$ | 5.24 | | | | 1.30 $e^5$ | 1.86 $e^4$ | 14.31 |
| S-trans,trans-abscisic acid methyl ester | $C_{16}H_{22}O_4$ | 7.25 | | | | 4.90 $e^4$ | 8.87 $e^3$ | 18.10 |
| Abscisic acid methyl ester | $C_{16}H_{22}O_4$ | 7.25 | | | | 4.90 $e^4$ | 8.87 $e^3$ | 18.10 |
| S-abscisic acid methyl ester | $C_{16}H_{22}O_4$ | 7.25 | | | | 4.90 $e^4$ | 8.87 $e^3$ | 18.10 |
| Abscisic acid glucosyl ester | $C_{21}H_{30}O_9$ | 3.42 | 8.51 $e^4$ | 1.78 $e^4$ | 20.92 | | | |
| Jasmonic acid | $C_{12}H_{18}O_3$ | 5.73 | 1.62 $e^5$ | 1.91 $e^4$ | 11.79 | 2.14 $e^5$ | 2.61 $e^4$ | 12.16 |
| Jasmonic acid methyl ester | $C_{13}H_{20}O_3$ | 6.24 | | | | 2.14 $e^4$ | 2.41 $e^3$ | 11.26 |
| 9,10-Dihydrojasmonic acid | $C_{12}H_{20}O_3$ | 6.44 | 2.66 $e^6$ | 3.58 $e^5$ | 13.46 | | | |
| 3-Oxo-2-(2-(Z)-pentenyl)cyclopentane-1-octanoic acid | $C_{18}H_{30}O_3$ | 6.35 | 8.63 $e^5$ | 1.61 $e^5$ | 18.66 | | | |
| Cis-12-oxo-phytodienoic acid | $C_{18}H_{28}O_3$ | 6.15 | 7.68 $e^5$ | 1.48 $e^5$ | 19.27 | 1.54$e^4$ | 1.72 $e^3$ | 11.17 |
| N-(jasmonoyl)-(S)-isoleucine | $C_{18}H_{29}NO_4$ | 6.32 | 1.05 $e^5$ | | | 1.60 $e^5$ | | |
| Cucurbic acid | $C_{12}H_{20}O_3$ | 6.44 | 2.66 $e^6$ | 3.58 $e^5$ | 13.47 | | | |
| Tuberonic acid | $C_{12}H_{18}O_4$ | 3.93 | 1.39 $e^5$ | 1.91 $e_4$ | 13.74 | 2.28 $e^5$ | 2.06 $e^4$ | 9.04 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Tuberonic acid methyl ester | $C_{13}H_{20}O_4$ | 6.40 | 1.20 $e^5$ | 1.54 $e^4$ | 12.79 | 2.10 $e^5$ | 2.19 $e^4$ | 10.43 |
| Norbrassinolide | $C_{27}H_{46}O_6$ | 6.32 | 3.83 $e^4$ | 7.56 $e^3$ | 19.74 | | | |
| Ponasterone A | $C_{27}H_{44}O_6$ | 6.15 | 1.59 $e^4$ | 3.18 $e^3$ | 20.00 | | | |
| Salicylic acid | $C_7H_6O_3$ | 4.98 | | | | 3.73 $e^5$ | 2.22 $e^4$ | 5.95 |

* Peak intensity of the $[^{37}Cl$ isotope + $H^+]^+$ ion instead of the $[^{13}C$ isotope + $H^+]^+$ ion.

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# CHAPTER IV

## UHPLC-HRMS/MS BASED CHEMICAL ANNOTATION OF PHYTOHORMONES FROM TOMATO FRUIT

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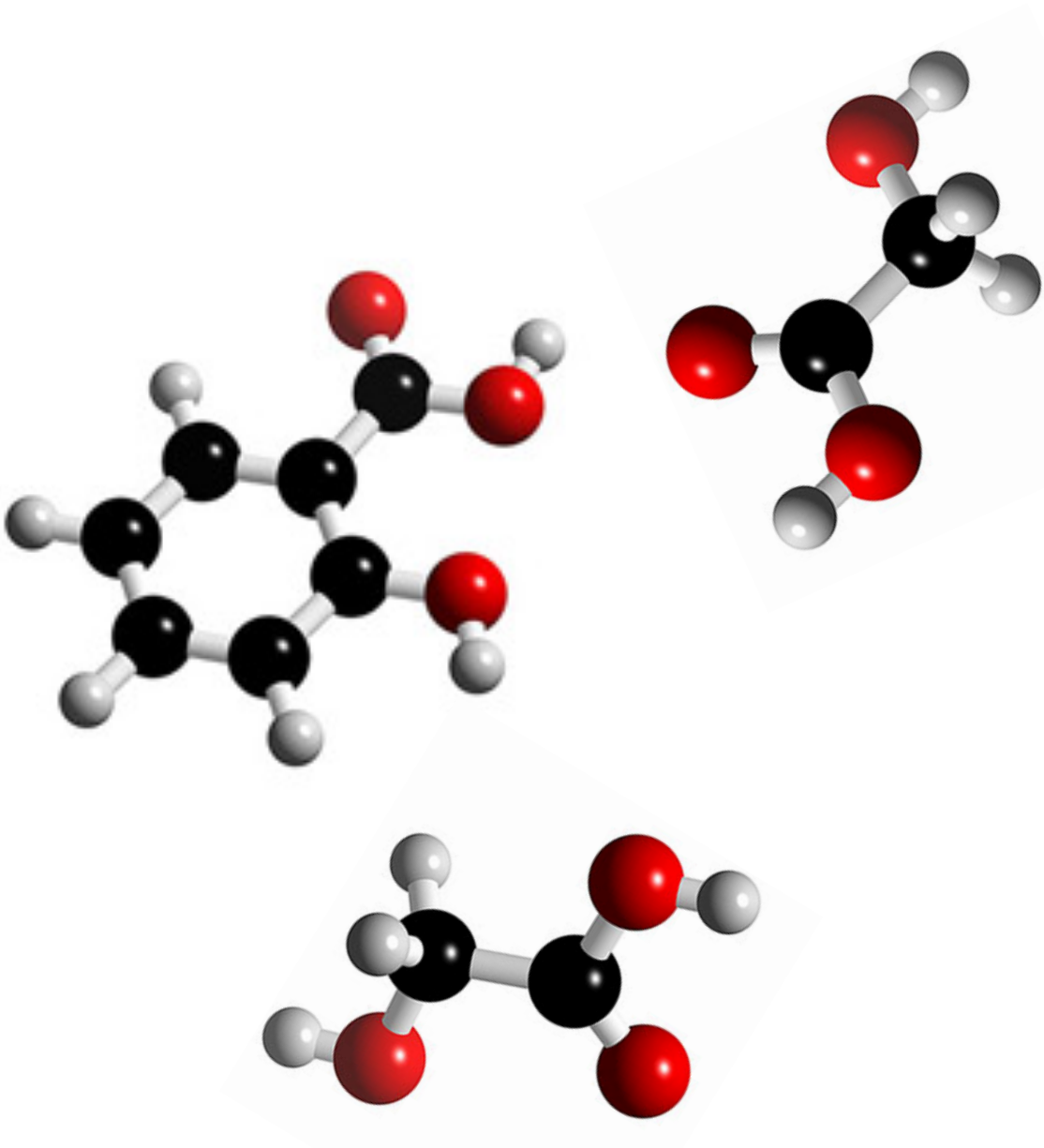

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# ABSTRACT

Phytohormones are key signaling biomolecules, which are assigned significant value because of their regulating functions in numerous physiological and developmental plant processes. Deepening the knowledge about phytohormone functioning is, however, strongly hampered by the complex signaling networks and crosstalk. Although metabolomics experiments may be of significant value within this context, the identification of metabolites is still seen as a major bottleneck in the interpretation of these experiments. In this study, it was aimed to define the phytohormonal profiles of tomato fruits, covering various developmental stages. For this purpose, ultra-high performance liquid chromatography (UHPLC) and high-resolution hybrid quadrupole Q-Exactive Orbitrap mass spectrometry (HRMS/MS) were employed. This analytical platform combines the capabilities of tandem mass spectrometry and the strengths of high-resolution mass measurement, which offers great identification potential towards unknown metabolites. This potential was fully exploited by an integrated identification strategy of chemical formula prediction and *in silico* based structure elucidation. Using this strategy, 52 metabolites were annotated as phytohormonal compounds and recognized as typifying the tomato fruit hormonal profile. These metabolites represent various hormonal classes, i.e. gibberellines (12), auxins (12), cytokinins (10), jasmonates (10), abscisates (2), strigolactones (2), brassinosteroids (2), salicylates (1) and polyamines (1). The intrinsic value of such profiling towards the unraveling of developmental, physiological or metabolic plant processes was demonstrated by unsupervised pattern recognition, i.e. principal component analysis.

# 1. INTRODUCTION

As a minor component of the metabolome, phytohormones are of particular interest because of their regulating functions in numerous physiological and developmental plant processes (1). Herewith, it has been recognized that the regulating actions from these key signaling biomolecules are not effectuated in a mono-causal manner (1,2). In contrast, plant metabolism is mediated through complex crosstalk including additive, synergistic and antagonistic actions between multiple phytohormones (1-3). As a consequence, the development of methods for the simultaneous analysis of multiple phytohormones has been assigned critical importance for elucidating hormone functions and signaling networks (4-6). In this context, considerable efforts have been made to establish multi-targeted phytohormone profiling whereby liquid chromatography coupled to mass spectrometry (LC-MS) has emerged as the key analytical technique (7). Moreover, within this analytical setting, full-scan high-resolution MS approaches such as time-of-flight, Fourier-transform ion cyclotron resonance and Orbitrap-based instruments are ascribed true metabolomic profiling potential (8,9). These analytical platforms are indeed able to reveal a comprehensive view on the relative levels of hundreds to thousands of metabolites, present in the plant material of interest (10). However, the extraction of meaningful information from mass spectrometry metabolomics data is regarded as a challenging task because of the data complexity and richness (12).

An effective strategy for data handling is therefore needed and generally entails (i) collecting and preprocessing the data; (ii) processing and mining the data to extract those compounds of interest; and (iii) identifying relevant compounds (12,13). Within the presented strategy, structural elucidation and thus identification of metabolites is often seen as the main bottleneck in the interpretation of metabolomics experiments (14,15). A typical process of identification starts with interpretation of the mass spectrum to ensure that the signal of interest is corresponding to a monoisotopic ion and not to a natural isotopologue or adduct ion (16). Determining the elemental composition of the monoisotopic ion is the first next step in actual compound characterization. Unfortunately, despite very accurate ultra-high resolution mass measurements (sub-ppm errors), multiple elemental compositions may still be possible (17).

Therefore, evaluation of other data such as isotope patterns and MS/MS fragmentation spectra is recommended to determine the unique elemental composition. In addition, the search space may be greatly reduced by applying heuristic rules (17,18). With this rationale, various software tools such as Mass Frontier (Thermo Fisher Scientific), MassLynx (Waters), PeakView (AB Sciex), SmartFormula3D (Bruker Daltonics), and MZmine (18) were developed to effectuate such an integrated approach. However, for each molecular formula, a large number of candidate structures exist, which have to be ranked or filtered prior to ultimate identification by means of reference standards (19,20). MS/MS and $MS^n$ spectra enclose a wealth of structural information and may provide the next level of evidence, needed for structural elucidation (14). Searching spectral libraries of reference spectra (e.g. Metlin, MassBank, LipidMaps and HMDB) has been established as a reliable way to achieve putative metabolite annotation (14,21,22). The use of these mass spectral libraries is currently still rather limited because libraries for LC-(HR)MS data are still relatively small and the spectra comparability among different instruments is sometimes inadequate (20). Therefore, an *in silico* based strategy may represent a valuable alternative since measured spectra are interpreted towards hypothetically predicted fragmentation spectra of a given molecular structure. Rule-based fragmentation approaches (e.g. Mass Frontier and ACD Fragmenter (ACD/Labs)) apply cleavage rules, collected from literature, to predict fragment ions. The advantage of this approach relates to its potentially high specificity, which also implies that when a fragmentation rule is missed from the knowledge based, the corresponding fragment ions can never be predicted (22). Combinatorial fragmentation tools (e.g. Fragment Identificator (23) and MetFrag (15)) were developed under a different rationale, i.e. generation of fragment ions through combinatorial disconnection of chemical bonds and calculation of each bond's internal energy. This approach avoids the potential bias derived from a confined set of fragmentation rules, but may sometimes be time-consuming and lacking the specificity of the rule-based prediction (22).

In this study, a 'suspects screening' approach was performed in order to define the qualitative phytohormonal profile of tomato fruits (*Solanum lycopersicum* L.). More specifically, it was aimed to determine which phytohormones were, possibly only temporarily, present during tomato fruit development and ripening. To establish this suspect screening, compound-specific information about the suspects (i.e. known phytohormones) was utilized and an analytical method based on ultra-high performance liquid chromatography (UHPLC) hyphenated with high-resolution hybrid quadrupole Orbitrap mass spectrometry (HRMS/MS) was developed. This analytical platform encloses advanced potential for identification of unknown metabolites, founded on the capabilities of tandem mass spectrometry and the strengths of high-resolution accurate mass measurements. Phytohormones were identified up to the level of 'putatively annotated compounds' (21) using the acquired metabolomics data and an integrated strategy of highly accurate chemical formula prediction and *in silico* based fragmentation for structure elucidation.

# 2. MATERIALS AND METHODS

## 2.1 CHEMICALS AND REAGENTS

The phytohormone standards (±)-*cis,* trans-abscisic acid, gibberellic acid, indol-3-acetic acid, jasmonic acid, epibrassinolide, *trans*-zeatin and $N^6$-benzyladenine were all purchased from Sigma-Aldrich Co. (St. Louis, USA). Salicylic acid was obtained from Fisher Scientific (Loughborough, UK). Deuterium-labeled internal standards $d_5$-indol-3-acetic acid, $d_5$-*trans*-zeatin, $d_7$-$N^6$-benzyladenine and $d_6$-abscisic acid were from OlChemIm Ltd. (Olomouc, Czech Republic).

Reagents were of analytical grade when used for extraction purposes and of LC-MS grade for UHPLC-Q-Exactive™ hybrid quadrupole Orbitrap-MS applications. They were respectively purchased from VWR International (Merck, Darmstadt, Germany) and Fisher Scientific (Loughborough, UK). Ultra-pure water was obtained by usage of a purified-water system (VWR International, Merck, Darmstadt, Germany).

## 2.2 PLANT MATERIAL AND SAMPLE PREPARATION

Tomato plants (*Solanum lycopersicum* L. cv. Moneymaker) were grown in a greenhouse compartment of the Institute for Agricultural and Fisheries Research (ILVO, Melle, Belgium) and were subjected to normal cultural practices (24). Tomato fruits were obtained from five different plants and collected in such a way that various stages of development and ripening were enclosed. More specifically, five different developmental stages, defined by days after anthesis (DAA) and ranging from green immature to red-ripe, were considered. For each stage, three replicate fruits (coming from a single truss) were selected. Following characterization of the developmental stages was obtained (n = 3): (1) 7.3 ± 1.5 DAA; (2) 13.7 ± 1.5 DAA; (3) 19.7 ± 0.6 DAA; (4) 28.3 ± 0.6 DAA; (5) 38.6 ± 0.6 DAA. This approach was assumed to cover a maximum diversity with respect to tomato fruit phytohormone profiles. Sampling and preparation of samples prior to extraction was performed according to Van Meulebroek *et al.* (2012) (9). An overview of the entire profiling strategy, starting from the sampling and ending with the structural elucidation of screened out metabolites, is presented in Figure 4.1.

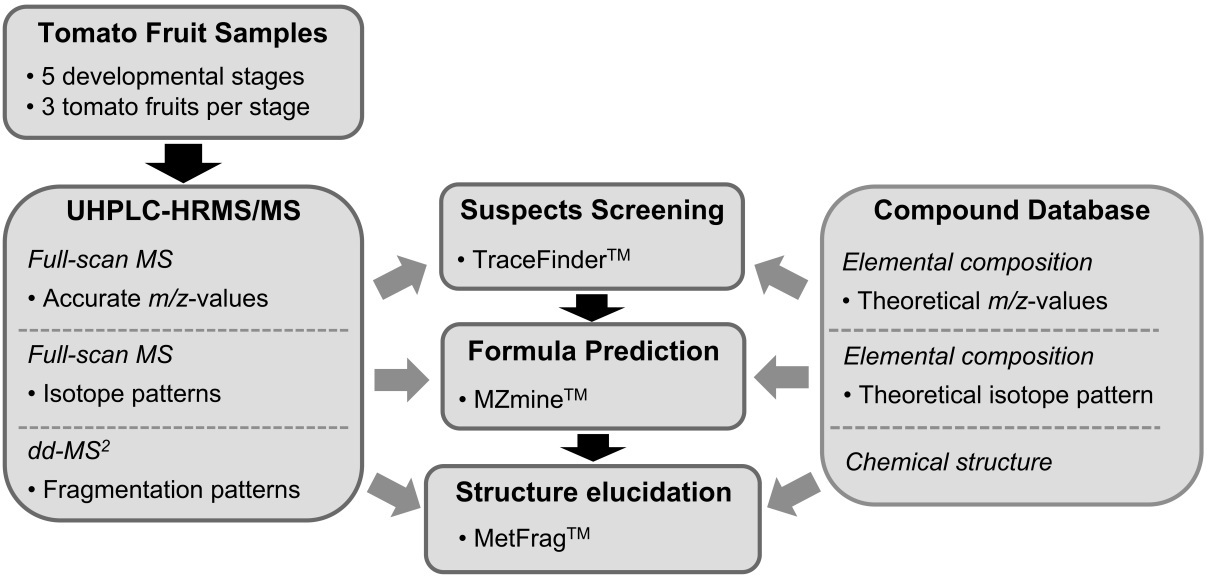


**FIGURE 4.1.** Overview of the strategy, implemented to establish the phytohormonal profiles of tomato fruits. This strategy is founded on the use of a database, comprising information about known phytohormones (i.e. suspects).

## 2.3 PHYTOHORMONE ANALYSIS

Extraction of phytohormones from tomato fruit tissue was performed as described by Van Meulebroek *et al.* (2012) (9). Mass analysis was established by UHPLC-Q-Exactive™ hybrid quadrupole Orbitrap-MS (Thermo Fisher Scientific, Breda, the Netherlands). Chromatographic separation was thereby achieved on an Ultimate LPG-3400XRS pumping system (Thermo Fisher Scientific, Breda, the Netherlands), equipped with a Nucleodur Gravity C18 column (1.8 μm, 50 mm x 2.1 mm internal diameter) (Macherey-Nagel, Düren, Germany). The gradient elution program, flow rate, and column oven temperature were adopted from Van Meulebroek *et al.* (2012) (9). The Q-Exactive™ Orbitrap mass spectrometer was equipped with a heated electrospray ionization source (HESI-II), operating in both positive and negative ionization mode. Ion source working parameters were adopted from Van Meulebroek *et al.* (2012) (9), with an additional S-lens RF level of 50. The mass spectrometer was operated in data-dependent fragmentation mode (dd-$MS^2$), using an inclusion list of specified suspects (i.e. 294 known phytohormones). Information about the theoretical molecular masses, calculated by Xcalibur™ 2.2 software (Thermo Fisher Scientific, San Jose, USA), was as such provided. The main parameter settings for the dd-$MS^2$ scans included a parent mass isolation width of 4.0 *m/z*, an automatic gain control (AGC) target of 5 x $e^4$ ions, a maximum injection time (IT) of 100 ms, and a mass resolution of 17,500 full width at half maximum (FWHM). Fragmentation of selected ions was performed by high-energy collisional dissociation with a normalized collision energy (NCE) and stepped NCE setting of 30 eV and 33%, respectively. Survey scans (full-scan experiments), using a mass resolution of 140,000 FWHM and an AGC target of 5 x $e^6$ ions, were combined with the dd-$MS^2$ experiments. The mass scan range was thereby set at 100 to 800 *m/z*. A dd-$MS^2$ scan was initiated when a minimum percentage, i.e. 1.0%, of the full-scan AGC target was reached by any ion. The full-scan/dd-$MS^2$ analytical experiment was implemented for each ionization mode separately. As such, each tomato fruit sample was analyzed twice. Instrument control and data processing were carried out by Xcalibur™ 2.2 software.

## 2.4 COMPOUND DATABASE CONSTRUCTION

A database was constructed for suspects screening in which the elemental compositions, molecular weights, and chemical structures of 294 phytohormonal compounds were included (25,26). Molecular weights were calculated with Xcalibur™ 2.2 software and chemical structures (.mol files) were drawn with ChemBioDraw 13.0 (PerkinElmer Inc., Massachusetts, USA). The targeted phytohormones represented various hormonal classes including abscisates, auxins, brassinosteroids, cytokinins, gibberellins, jasmonates, polyamines, salicylates and strigolactones.

## 2.5 DATABASE-BASED SCREENING FOR SUSPECTS IN UHPLC-HRMS/MS DATA

To reveal the ions (characterized by their *m*/*z*-value and retention time) that could be potentially assigned a phytohormonal identity, the *m*/*z*-values of the detected ions were matched against these of known phytohormones, included in the database. For this purpose, TraceFinder™ software (Thermo Fisher Scientific, San Jose, USA) was used. During this suspects screening, $[M+H^+]^+$ and $[M-H^+]^-$ adducts were taken into consideration and a 5 ppm mass tolerance was allowed. In addition, signal-to-noise ratio (S/N, minimum of 10), chromatographic peak performance (valley rise of 2, valley S/N of 1.10), and signal intensity (minimum of 20,000) were used as secondary criteria to retain a particular ion for further identification purposes. Since this screening is mainly based on accurate mass features, a retained ion may be linked to multiple phytohormonal identities if these are sharing identical chemical formulas.

## 2.6 CHEMICAL FORMULA PREDICTION

A universal software tool, developed within the MZmine 2 framework (18), was used for the prediction, filtering and ranking of candidate formulas. The elements that were allowed for formula prediction were restricted to the basic building blocks of natural metabolites, i.e. hydrogen (H), carbon (C), oxygen (O), nitrogen (N), phosphorus (P), and sulfur (S). The chemical formulas were searched within a 5 ppm or 0.001 Da window towards the detected mass after removal of the ionization adduct. Filtering the list of candidate formulas was established by using a number of heuristic rules. A set of empirical restrictions regarding element counts and ratios (NOPS/C ratio, H/C ratio, and multiple element counts) was applied to verify chemical formula validity. In addition, the ring double bond equivalent (RDBE), which estimates the number of rings and unsaturated bonds in a molecule, was allowed to range between 0 and 40 and had to be an

integer (27). Remaining candidate formulas were further ranked on the basis of their isotope pattern score. These scores were determined by considering an isotope *m/z* tolerance of 5 ppm and 0.001 Da, a retention time tolerance of 0.05 min, a minimum relative abundance of 0.1%, and a minimum absolute intensity of 1 x $e^3$. At this stage, no discriminating value was assigned to the mass difference or MS/MS score (i.e. a minimum MS/MS score of 0%).

## 2.7 STRUCTURE ELUCIDATION BY COMBINATORIAL-BASED PREDICTION OF MS/MS PRODUCT IONS

The next step within the identification strategy was based on *in silico* prediction of fragmentation patterns for phytohormone candidate structures. More specifically, MS/MS ion fragments were predicted for the candidate structures and matched towards the experimentally, collision-induced dissociation fragmentation spectrum of the ion under investigation. For this purpose, a combinatorial-based prediction strategy, enclosing a systematic bond disconnection method, was applied by usage of the MetFrag software tool (15). Generated product ions from a candidate structure were matched against the measured fragments, whereby a mass tolerance window of 20 ppm or 0.02 Da (based on the mass resolution of 17,500 FWHM) was allowed for obtaining a positive match. During this identification step, only the 30 most intense measured fragments were considered.

## 2.8 PHYTOHORMONE PROFILING TO UNRAVEL PLANT METABOLISM

To demonstrate the intrinsic value of the profiling strategy, the initial set of tomato fruit samples and their associated phytohormone profiles were used. As earlier described, these tomato fruits were classified according to their DAA and prevailing developmental stage. Hereby, five developmental stages (a to e) with three biological replicates (1 to 3) for each stage were considered. For each tomato fruit, the relative abundances (i.e. peak areas) of the annotated phytohormones were acquired by means of Xcalibur™ 2.2. Subsequent multivariate data analysis was performed by means of principal component analysis (PCA) and hierarchical cluster analysis (HCA) (single linkage). This clustering strategy characterizes similarities among samples by examining interpoint distances representing all possible sample pairs in high dimensional space (27). This unsupervised statistical approach was realized using Simca™ 13.0 software (Umetrics, Malmö, Sweden), whereby data were preprocessed by pareto scaling and log-transformation in order to standardize the range of ion peak areas and induce normality, respectively.

# 3. RESULTS AND DISCUSSION

## 3.1 DATABASE-BASED SCREENING FOR SUSPECTS IN UHPLC-HRMS/MS DATA

A database-based evaluation of the metabolomics full-scan data allowed to determine ions, which could be assigned a potential phytohormonal identity. Screening of all samples, either acquired in positive or negative ionization mode, revealed that 149 of 294 phytohormonal identities were potentially represented by one or more ions. It was observed that the majority of these phytohormones (i.e. 66.4%) were solely represented by positive ions. Only a small number was solely represented by negative ions (i.e. 11.4%). Furthermore, it should be noted that a phytohormonal assignment was only made if the respective ion was observed in at least two of the three replicate samples, considered within a developmental stage. Imposing this condition implied an improved reliability regarding the obtained results, i.e. the screened out ions. The tree replicate fruits of a particular developmental stage were indeed coming from a single truss and were thus expected to present quite similar phytohormone profiles.

## 3.2 CHEMICAL FORMULA PREDICTION

The potential phytohormonal identity of the retained ions was further verified by predicting their elemental composition. Although the accurate mass may provide a first indication about an ion's elemental composition, the use of heuristic rules and isotope pattern scores was needed for filtering and ranking the candidate formulas. In the study of Pluskal *et al*. (2012) (18), it was demonstrated that the applied isotopic pattern-scoring algorithm was able to correctly predict the chemical formula for 79% of the 48 analyzed compounds. Moreover, the correct chemical formula was among the top 10 candidates in all cases. Therefore, it was opted to use in this study a 'top 10 rank' criterion to include ions for further identification purposes. This implied that a hypothesized phytohormonal identity regarding a screened out ion was rejected if the associated chemical formula was not ranked in the top 10 of candidate formulas. If the proposed criterion was met, no further conclusions were made based on the isotope pattern score or ranking. For example, screening for 2-methylthio-$N^6$-isopentenyladenine ($C_{11}H_{15}N_5S$) resulted in two positive hits (i.e. two ions). For both ions, the hypothesized $C_{11}H_{15}N_5S$ elemental composition was among the top 10 candidate molecular formulas. Based on the isotope pattern scores (87.96% versus 68.46%) and ranking (ranked $4^{th}$ versus $10^{th}$), preference could be given to one of these ions. However, as stated

by Pluskal *et al.* (2012) (18), the intensity range of the chromatographic peak has a significant influence on the quality of the isotope patterns. Therefore, it would not be opportune to compare isotope pattern scores and ranks between peaks. Both ions were thus retained for subsequent structural elucidation. This strategy of chemical formula prediction, filtering and ranking showed that various chemical formulas, associated with in total 27 phytohormonal identities, were not among the top 10 candidate formulas of any ion. As a consequence, these particular phytohormones were no longer retained. For those ions that could still be recognized as potential phytohormones, the average rank of the hypothesized formula was 3.2 ± 2.5 (n = 144). For 33 ions, the hypothesized formula was ranked first. It should be noted that various phytohormonal identities could at this stage still be represented by multiple ions.

### 3.3 STRUCTURE ELUCIDATION BY COMBINATORIAL-BASED PREDICTION OF MS/MS PRODUCT IONS

Elucidating the chemical structure of the retained ions was conducted by interpretation of the associated collision-induced dissociation fragmentation spectra. These spectra were matched against the MS/MS product ions that were predicted for the hypothesized phytohormonal structures. For this purpose, a combinatorial-based approach was performed using MetFrag. With MetFrag, candidate structures are retrieved from compound libraries (KEGG Compound, ChemSpider and PubChem) based on the accurate mass or elemental composition and subsequently ranked according to matching fragmentation spectra. As such, information about the assessed phytohormonal structure in terms of probability among candidate structures could be obtained (Figure 4.2). Using the PubChem compound library, the study of Wolf *et al.* (2010) (15) indicated that the correct candidate structure occurred at median rank 31.5 (n = 102). However, it was also noted by Wolf *et al.* (2010) (15) that in several cases MetFrag was not able to rank the correct compound even among the top 100 candidates. One reason for this was the high number of very similar candidate structures, and the difficulty to distinguish them based on the predicted spectra. In addition, the selected compound library, fragmentation tree depth, applied collision energy, and initial number of retrieved candidate structures appeared to significantly affect the final ranking of the correct compound.

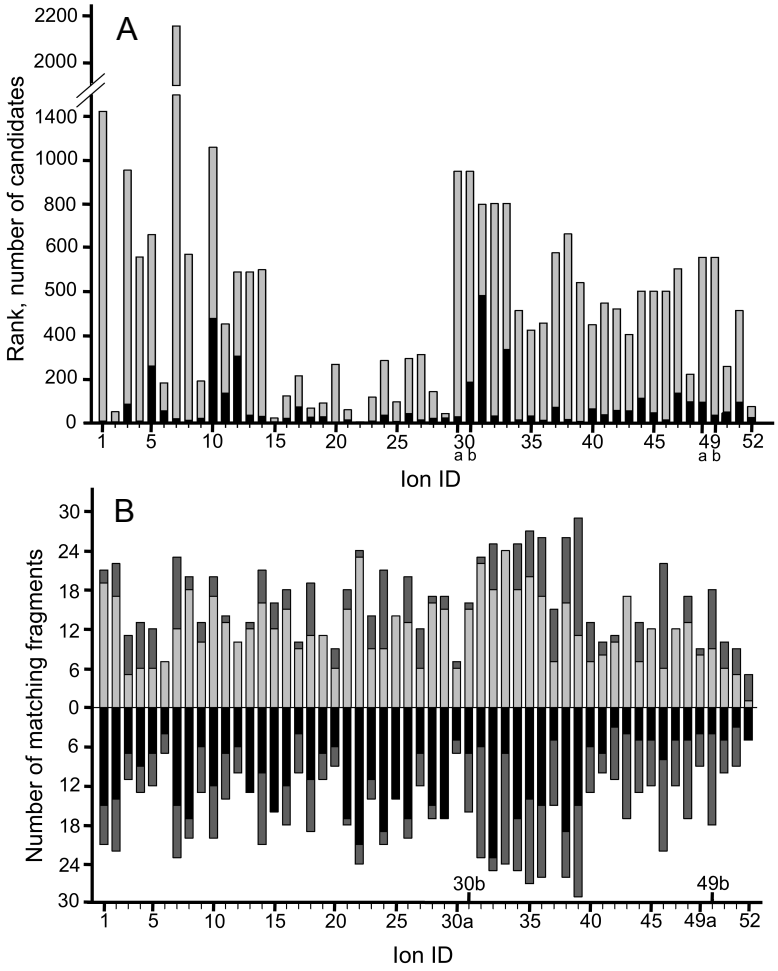


**FIGURE 4.2.** (A) Total number of candidate structures (light grey), obtained for a certain ion (Ion ID refers to Table 1), and obtained rank of the phytohormonal structure on the basis of the fragmentation score (black). (B) Number of measured fragment ions that matches the *in silico* predicted fragments for the first ranked structure (light grey) and phytohormonal structure (black). The maximum number of matching fragments among all candidate structures is indicated as well (dark grey). Herewith, 'a' and 'b' represent a same phytohormonal structure, acquired in negative and positive ionization mode, respectively.

Therefore, proposing a minimum requirement or exclusion criterion regarding the rank of the hypothesized compound would be rather risky. For this reason, our structural interpretation was mainly based on the number of matching fragment ions. It was opted to reject ions with less than three matching fragments as potentially contributing to the tomato fruit phytohormonal profile. This threshold-value was determined based on the results, obtained for 8 reference standards. It was indeed observed that the minimum number of matching fragments for these standards was 3 (i.e. for gibberellic acid). Based on this strategy, 82 phytohormonal identities were retained (Table 4.1).

As the proposed strategy is not able to distinguish between stereoisomers (*cis* versus *trans*, S versus R), only 69 phytohormonal compounds were concluded to be potentially present in tomato fruit. In addition, the database also included a number of two-dimensional isomers. Matching measured versus predicted fragment ions do has the potential of distinguishing these types of isomers. For example (Figure 4.3), an unknown metabolite was assigned a potential phytohormonal identity because the accurate mass and predicted chemical formula positively matched the database. With respect to the hypothesized elemental composition ($C_{11}H_{11}NO_2$), an isotope pattern score of 80.88% was obtained and a first place rank among the candidate formulas was observed. Elucidation of the metabolite's structure was realized by comparing the collision-induced dissociation MS/MS spectrum against the *in silico* predicted fragment ions from various isomeric phytohormonal compounds (e.g. 1-methylindole-3-acetic acid, 5-methylindole-3-acetic acid, and indole-5-carboxylic acid ethyl ester). This strategy was able to exclude indole-5-carboxylic acid ethyl ester as a possibility, but was not able to distinguish between 1-methylindole-3-acetic acid and 5-methylindole-3-acetic acid. Indeed, the obtained fragment *m*/z-values for these latter phytohormones were identical. With this rationale, some ions (Table 4.1) were assigned multiple phytohormonal identities. This eventually resulted in 52 ions, which were putatively annotated. An example of the complete strategy, elaborated for one compound, is presented in Figure 4.4. As for this particular compound an authentic standard was available (i.e. *cis,trans*-abscisic acid), identity could as a result be confirmed.

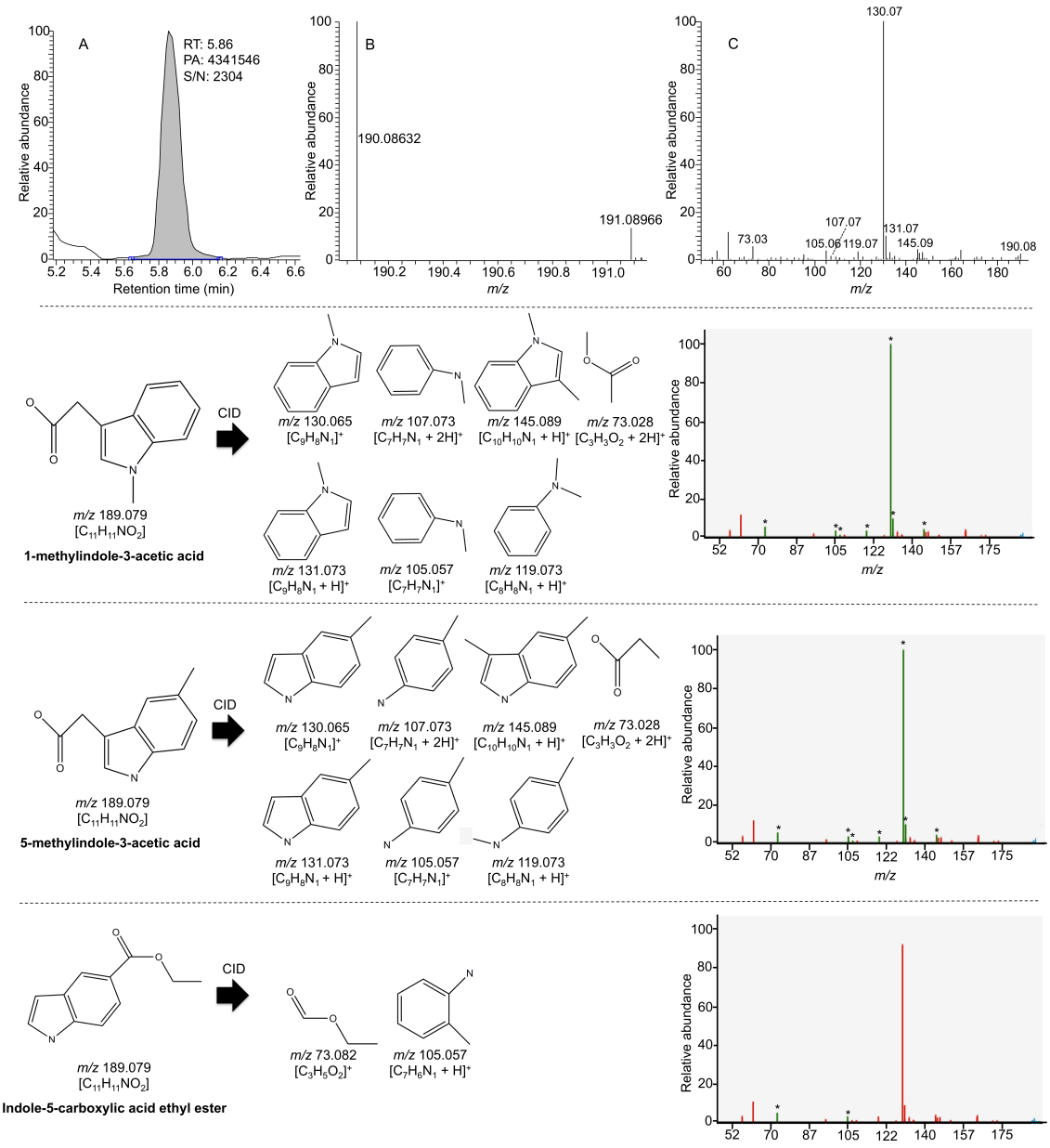


**FIGURE 4.3.** Chromatogram (A), full scan spectrum (B) and MS/MS spectrum (C) from a metabolite, which was assigned a potential phytohormonal identity. Chemical formula prediction ranked the hypothesized chemical formula (i.e. $C_{11}H_{11}NO_2$) first and calculated an isotope pattern score of 80.88%. Various phytohormonal compounds (e.g. 1-methylindole-3-acetic acid, 5-methylindole-3-acetic acid, and indole-5-carboxylic acid ethyl ester) were evaluated by comparison of the *in silico* predicted fragment ions and the collision-induced dissociation fragmentation spectrum. Matching fragments were presented and indicated by an asterisk in the measured MS/MS spectrum.

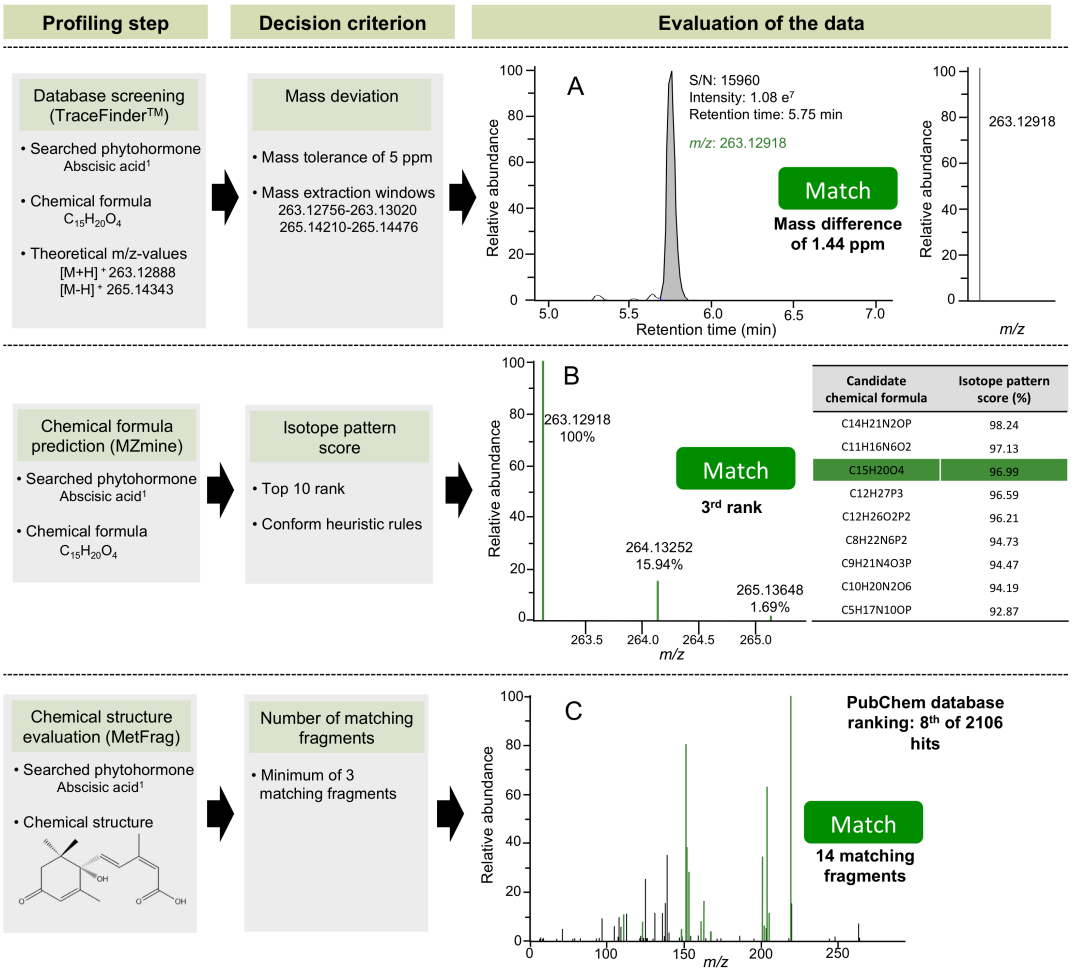


**FIGURE 4.4.** Overview of the applied phytohormone annotation strategy, which enfolds three major steps. These steps include database screening, chemical formula prediction, and chemical structure evaluation. The suggested decision criteria for the various steps are evaluated towards the chromatogram and associated mass spectrum (A), the mass spectrum (B), and the fragmentation profile (C), respectively. [1]The used abscisic acid terminology refers to various isomeric configurations: (±)-*trans,trans*-abscisic acid; (±)-*cis,trans*-abscisic acid; (+)-*trans,trans*-abscisic acid; (+)-*cis,trans*-abscisic acid; (-)-*cis,trans*-abscisic acid.

**TABLE 4.1** List of phytohormonal compounds that were recognized as potentially present in tomato fruit tissue and typifying the inherent phytohormone profiles. For the associated ions, the ionization mode and retention time (min) are given. The results of the chemical formula prediction are described by the isotope pattern score (%) and obtained rank among the candidate formulas. Structural elucidation by *in silico*-based prediction of MS/MS fragments is indicated by the number of matching fragments, fragmentation score, and the *m*/*z*-value of the 4 major fragments. The numbers in superscript are grouping the phytohormone identities that can be annotated to a single ion. For dihydrozeatin-O-glucoside riboside (Ion ID 22) no (relative) fragmentation score could be calculated since the considered phytohormone structure was not present in the MetFrag databases.

| Phytohormone trivial name | ID | Elemental composition | Ioniz. mode | RT (min) | Isotope pattern score %(rank) | Matching fragments | Fragment. score | Fragment *m*/z-values |
|---|---|---|---|---|---|---|---|---|
| Abscisates | | | | | | | | |
| (±)-*Trans,trans*-abscisic acid[1] | 1 | $C_{15}H_{20}O_4$ | - | 5.75 | 96.99 ($3^{rd}$) | 14 | 0.949 | 219.14, 205.12, 204.12, 203.11 |
| (+)-*Trans,trans*-abscisic acid[1] | 1 | $C_{15}H_{20}O_4$ | - | 5.75 | 96.99 ($3^{rd}$) | 14 | 0.949 | 219.14, 205.12, 204.12, 203.11 |
| (±)-*Cis,trans*-abscisic acid[1] | 1 | $C_{15}H_{20}O_4$ | - | 5.75 | 96.99 ($3^{rd}$) | 14 | 0.949 | 219.14, 205.12, 204.12, 203.11 |
| (+)-*Cis,trans*-abscisic acid[1] | 1 | $C_{15}H_{20}O_4$ | - | 5.75 | 96.99 ($3^{rd}$) | 14 | 0.949 | 219.14, 205.12, 204.12, 203.11 |
| (-)-*Cis,trans*-abscisic acid[1] | 1 | $C_{15}H_{20}O_4$ | - | 5.75 | 96.99 ($3^{rd}$) | 14 | 0.949 | 219.14, 205.12, 204.12, 203.11 |
| (±)-*Cis,*trans-abscisic acid glucosyl ester | 2 | $C_{21}H_{30}O_9$ | + | 4.79 | 93.13 ($9^{th}$) | 6 | 0.894 | 247.13, 229.12, 217.12, 201.13 |
| Auxins and indols | | | | | | | | |
| 1-Methylindole-3-acetic acid[2] | 3 | $C_{11}H_{11}NO_2$ | + | 5.86 | 80.88 ($1^{st}$) | 8 | 0.934 | 145.09, 131.07, 130.07, 73.03 |
| 5-Methylindole-3-acetic acid[2] | 3 | $C_{11}H_{11}NO_2$ | + | 5.86 | 80.88 ($1^{st}$) | 8 | 0.934 | 145.09, 131.07, 130.07, 73.03 |
| 7-Methylindole-3-acetic acid[2] | 3 | $C_{11}H_{11}NO_2$ | + | 5.86 | 80.88 ($1^{st}$) | 8 | 0.934 | 145.09, 131.07, 130.07, 73.03 |
| Indole-3-acetic acid | 4 | $C_{10}H_9NO_2$ | + | 5.50 | 93.00 ($1^{st}$) | 5 | 0.970 | 159.07, 131.07, 130.04, 91.04 |
| Indole-3-acetamide | 5 | $C_{10}H_{10}N_2O$ | + | 4.87 | 76.84 ($1^{st}$) | 7 | 0.486 | 149.07, 130.04, 93.05, 85.05 |
| Indole-3-acetonitrile | 6 | $C_{10}H_8N_2$ | + | 4.01 | 54.22 ($1^{st}$) | 4 | 0.929 | 142.07, 132.08, 130.07, 67.04 |
| Indole-3-acetyl-L-alanine | 7 | $C_{13}H_{14}N_2O_3$ | - | 5.40 | 97.21 ($2^{nd}$) | 13 | 0.983 | 201.10, 142.05, 130.05, 129.04 |
| Indole-3-acetyl-L-aspartic acid | 8 | $C_{14}H_{14}N_2O_5$ | + | 7.26 | 79.23 ($3^{rd}$) | 17 | 0.929 | 273.09, 255.08, 246.10, 245.09 |
| Indole-3-carboxaldehyde | 9 | $C_9H_7NO$ | + | 4.01 | 98.51 ($1^{st}$) | 6 | 0.691 | 130.07, 130.04, 119.05, 118.07 |
| 5-Methoxytryptamine[3] | 10 | $C_{11}H_{14}N_2O$ | + | 5.84 | 97.53 ($2^{nd}$) | 6 | 0.582 | 165.10, 164.09, 149.11, 135.09 |
| 6-Methoxytryptamine[3] | 10 | $C_{11}H_{14}N_2O$ | + | 5.84 | 97.53 ($2^{nd}$) | 6 | 0.582 | 165.10, 164.09, 149.11, 135.09 |
| 7-Methoxytryptamine[3] | 10 | $C_{11}H_{14}N_2O$ | + | 5.84 | 97.53 ($2^{nd}$) | 6 | 0.582 | 165.10, 164.09, 149.11, 135.09 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 1-Methylindole-3-carboxaldehyde | 11 | $C_{10}H_9NO$ | + | 5.43 | 95.82 ($3^{rd}$) | 4 | 0.232 | 145.05, 132.08, 118.07, 84.04 |
| 2-Oxindole-3-acetic acid | 12 | $C_{10}H_9NO_3$ | + | 4.35 | 97.80 ($3^{rd}$) | 5 | 0.265 | 147.03, 146.06, 139.03, 133.05 |
| 5-Hydroxyindole-3-acetic acid | 13 | $C_{10}H_9NO_3$ | + | 4.97 | 97.27 ($2^{nd}$) | 4 | 0.861 | 174.06, 103.04, 101.05, 76.03 |
| 1-acetylindole-3-carboxaldehyde | 14 | $C_{11}H_9NO_3$ | + | 3.96 | 98.22 ($1^{st}$) | 10 | 0.868 | 171.07, 160.08, 148.06, 147.07 |
| Polyamines | | | | | | | | |
| Spermine | 15 | $C_{10}H_{26}N_4$ | + | 4.18 | 97.23 ($1^{st}$) | 16 | 0.947 | 131.15, 130.15, 129.14, 127.12 |
| Strigolactons | | | | | | | | |
| Smoke 11 | 16 | $C_7H_8O_5$ | + | 5.51 | 99.78 ($1^{st}$) | 12 | 0.509 | 130.03, 128.05, 127.04, 133.02 |
| 1,2-O-isopropylidene-$\alpha$-D-xylofuranose | 17 | $C_8H_{14}O_5$ | - | 4.81 | 98.11 ($2^{nd}$) | 4 | 0 | 171.06, 129.02, 125.06, 59.01 |
| Brassinosteroids | | | | | | | | |
| Epibrassinolide[4] | 18 | $C_{28}H_{48}O_6$ | + | 7.08 | 88.71 ($3^{rd}$) | 11 | 0.757 | 445.33, 349.24, 331.23, 321.21 |
| Tris-epibrassinolide[4] | 18 | $C_{28}H_{48}O_6$ | + | 7.08 | 88.71 ($3^{rd}$) | 11 | 0.757 | 445.33, 349.24, 331.23, 321.21 |
| SS-epibrasinolide[4] | 18 | $C_{28}H_{48}O_6$ | + | 7.08 | 88.71 ($3^{rd}$) | 11 | 0.757 | 445.33, 349.24, 331.23, 321.21 |
| Epicastasterone[5] | 19 | $C_{28}H_{48}O_5$ | + | 7.52 | 88.26 ($2^{nd}$) | 7 | 0.786 | 447.35, 430.34, 429.34, 172.11 |
| Tris-epicastasterone[5] | 19 | $C_{28}H_{48}O_5$ | + | 7.52 | 88.26 ($2^{nd}$) | 7 | 0.786 | 447.35, 430.34, 429.34, 172.11 |
| 3,24-Diepicastasterone[5] | 19 | $C_{28}H_{48}O_5$ | + | 7.52 | 88.26 ($2^{nd}$) | 7 | 0.786 | 447.35, 430.34, 429.34, 172.11 |
| SS-epicastasterone[5] | 19 | $C_{28}H_{48}O_5$ | + | 7.52 | 88.26 ($2^{nd}$) | 7 | 0.786 | 447.35, 430.34, 429.34, 172.11 |
| Cytokinins | | | | | | | | |
| $N^6$-benzyladenine | 20 | $C_{12}H_{11}N_5$ | + | 5.26 | 96.51 ($3^{rd}$) | 6 | 1 | 148.06, 107.07, 93.07, 92.06 |
| Dihydrozeatin-9-glucoside[6] | 21 | $C_{16}H_{25}N_5O_6$ | + | 4.38 | 94.24 ($5^{th}$) | 17 | 0.880 | 222.13, 221.13, 220.12, 203.12 |
| Dihydrozeatin-7-glucoside[6] | 21 | $C_{16}H_{25}N_5O_6$ | + | 4.38 | 94.24 ($5^{th}$) | 17 | 0.880 | 222.13, 221.13, 220.12, 203.12 |
| Dihydrozeatin-O-glucoside[6] | 21 | $C_{16}H_{25}N_5O_6$ | + | 4.38 | 94.24 ($5^{th}$) | 17 | 0.880 | 222.13, 221.13, 220.12, 203.12 |
| Dihydrozeatin-O-glucoside riboside | 22 | $C_{21}H_{33}N_5O_{10}$ | + | 5.31 | 88.11 ($9^{th}$) | 16 | / | 163.06, 146.06, 145.05, 135.05 |
| N6-isopentenyladenine-9-glucoside | 23 | $C_{16}H_{23}N_5O_5$ | + | 4.73 | 90.23 ($8^{th}$) | 11 | 0.883 | 321.14, 204.12, 143.06, 136.07 |
| Meta-topolin riboside[7] | 24 | $C_{17}H_{19}N_5O_5$ | + | 4.76 | 94.25 ($10^{th}$) | 19 | 0.866 | 213.08, 196.07, 195.07, 185.05 |
| Ortho-topolin riboside[7] | 24 | $C_{17}H_{19}N_5O_5$ | + | 4.76 | 94.25 ($10^{th}$) | 19 | 0.866 | 213.08, 196.07, 195.07, 185.05 |
| Para-topolin riboside[7] | 24 | $C_{17}H_{19}N_5O_5$ | + | 4.76 | 94.25 ($10^{th}$) | 19 | 0.866 | 213.08, 196.07, 195.07, 185.05 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Meta-topolin-9-glucoside[8] | 25 | $C_{18}H_{21}N_5O_6$ | - | 4.51 | 85.15 ($10^{th}$) | 14 | 0.741 | 239.08, 197.06, 179.06, 178.04 |
| Ortho-topolin-9-glucoside[8] | 25 | $C_{18}H_{21}N_5O_6$ | - | 4.51 | 85.15 ($10^{th}$) | 14 | 0.741 | 239.08, 197.06, 179.06, 178.04 |
| Para-topolin-9-glucoside[8] | 25 | $C_{18}H_{21}N5O_6$ | - | 4.51 | 85.15 ($10^{th}$) | 14 | 0.741 | 239.08, 197.06, 179.06, 178.04 |
| Meta-methoxytopolin-9-riboside[9] | 26 | $C_{18}H_{21}N_5O_5$ | - | 4.46 | 86.43 ($3^{rd}$) | 17 | 0.552 | 352.14, 252.09, 189.09, 134.06 |
| Ortho-methoxytopolin-9-riboside[9] | 26 | $C_{18}H_{21}N_5O_5$ | - | 4.46 | 86.43 ($3^{rd}$) | 17 | 0.552 | 352.14, 252.09, 189.09, 134.06 |
| Para-methoxytopolin-9-riboside[9] | 26 | $C_{18}H_{21}N_5O_5$ | - | 4.46 | 86.43 ($3^{rd}$) | 17 | 0.552 | 352.14, 252.09, 189.09, 134.06 |
| *Cis*-zeatin[10] | 27 | $C_{10}H_{13}N_5O$ | + | 4.13 | 95.63 ($4^{th}$) | 7 | 0.958 | 204.09, 203.12, 202.11, 148.06 |
| *Trans*-zeatin[10] | 27 | $C_{10}H_{13}N_5O$ | + | 4.13 | 95.63 ($4^{th}$) | 7 | 0.958 | 204.09, 203.12, 202.11, 148.06 |
| *Cis*-zeatin riboside[11] | 28 | $C_{15}H_{21}N_5O_5$ | + | 4.88 | 94.97 ($8^{th}$) | 15 | 0.607 | 220.12, 205.11, 188.07, 181.06 |
| *Trans*-zeatin riboside[11] | 28 | $C_{15}H_{21}N_5O_5$ | + | 4.88 | 94.97 ($8^{th}$) | 15 | 0.607 | 220.12, 205.11, 188.07, 181.06 |
| *Cis*-zeatin-7-glucoside[12] | 29 | $C_{16}H_{23}N_5O_6$ | - | 4.50 | 93.45 ($9^{th}$) | 17 | 0.236 | 290.13, 218.10, 200.09, 161.05 |
| *Cis*-zeatin-9-glucoside[12] | 29 | $C_{16}H_{23}N_5O_6$ | - | 4.50 | 93.45 ($9^{th}$) | 17 | 0.236 | 290.13, 218.10, 200.09, 161.05 |
| *Trans*-zeatin-7-glucoside[12] | 29 | $C_{16}H_{23}N_5O_6$ | - | 4.50 | 93.45 ($9^{th}$) | 17 | 0.236 | 290.13, 218.10, 200.09, 161.05 |
| *Trans*-zeatin-9-glucoside[12] | 29 | $C_{16}H_{23}N_5O_6$ | - | 4.50 | 93.45 ($9^{th}$) | 17 | 0.236 | 290.13, 218.10, 200.09, 161.05 |
| Jasmonates | | | | | | | | |
| (±)-Jasmonic acid[13] | 30 | $C_{12}H_{18}O_3$ | + | 6.00 | 98.43 ($2^{nd}$) | 7 | 0.561 | 193.12, 155.07, 151.11, 112.09 |
| (±)-Jasmonic acid | 30 | $C_{12}H_{18}O_3$ | - | 6.00 | 98.08 ($2^{nd}$) | 5 | 0.959 | 165.13, 163.11, 109.07, 81.03 |
| (-)-Jasmonic acid[13] | 30 | $C_{12}H_{18}O_3$ | + | 6.00 | 98.43 ($2^{nd}$) | 7 | 0.561 | 193.12, 155.07, 151.11, 112.09 |
| (-)-Jasmonic acid | 30 | $C_{12}H_{18}O_3$ | - | 6.00 | 98.08 ($2^{nd}$) | 5 | 0.959 | 165.13, 163.11, 109.07, 81.03 |
| (±)-Jasmonic acid methyl ester[14] | 31 | $C_{13}H_{20}O_3$ | + | 6.38 | 98.47 ($2^{nd}$) | 6 | 0.170 | 209.12, 165.13, 125.10, 123.08 |
| (-)-Jasmonic acid methyl ester[14] | 31 | $C_{13}H_{20}O_3$ | + | 6.38 | 98.47 ($2^{nd}$) | 6 | 0.170 | 209.12, 165.13, 125.10, 123.08 |
| (+)-Cucurbic acid | 32 | $C_{12}H_{20}O_3$ | + | 6.55 | 97.90 ($2^{nd}$) | 12 | 0.841 | 195.14, 182.13, 181.12, 164.12 |
| (±)-9,10 dihydrojasmonic acid | 33 | $C_{12}H_{20}O_3$ | + | 6.19 | 98.12 ($2^{nd}$) | 7 | 0.393 | 195.14, 181.12, 125.06, 111.04 |
| (±)-3-Oxo-2-(2-(Z)-pentenyl)cyclopentane-1-hexanoic acid | 34 | $C_{16}H_{26}O_3$ | + | 6.36 | 94.47 ($2^{nd}$) | 17 | 0.937 | 251.20, 249.18, 213.15, 153.09 |
| (±)-3-Oxo-2-(2-(Z)-pentenyl)cyclopentane-1-octanoic acid | 35 | $C_{18}H_{30}O_3$ | + | 8.18 | 97.12 ($2^{nd}$) | 14 | 0.885 | 165.13, 151.11, 135.12, 125.10 |
| *Cis*-12-oxo-phytodienoic | 36 | $C_{18}H_{28}O_3$ | + | 7.66 | 96.79 ($2^{nd}$) | 15 | 0.868 | 277.22, 276.21, 275.21, 149.10 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| acid[15] | | | | | | | | |
| *Trans*-12-oxo-phytodienoic acid[15] | 36 | $C_{18}H_{28}O_3$ | + | 7.66 | 96.79 ($2^{nd}$) | 15 | 0.640 | 277.22, 276.21, 275.21, 149.10 |
| Dinor-12-oxo-phytodienoic acid | 37 | $C_{16}H_{24}O_3$ | + | 6.64 | 87.95 ($2^{nd}$) | 5 | 0.640 | 209.12, 122.07, 113.06, 81.07 |
| (±)-Tuberonic acid | 38 | $C_{12}H_{18}O_4$ | + | 5.01 | 97.40 ($2^{nd}$) | 19 | 0.905 | 210.13, 209.12, 192.11, 191.11 |
| (±)-Tuberonic acid methyl ester | 39 | $C_{13}H_{20}O_4$ | + | 6.51 | 96.80 ($2^{nd}$) | 15 | 0.985 | 182.13, 181.12, 156.08, 155.07 |
| | | | | | | | | |
| Gibberellins | | | | | | | | |
| Gibberellin A1 | 40 | $C_{19}H_{24}O_6$ | + | 4.76 | 87.71 ($8^{th}$) | 8 | 0.191 | 304.17, 303.16, 256.15, 255.14 |
| Gibberellin A1methyl ester | 41 | $C_{20}H_{26}O_6$ | - | 6.02 | 98.91 ($3^{rd}$) | 7 | 0.890 | 343.16, 318.18, 317.18, 283.10 |
| Gibberellic acid (GA3) | 42 | $C_{19}H_{22}O_6$ | - | 5.37 | 94.35 ($4^{th}$) | 3 | 0.127 | 301.14, 283.13, 271.10 |
| Gibberellin A20 | 43 | $C_{19}H_{24}O_5$ | - | 5.88 | 96.40 ($3^{rd}$) | 4 | 0.680 | 298.16, 288.17, 287.17, 161.06 |
| Gibberellin A4 methyl ester | 44 | $C_{20}H_{26}O_5$ | - | 6.54 | 95.89 ($3^{rd}$) | 5 | 0.624 | 328.17, 302.19, 301.18, 283.17 |
| Gibberellin A24 | 45 | $C_{20}H_{26}O_5$ | - | 5.95 | 93.32 ($8^{th}$) | 5 | 0.581 | 301.18, 299.17, 242.12, 228.12 |
| Gibberellin A44 | 46 | $C_{20}H_{26}O_5$ | + | 6.07 | 93.17 ($9^{th}$) | 8 | 0.976 | 329.17, 303.20, 302.19, 301.18 |
| Gibberellin A7 | 47 | $C_{19}H_{22}O_5$ | - | 5.19 | 97.80 ($1^{st}$) | 5 | 0.520 | 300.14, 286.12, 272.14, 175.08 |
| Gibberellin A8 | 48 | $C_{19}H_{24}O_7$ | + | 5.40 | 94.32 ($6^{th}$) | 5 | 0.323 | 302.11, 259.10, 122.11, 121.10 |
| Gibberellin A15 | 49 | $C_{20}H_{26}O_4$ | + | 6.64 | 96.18 ($2^{nd}$) | 4 | 0.532 | 313.18, 287.20, 286.19, 285.18 |
| Gibberellin A15 | 49 | $C_{20}H_{26}O_4$ | - | 6.64 | 96.02 ($2^{nd}$) | 4 | 0.410 | 301.18, 286.19, 285.19, 283.17 |
| Gibberellin A13 | 50 | $C_{20}H_{26}O_7$ | - | 6.07 | 92.78 ($9^{th}$) | 5 | 0.235 | 333.17, 315.16, 159.08, 101.02 |
| Gibberellin A53 | 51 | $C_{20}H_{28}O_5$ | - | 6.94 | 96.71 ($2^{nd}$) | 3 | 0.633 | 330.18, 302.19, 301.18 |
| | | | | | | | | |
| Salicylates | | | | | | | | |
| Salicylic acid | 52 | $C_7H_6O_3$ | - | 5.82 | 98.20 ($2^{nd}$) | 5 | 0.791 | 109.03, 108.02, 107.01, 93.03 |

## 3.4 PHYTOHORMONE PROFILING TO UNRAVEL PLANT METABOLISM

The potential of the presented profiling strategy with respect to metabolomics experiments was indicated by a multivariate data analysis approach. In first instance, it was aimed to reveal any clustering, grouping or patterning among tomato fruit samples on the sole basis of the associated phytohormone profiles and in correspondence with the defined fruit developmental stages. For this particular purpose, quantitative data (peak areas) regarding the 52 annotated ions were gathered for each tomato fruit sample that has been analyzed. Good performance of the applied PCA-modeling (Figure 4.5 A) was indicated by the values of $R^2(X)$ (0.96, representing the explained variation in X) and $Q^2$ (X) (0.55, representing the goodness of prediction calculated by full 7-fold cross validation). At first sight, some clustering of tomato fruit samples was revealed by the PCA score scatterplot (Figure 4.5 A). In this regard, unsupervised HCA was performed to investigate the real clustering and potential similarities between the different tomato fruit samples. Hereby, sample similarities are designated based on a two-dimensional diagram, i.e. dendrogram (Figure 4.5 B). This approach indicated a clustering that was strongly related to the defined developmental stages. More specifically, tomato fruit samples were assigned to clusters, which could be considered as enclosing a particular developmental stage. This with the exception of fruit sample $d_3$, which was ascribed a more advanced stage of development than assumed according to DAA. It may thus generally be stated that the established phytohormone profile has the potential to discriminate between tomato fruits in terms of developmental stage or ripening degree. This finding reflects as such a significant role of phytohormones towards these processes, which has been reported (29-32). In this context, a subsequent elaborated supervised approach of partial least squares (PLS) or orthogonal PLS (OPLS) discriminant analysis may eventually reveal those phytohormones with a leading contributing role. However, the generated loading plot (Figure 4.5 C) may already give a good indication about which phytohormones or phytohormonal classes are most likely to be involved in tomato fruit development and ripening.

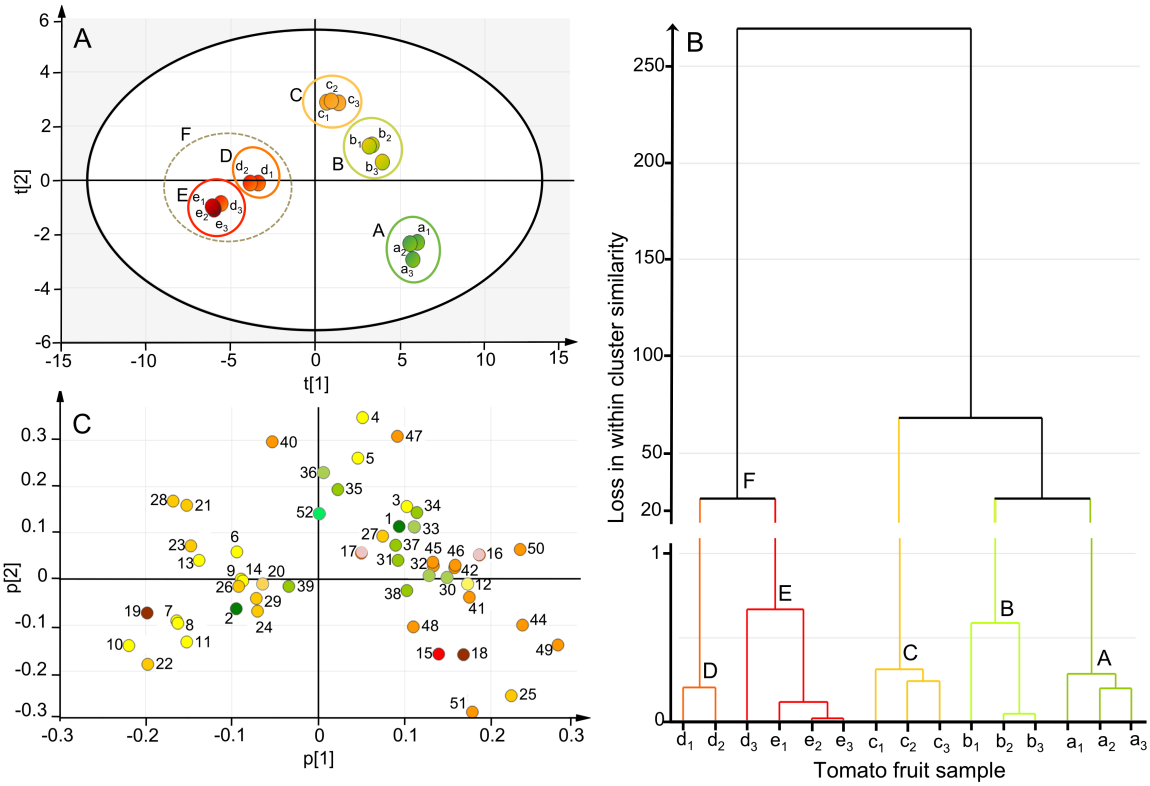


**FIGURE 4.5.** Principal component analysis score plot (A) for tomato fruits, representing various developmental stages: Stage 1, 7.3 ± 1.5 DAA; Stage 2, 13.7 ± 1.5 DAA; Stage 3, 19.7 ± 0.6 DAA; Stage 4, 28.3 ± 0.6 DAA; Stage 5, 38.6 ± 0.6 DAA. The dendrogram encloses information about the similarities of samples (i.e. clusters), which has been transferred into panel A. The loading plot (C) provides a first indication about the involvement of individual ions towards tomato fruit development and ripening. Numbering of metabolite ions refers to the ion IDs, presented in Table 4.1. Coloration is performed according to the various phytohormonal classes ( ● abscisates (1-2); ● auxins (3-14); ● polyamines (15); ● strigolactones (16-17); ● brassinosteroids (18-19); ● cytokinins (20-29); ● jasmonates (30-39); ● gibberellins (40-51); ● salicylates (52)).

This example nicely demonstrates how the presented profiling strategy may assist in efficiently screening for a possible involvement of phytohormones in the biological process under investigation and to determine the significance of specific hormonal classes or individual phytohormones. Hereby, the investigated process may relate to tomato fruit development, physiology, metabolism, biotic and abiotic responses, physiological disorders, etc. Moreover, as demonstrated by the example above, (part of) the established phytohormone profile may be used as an indicator of fruit age, which might be more accurate than DAA. Evidently, the profiling strategy would also be of interest for and could therefore be focused on other plant organs such as leafs and roots. In addition, this strategy may have its use towards other plant species as well. In conclusion, the main underlying reason for applying such a phytohormone profiling strategy relates

to a more efficient unraveling of plant processes in which a regulating action of phytohormones could be assumed. Although other metabolites may have a significant role in the targeted biological processes, the available knowledge on the regulation actions of specific compounds offers great opportunities to obtain valuable results rapidly or to indicate the general research orientation. In this context, it should be kept in mind that the acquired phytohormone profile is only an approximation of what the real fingerprint could be. Firstly, one must be aware that there are limits to the detection capability of the applied analytical platform (ng $g^{-1}$ or pg $g^{-1}$ fresh weight) and that phytohormones are typically present and active in plants at very low concentrations (ng $g^{-1}$ fresh weight) (33). Secondly, decisive answers about the phytohormonal identities of the targeted metabolites should be established by means of reference standards (21). Thirdly, as research on the biosynthesis and activity of phytohormononones progresses, it is becoming clear that a diversity of metabolites, related to the different classical phytohormones (e.g. by hydroxylation, methylation, glycosylation, amino acid conjugation), and newly recognized phytohormones might enlarge the established profile.

# 4. CONCLUSIONS

In this study, an analytical method based on UHPLC and high-resolution hybrid quadrupole Q-Exactive Orbitrap-MS was developed for the metabolic profiling of phytohormones in tomato fruit. The possibility of tandem mass spectrometry offers additional identification power compared to single-stage high-resolution mass analyzers. This more extensive identification potential is regarded as critically important for holistic metabolomic experiments in which the identification of unknown metabolites is often considered as the main bottleneck. Using this analytical platform and its inherent identification capabilities, profiles of putatively annotated phytohormones were established for tomato fruits, representing various developmental stages and thus covering divergent phytohormone profiles. However, these profiles were founded on the current knowledge regarding phytohormonal active metabolites. One should be aware that progress about known and unknown metabolites, which are assigned hormonal activity, is still on going.

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# CHAPTER V

## A METABOLOMICS APPROACH TO UNRAVEL THE REGULATING ROLE OF PHYTOHORMONES TOWARDS CAROTENOID METABOLISM IN TOMATO FRUIT

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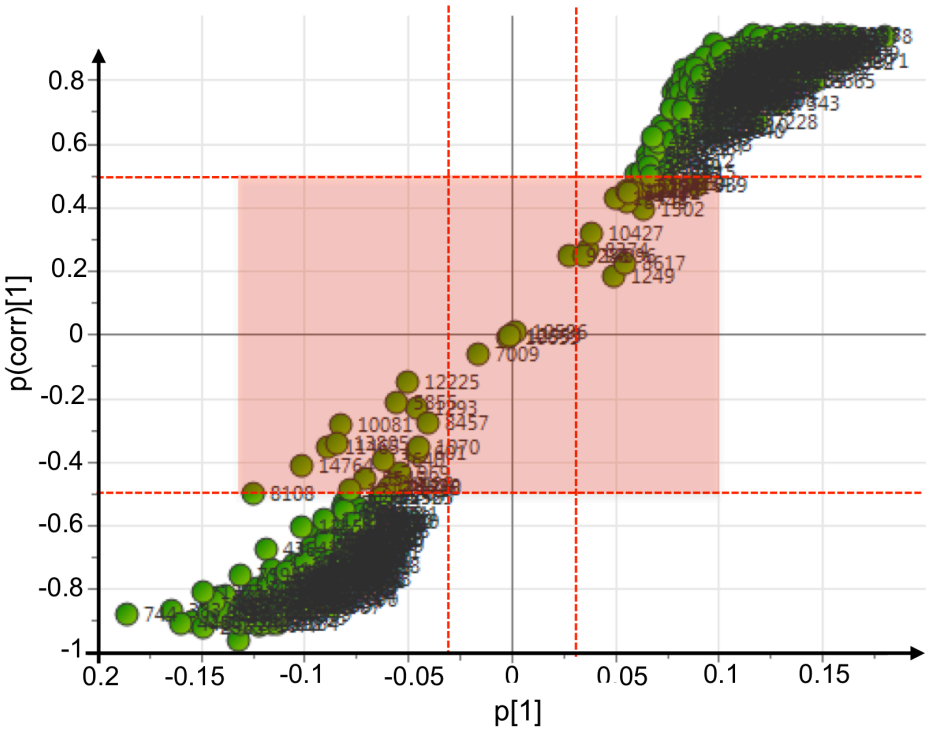

**Adapted from:**

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# ABSTRACT

Carotenoids are important secondary metabolites, which have been recognized as an essential component of the human diet because of their valuable beneficial health effects. With this rationale, there is a continuous aim to define the distribution of these compounds in plants, to better understand their metabolism and to increase their concentration levels in fruits and vegetables. This study aimed at deepening the knowledge on the regulatory role of phytohormones in carotenoid metabolism. More specifically, it was envisaged to reveal the phytohormones involved in the metabolism of $\alpha$-carotene, $\beta$-carotene, lycopene, lutein and zeaxanthin. To this purpose, the phytohormone profiles of 50 tomato fruits were determined by high-resolution Orbitrap mass spectrometry and evaluated towards the associated carotenoid levels. Data mining was performed by differential expression and orthogonal partial least squares analyses. This metabolomics approach revealed 5 phytohormonal metabolites, which significantly influenced (Variable Importance in Projection scores $\geq$ 0.80) carotenoid metabolism. These metabolites were identified as *cis*-12-oxo-phytodienoic acid, cucurbic acid, 2-oxindole-3-acetic acid, 1-acetylindole-3-carboxaldehyde, and *cis*-zeatin-O-glucoside. The involvement of the individual phytohormones towards carotenoid metabolism was investigated by regression analysis (P-values $\leq$ 0.05, $R^2$ varying between 0.280 and 0.760) and statistical correlation (P-values $\leq$ 0.01, correlation varying between 0.403 and 0.846). It was concluded that these phytohormones all have significant contributing value in the regulation of carotenoid metabolism, thereby exhibiting down- and up-regulating influences. As a result, this knowledge encloses the potential for improving tomato fruit nutritional quality by targeted control of agronomic conditions, exogenous use of plant bioregulators, or genetic engineering.

# 1. INTRODUCTION

Carotenoids represent a diverse group of secondary metabolites, which are widely distributed in nature (1). The diversity within this group is reflected by the more than 700 different carotenoids, which have already been isolated and identified from various natural sources (2). The primary sources, involved in carotenoid biosynthesis, are predominantly photosynthesizing organisms such as green plants, algae and certain bacteria (3). Although not all the biosynthetic steps have been elucidated, a detailed outline of the current understanding with respect to carotenogenesis is described in Bramley (2002) (4) and Shumskaya and Wurtzel (2013) (5). With respect to the structural properties, carotenoids are typically characterized by a conjugated double bound system, which is determinative for their various natural functions and actions (6). In plants, carotenoids are of importance for photosynthesis since they are defined as being ancillary light pigments, photo-protectors and basic units of the photosynthetic apparatus (7). Furthermore, these hydrophobic compounds are also involved in the stabilization of membrane lipids, are ascribed antioxidant properties, and serve as attractants of insects and animals for pollination and seed dispersal (1,7). In addition, these compounds have been recognized as an essential component of human diet because of the associated health benefits (8).

In epidemiological and clinical studies, the intake of carotenoids has indeed been associated with a reduced prevalence of chronic degenerative diseases, including cancers, cardiovascular disorders and age-related macular degeneration (9). This finding was mainly attributed to a number of biological functions of carotenoids, i.e. provitamin A activity, immune response modulation, antioxidant effects and induction of gap junction communication (10). Because of the indispensible roles of carotenoids in plants and their important health benefits to humans, intensive efforts have been made to understand their metabolism, define their distribution in plants, and increase their levels in fruits and vegetables (11).

With respect to carotenoid metabolism, significant progress has been made in understanding biosynthesis and catabolism in plants. However, the current challenge regarding carotenoid metabolism relates to the identification and deepening of regulatory mechanisms and processes (1,4,6,12). Within this context, phytohormones are of particular interest because of their

regulatory functions in various physiological and developmental plant processes (5). As reported by Srivastava and Handa (2005) (13), various phytohormones are involved in fruit development and ripening, which are both accompanied by quantitative and qualitative changes in the carotenoid profile (6). Although the relevance of phytohormones towards carotenoid metabolism has been described, inadequate knowledge about the specific involvement of phytohormones and their modes of actions is available (13).

This study aimed at deepening the knowledge on the regulatory role of phytohormones towards carotenoid metabolism in tomato fruit. It is indeed stated that tomato (*Solanum lycopersicum* L.) is an excellent model for fleshy fruit research. Moreover, tomato is among the most consumed vegetable crops worldwide and of great significance because of its high nutritional value (14). During this study, it was opted to particularly investigate the regulating role of phytohormones towards the metabolism of $\alpha$-carotene, $\beta$-carotene, lycopene, lutein, and zeaxanthin since these are among the major carotenoids in tomato fruit (15). Furthermore, because of the complex crosstalk among phytohormone-signalling pathways, a metabolomics (i.e. hormonome) approach was undertaken (16,17). Metabolomic profiling techniques are of particular interest since they can reveal a comprehensive view on the relative levels of hundreds to thousands of metabolites, present in the plant material under investigation (18,20). As a consequence, based on the overall metabolome differences and similarities between well-chosen sample types, a metabolomics approach has the potential to indicate the significance of a certain phytohormone towards the metabolism of specific carotenoids. With regard to the metabolomic profiling aims, full-scan mass spectrometry (MS) appears to be most designated because of the associated properties (21-24). Full-scan mass analysis offers indeed the possibility to simultaneously analyse a virtually unlimited number of compounds. Additionally, the retrospective post-acquisition evaluation of data allows screening for analytes that were not *a priori* selected (25). In this context, Fourier Transform Orbitrap-MS is extremely suited since the applied technology provides precise mass accuracy (mass deviations < 2 ppm, mass resolving power up to 100,000 full width at half maximum (FWHM)), resulting in both high selectivity and sensitivity for analysis of samples with complex matrix co-extracts (18,20,26). The proposed research approach is believed to be most adequate for deepening the knowledge about the plant hormone network and its association with biological phenomena, i.e. carotenoid metabolism.

# 2. MATERIALS AND METHODS

## 2.1 PLANT MATERIAL AND SAMPLE PREPARATION

Tomato plants (*Solanum lycopersicum* L. cv. Moneymaker) were grown in a greenhouse compartment of the Institute for Agricultural and Fisheries Research (ILVO, Melle, Belgium) and were subjected to normal cultural practices (27). When tomato plants were 29 weeks old, 108 tomato fruits were collected from 4 tomato plants during a single harvest moment. Considering the days after anthesis (DAA), the harvested tomato fruits were classified into five groups (Table 5.1), representing various developmental stages.

**Table 5.1.** Grouping of tomato fruits on the basis of their developmental stage, reflected by days after anthesis (DAA). The number of different plants and trusses from which selected tomato fruits originated, is indicated as well. Based on this grouping, tomato fruits (total n = 50) were selected for subsequent carotenoid and phytohormone analysis.

| Classification | Days after anthesis (DAA) | Origin of fruits (n = 10), selected for analysis |
|---|---|---|
| Group 1 | 22 – 30 | 3 plants, 3 trusses |
| Group 2 | 31 – 39 | 3 plants, 3 trusses |
| Group 3 | 40 – 48 | 4 plants, 4 trusses |
| Group 4 | 49 – 57 | 4 plants, 6 trusses |
| Group 5 | 58 – 68 | 4 plants, 5 trusses |

Next, from each group, 10 fruits were selected for analysis. As such, different stages of fruit development and ripening were enclosed, whereby a wide range of carotenoid concentrations and divergent phytohormone profiles were covered. Selection of these fruits was each time realized in such a way that the number of different plants and trusses, from which the fruits originated, was maximized. For each single batch of 10 fruits, at least three different plants and thus at least three different trusses were considered. The consecutive cutting, lyophilization, grinding and sieving of each individual tomato fruit resulted in a homogenous powder, which allowed representative sampling. It should be noted that from the moment that the tomato fruits were harvested, they were kept cold (-20 °C) and shielded from light in order to prevent degradation of any fruit component.

## 2.2 CHEMICALS AND REAGENTS

The carotenoid analytical standard all-*trans*-α-carotene was purchased from Wako Chemicals GmbH (Neuss, Germany), all-*trans*-β-carotene was from Sigma-Aldrich Co. (St. Louis, MO, USA), all-*trans*-lycopene was from Phytolab GmbH & Co. KG (Vestenbergsgreuth, Germany), all-*trans*-lutein was from Extrasynthese (Genay, France) and all-*trans*-zeaxanthin was from TRC Inc. (Eching, Germany). The internal standard β-apo-8'-carotenal was obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). The phytohormone analytical standards *trans*-zeatin-O-glucoside, 2-oxindole-3-acetic acid, *cis*-12-oxo-phytodienoic acid, tuberonic acid, tuberonic acid methyl ester, jasmonic acid methyl ester and cucurbic acid were purchased from OlChemIm Ltd. (Olomouc, Czech Republic), whereas 1-acetylindole-3-carboxaldehyde was obtained from Acros Organics (Geel, Belgium). The deuterium-labelled internal standards $d_5$-indole-3-acetic acid, $d_5$-zeatin, $d_7$-$N^6$-benzyladenine and $d_6$-abscisic acid were obtained from OlChemIm Ltd. (Olomouc, Czech Republic). Reagents were of analytical grade when used for extraction purposes and of LC-MS grade for (U)HPLC-Orbitrap-MS applications. They were respectively purchased from VWR International (Merck, Darmstadt, Germany) and Fisher Scientific (Loughborough, UK). Ammonium acetate and formic acid were obtained from VWR International (Merck, Darmstadt, Germany), magnesium carbonate ($MgCO_3$) was from Sigma Aldrich (St. Louis, MO, USA). Ultrapure water was obtained by usage of a purified-water system (VWR International, Merck, Darmstadt, Germany).

## 2.3 CAROTENOID ANALYSIS

The carotenoids, which were selected for targeted analysis, included both carotenes (i.e. α-carotene, β-carotene, lycopene) and xanthophylls (i.e. lutein, zeaxanthin). Analysis was performed according to the procedures, described by Van Meulebroek *et al.* (2014) (20) (Chapter II). Although the employed analytical platform, consisting of HPLC coupled to Exactive™ single-stage Orbitrap-MS (Thermo Fisher Scientific, San José, USA), had the capacity to effectuate a metabolomic screening, no such untargeted approach was aimed in this study. Concentrations of the targeted carotenoids were calculated by means of matrix-matched calibration curves whereby potential fluctuations during analysis were counteracted by using an internal standard, i.e. β-apo-8'-carotenal. Good quantitative performance of the applied analytical method has earlier been demonstrated (Chapter II).

## 2.4 PHYTOHORMONE ANALYSIS

Extraction and detection of phytohormones and related metabolites from tomato fruit tissue were performed as described by Van Meulebroek *et al.* (2012) (18) (Chapter III). Detection of phytohormones was achieved by means of UHPLC coupled to Exactive$^{TM}$ single-stage Orbitrap-MS (Thermo Fisher Scientific, San José, USA). The full-scan operating principle of this type of mass spectrometer and the associated metabolomic screening possibilities were exploited to establish the phytohormone (and related metabolites) profiles of the individual tomato fruits. To ensure data quality for metabolomic screening purposes, system stability during analysis was verified by using matrix-matched calibration curves (18). These curves (6 calibration levels) were set up in early-stage tomato fruit matrix and were run at the beginning and end of the batch of samples. For each of the major hormonal classes, at least one representative was considered. The following average coefficients of variance (n = 6) were obtained: 6.25% for (±)-*cis, trans*-abscisic acid, 5.07% for $N^6$-benzyladenine, 6.93% for epibrassinolide, 6.26% for gibberellic acid, 5.97% for jasmonic acid, 9.26% for salicylic acid, 0.77% for *trans*-zeatin, and 7.43% for indol-3-acetic acid. Since these values were well below 15% (28), appropriate stability during analysis was concluded.

## 2.5 CHEMOMETRIC DATA ANALYSIS

### 2.5.1 GENERAL WORK METHODOLOGY

The objective of this study was to define phytohormonal metabolites, which are significantly involved in the metabolism of specific carotenoids. Therefore, 50 tomato fruits were individually analysed to gather information about both their prevailing carotenoid concentrations (targeted approach) and phytohormone profile (untargeted approach). Subsequently, to investigate the potential involvement of a phytohormonal metabolite in the metabolism of a specific carotenoid, it was verified to what extent the abundance of that phytohormone could be related with the concentration of that carotenoid. To this purpose, a general work methodology (Figure 5.1), including four steps, was implemented. It should be stressed that for each of the selected carotenoids, the proposed work methodology was each time re-implemented from the beginning. For clarity reasons, the various steps of the proposed work methodology are discussed on the basis of a single carotenoid.

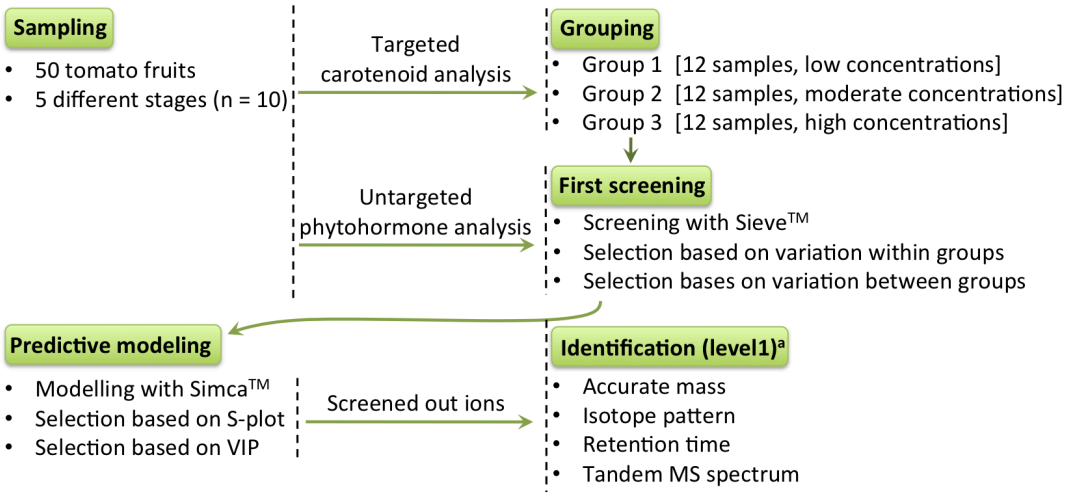


**FIGURE 5.1.** Overview of the applied metabolomic strategy, which aimed at revealing phytohormones that are involved in the metabolism of the carotenoids α-carotene, β-carotene, lycopene, zeaxanthin and lutein. Identification of potentially interesting compounds was based on accurate mass, isotopic pattern, retention time, and high-resolution tandem MS data. Using these criteria, compounds could be identified at the highest level ([a]level 1) of confidence, as defined by Sumner *et al.* (2007) (19).

### 2.5.2 STEP 1: GROUPING OF SAMPLES BASED ON CAROTENOID CONCENTRATION

During the first step of the applied work methodology, three groups of 12 samples (i.e. tomato fruits) were virtually created. Allocation of a sample into a specific group was based on the carotenoid concentration of that sample. Since carotenoid concentrations are strongly correlated with fruit development and ripening (13), it can be stated that the groups were in essence reflections of various fruit developmental stages. In this regard, DAA could also be envisaged as a grouping factor. However, this would evidently be less representative for the actual carotenoid concentrations since these are strongly depending on passed and prevailing growth conditions, fruit load, fruit position, etc. (15,29). The groups were created to enable a subsequent screening for (phytohormonal) metabolites, which may explain carotenoid concentration differences between groups. For this reason, statistical differences between groups in terms of carotenoid concentration were pursued. Statistical differences (SPSS™ statistics 21.0) were evaluated using one-way ANOVA and post-hoc Tukey's multiple comparisons test (P-values ≤ 0.05).

### 2.5.3 STEP 2: FIRST SCREENING USING SIEVE™ SOFTWARE

During the second step, a first screening for metabolites potentially involved in carotenoid metabolism was realized. For this purpose, the full-scan data files, enclosing the phytohormone profiles, were used and classified according to the previously created groups. The underlying

motivation for this particular approach lays in the fact that the probability is low that a metabolite, whose abundance is hardly depending on the allocated group, is of great significance with respect to carotenoid metabolism or concentration. For this untargeted screening of relevant metabolites, i.e. metabolites whose abundance is thus strongly depending on the considered group, Sieve™ 2.1 (Thermo Fisher Scientific, San José, USA) was used. Processing of the full-scan data by Sieve™ followed a multiple step strategy. The first step involved the selection of appropriate parameter settings in order to perform the differential expression analysis. The following settings were selected: a *m/z*-range of 100 - 800 Da, a *m/z* width of 5 ppm, a retention time range of 1.5 - 9.0 min, a peak intensity threshold of 25,000 arbitrary units, a maximum peak width of 0.5 min, and a maximum number of 15,000 frames. The second step included the peak alignment process during which corrections for inherent chromatographic variability were made. In the third step, Sieve™ reported the signal abundance for each ion $[m/z, RT]_i$, in each of the considered samples. During the search, all peaks that were above a given peak intensity threshold were taken into consideration. The ions, associated with these peaks, were collected from all raw LC-HRMS data to prevent any loss of information. During the final step, an actual screening for potential valuable metabolite ions was realized through a number of discriminative parameters. The inclusion of these parameters allowed reducing the number of metabolite ions that were eligible for further assessment. A first parameter referred to the differences between groups regarding the average ion abundances, i.e. the ratio. Only metabolite ions with at least an average 2-fold difference in peak abundance were retained. A second parameter concerned the coefficient of variance (CV), considered within each group. Selection of the maximum allowable CV was based on the CV-profile, which graphically represents the relation between the percentage of metabolite ions and the CV-value within a certain group. The maximum allowable CV-value varied between 60 and 80%. Metabolite ions were thus screened and selected on the basis of their behaviour within and between groups.

### 2.5.4 STEP 3: PREDICTIVE MODELLING USING SIMCA™ SOFTWARE

During the third step of the proposed work methodology, the abundances of the retained metabolite ions were used to construct a prediction model, using SIMCA™ 13 software (Umetrics, Malmö, Sweden). Such a model intends to explain and predict one Y-variable (i.e. carotenoid concentration) from the X-matrix (i.e. metabolite ion abundances). Based on this model, the

significance of the selected metabolite ions with respect to carotenoid metabolism could be revealed. For this purpose, partial least squares (PLS) models, which are commonly used to mathematically describe the quantitative relationship between the Y-variable and X-matrix (30), are highly suitable. In addition, a recent modification of the PLS method, i.e. orthogonal PLS (OPLS), was introduced. The main idea of OPLS is to separate the systematic variation in X into two elements: one that is linearly related (predictive) to Y and one that is unrelated (orthogonal) to Y (31). Therefore, during model optimization both PLS and OPLS were evaluated by means of SIMCA$^{TM}$. Furthermore, the implementation of logarithmic data transformation was assessed with respect to data normality, which is described in SIMCA$^{TM}$ by skewness and Min/Max. Another data pre-processing parameter concerned scaling, whereby the range of each variable was taken into account. Three types of scaling were taken into consideration, i.e. unit variance, pareto and centering.

During optimization, the model-validity was verified by CV-ANOVA, permutation testing, and considering three model characteristics: 1) $R^2(X)$ corresponding to the predictive and orthogonal variation in X that is explained by the model, 2) $R^2(Y)$ defining the total sum of variation in Y that is explained by the model, 3) $Q^2(Y)$ referring to the goodness of prediction calculated by full cross-validation. In SIMCA$^{TM}$, a typical 7-fold cross-validation procedure was conducted to validate the (O)PLS models against over-fitting (32,33). The estimated predictive ability of the model was evaluated by cross-validated ANOVA, using the cross-validated predictive Y-residuals and denoting significant models with P-values < 0.05. Response permutation testing was performed to estimate the significance of the generated models whereby the order of elements in the Y-vector was randomly permutated 200 times (34).

### 2.5.5 STEP 4: IDENTIFICATION OF RELEVANT METABOLITE IONS

A first step within the identification of relevant ions, up to now solely described by their accurate *m/z*-value and retention time, was completed by Sieve$^{TM}$ Database Lookup. With this feature, a specific ion could be recognized as a potential phytohormonal compound. For this purpose, a database was constructed by implementing the molecular formula of 275 phytohormonal compounds (35). Moreover, the isotopic pattern was simultaneously consulted as a secondary identification parameter. A second step within the identification process focused on the chemical structure of the retained ions and was performed by matching experimental collision-induced

dissociation fragmentation spectra with computational spectra (39). To this extent, all candidate compound structures were processed by Mass Frontier 5.0 (Thermo Fisher Scientific, San José, USA), predicting fragmentation spectra of protonated and deprotonated molecules, using the general and library fragmentation mechanisms. The maximum number of reaction steps was 4. Experimental fragmentation spectra were generated by Q Exactive™ hybrid quadrupole-Orbitrap mass spectrometry (Thermo Fisher Scientific, San José, USA). In addition to the common features of an Orbitrap mass analyser, there is amongst others the possibility of data-dependent fragmentation (dd-$MS^2$). This feature is founded on precursor ion selection, which is enabled by the presence of a quadrupole mass filter between the ion source and the C-trap. Although these dd-$MS^2$ experiments proved extremely suited for selective fragmentation and the intended structure elucidation, other hybrid quadrupole Orbitrap MS experiments such as full MS/AIF (all ion fragmentation), full MS + $tMS^2$ (targeted $MS^2$), full MS/dd-$MS^2$ and tSIM (targeted single ion monitoring)/dd-$MS^2$ are ascribed significant value as well (40). Prior to the actual mass analysis, chromatographic separation of the analytes was achieved on an UltiMate LPG-3400XRS pumping system (Thermo Fisher Scientific, Breda, the Netherlands) and followed the method, described in section 2.4. Instrument control and data processing were carried out by Xcalibur 2.2 software (Thermo Fisher Scientific, San José, USA). The instrument was equipped with a heated electrospray ionization source (HESI-II), operating in polarity switching mode. Ionization source working parameter settings were identical as for Exactive™ Orbitrap mass analysis (18). With respect to dd-$MS^2$, it was opted to use an inclusion list in which parent ions were specified for fragmentation. The inclusion list was constructed by considering the theoretical molecular masses, calculated by Xcalibur 2.2 software, associated with the metabolites of interest. Full-scan experiments were combined with dd-$MS^2$ experiments. The data-dependent scan was initiated when a minimum percentage, i.e. 1.0%, of the full-scan AGC target was reached by any ion. This specifically implies a minimum number of $5 \times 10^4$ ions. Although the criteria above provided a first indication regarding a compound's identity, additional confirmation was found crucial. Therefore, analytical standards were purchased, whereby relative retention times could be used as an additional identification parameter. Furthermore, the fragmentation spectra of the analytical standards enclose supportive value in confirming a compound's assigned identity. It should be noted that fragmentation spectra of authentic standards were obtained by Orbitrap Exactive™ HCD fragmentation (30 eV).

# 3. RESULTS AND DISCUSSION

## 3.1 CHEMOMETRIC DATA ANALYSIS

### 3.1.1 STEP 1: GROUPING OF SAMPLES BASED ON CAROTENOID CONCENTRATION

For each single carotenoid, a grouping of samples was implemented by using the respective carotenoid concentration as grouping factor. As a consequence, for each individual carotenoid, the samples and associated data files were differently grouped. When all 50 fruits were used for grouping, no significant differences could be obtained for all carotenoids between the different groups. Subsequent screening for relevant compounds would have no significance in that case. Therefore, the number of samples within each group had to be reduced. The number of samples within each group was determined by considering the variance within and the concentration differences between groups. Since both parameters are inversely proportional to the number of samples, a compromise was sought. It was found that by including 12 samples per group, significantly different groups could be established for each single carotenoid. This was reflected by the calculated P-values, which were maximally 0.047. It should be noted that this manipulation arises from the fact that the harvested fruits enclosed various developmental stages, which resulted in continuous carotenoid concentration profiles within groups. The created groups, compositionally dependent on the considered carotenoid, were characterized by average concentration levels as reported in Table 5.2. The methodology used for grouping of the samples is exemplified for lycopene and presented in Figure 5.2.

**Table 5.2** To enable a first screening for relevant phytohormones, tomato fruit samples were virtually classified into 3 groups. Each group included 12 samples and was created based on the carotenoid concentration. For each carotenoid, the average concentration is reported. It should be noted that the specific sample composition of the three groups differs as a function of considered carotenoid.

| Carotenoid | Classification | Average conc. (± stdev) (ng $mg^{-1}$ dry weight) |
|---|---|---|
| *α-carotene* | Group 1 | 0.10 ± 0.05 |
| | Group 2 | 0.33 ± 0.03 |
| | Group 3 | 0.7 ± 0.2 |
| *β-carotene* | Group 1 | 26.1 ± 3.4 |
| | Group 2 | 42.3 ± 3.4 |
| | Group 3 | 70.5 ± 21.3 |
| *Lycopene* | Group 1 | 0.7 ± 0.4 |
| | Group 2 | 17.3 ± 8.1 |
| | Group 3 | 54.0 ± 18.8 |
| *Lutein* | Group 1 | 17.9 ± 2.9 |
| | Group 2 | 32.6 ± 5.2 |
| | Group 3 | 60.8 ± 13.5 |
| *Zeaxanthin* | Group 1 | 8.9 ± 4.5 |
| | Group 2 | 26.8 ± 12.5 |
| | Group 3 | 87.6 ± 27.6 |

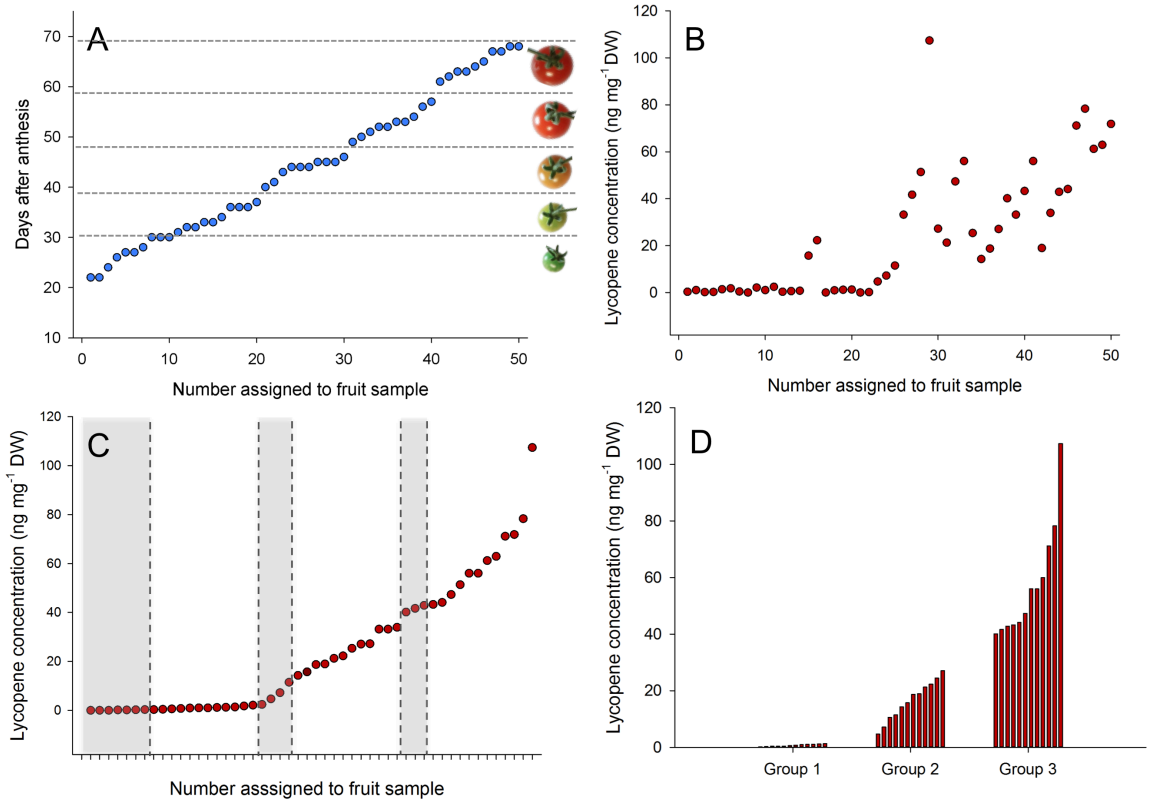


**FIGURE 5.2.** A) Available data set containing 50 tomato fruits, which are ranked according to DAA and subsequently assigned a number; B) Available data set of 50 fruits with information about the lycopene concentration levels; C) Available data set of 50 fruits ranked according to lycopene concentration levels, thereby shuffling the numbers on the X-axis (not presented due to lack of space); D) Created groups with 12 samples per group, composed based on figure panel C (grey shaded areas were fruits that were not selected). This procedure was specifically executed for each individual carotenoid.

### 3.1.2 STEP 2: FIRST SCREENING USING SIEVE™ SOFTWARE

To perform the differential expression analysis and associated first screening, the full-scan data from the phytohormone analysis were used. Since these data included information about compounds, obtained in both positive and negative mode, the differential analysis was performed for each ionization mode separately. This resulted in the generation of separate lists of ions, whereby each ion was characterized by a specific *m/z*-value and retention time. The number of detected ions varied for each of the considered carotenoids and for both ionization modes between about 12,500 and 15,000. A first screening for potential relevant ions was based on the mutual variance between groups, only retaining metabolite ions with at least an average 2-fold difference in peak abundance. A second selection of metabolites ions was based on the variance within the various groups. The maximum allowed coefficients of variance (CV) were a function of the CV-

profiles and varied between 60 and 80%. Furthermore, only $^{12}C$ ions were retained. The number of potentially relevant metabolite ions was as such strongly reduced for both ionization modes (negative and positive, respectively): 523 and 354 for $\alpha$-carotene, 585 and 414 for $\beta$-carotene, 724 and 598 for lycopene, 548 and 518 for lutein, and 506 and 581 for zeaxanthin.

### 3.1.3 STEP 3: PREDICTIVE MODELLING USING SIMCA™ SOFTWARE

To elucidate the metabolite ions that are actually involved in carotenoid metabolism, multivariate data-analysis, based on (O)PLS modelling, was performed. With this modelling, it was attempted to design models, which are able to predict carotenoid concentrations by using the peak intensities of the metabolite ions that were previously retained. For this modelling, the model type (PLS or OPLS), the way of data scaling (unit variance, pareto or centre scaling), and the use of logarithmic data transformation were optimized. Based on the skewness and Min/Max ratio of the data sets, it was opted to perform logarithmic data transformation in order to generate normally distributed data. In addition, pareto scaling was applied since substantially different ranges were observed among X-variables. The advantage of using pareto scaling compared to UV is that it reduces the impact of noise and artifacts in the models, which has a positive influence on the models' predictive ability (30). The validity of the generated models (Table 5.3) was ascertained by verifying the model quality with cross-validated Y-residuals (CV-ANOVA, P-values < 0.01), permutation testing, and three model characteristics, i.e. $R^2(X)$, $R^2(Y)$, and $Q^2(Y)$. According to Hawkins *et al.* (2003) (41), a large $Q^2(Y)$ (i.e. > 0.5) indicates good predictability of the model. Only for the $\alpha$-carotene models, $Q^2(Y)$ did not reach the target value. The inferior quality of the $\alpha$-carotene models was also reflected by the higher CV-ANOVA P-values, compared to the other models. However, since P-values were for these models still < 0.01, it was decided to further use these particular models (34).

**TABLE 5.3.** Description of the OPLS models with regard to data scaling (pareto (PAR), centre (Ctr), or unit variance (UV)) and transformation. For each model, the quality is indicated by three model characteristics. $R^2(X)$ and $R^2(Y)$ determine to which extent the model is able to explain the variance within the set of X- and Y-variables, respectively. $Q^2(Y)$ is indicative for the predictive value towards new data.

| *Model content* | | *Model parameters* | | *Model quality* | | |
|---|---|---|---|---|---|---|
| | | Scaling | Transformation | $R^2(X)$ | $R^2(Y)$ | $Q^2(Y)$ |
| Lycopene | ~ Negative metabolite ions | Par | Logarithmic | 0.780 | 0.926 | 0.753 |
| | ~ Positive metabolite ions | Par | Logarithmic | 0.781 | 0.974 | 0.922 |
| $\alpha$-carotene | ~ Negative metabolite ions | Par | Logarithmic | 0.644 | 0.492 | 0.446 |
| | ~ Positive metabolite ions | Par | Logarithmic | 0.748 | 0.489 | 0.338 |
| $\beta$-carotene | ~ Negative metabolite ions | Par | Logarithmic | 0.786 | 0.788 | 0.674 |
| | ~ Positive metabolite ions | Par | Logarithmic | 0.832 | 0.821 | 0.686 |
| Lutein | ~ Negative metabolite ions | Par | Logarithmic | 0.693 | 0.681 | 0.659 |
| | ~ Positive metabolite ions | Par | Logarithmic | 0.774 | 0.834 | 0.782 |
| Zeaxanthin | ~ Negative metabolite ions | Par | Logarithmic | 0.804 | 0.846 | 0.750 |
| | ~ Positive metabolite ions | Par | Logarithmic | 0.793 | 0.814 | 0.781 |

For all the other models, the outlined diagnostic validation approaches ensured that models were robust, significant and not over-fitted. The graphical output of the model implementations is presented in Figure 5.3.

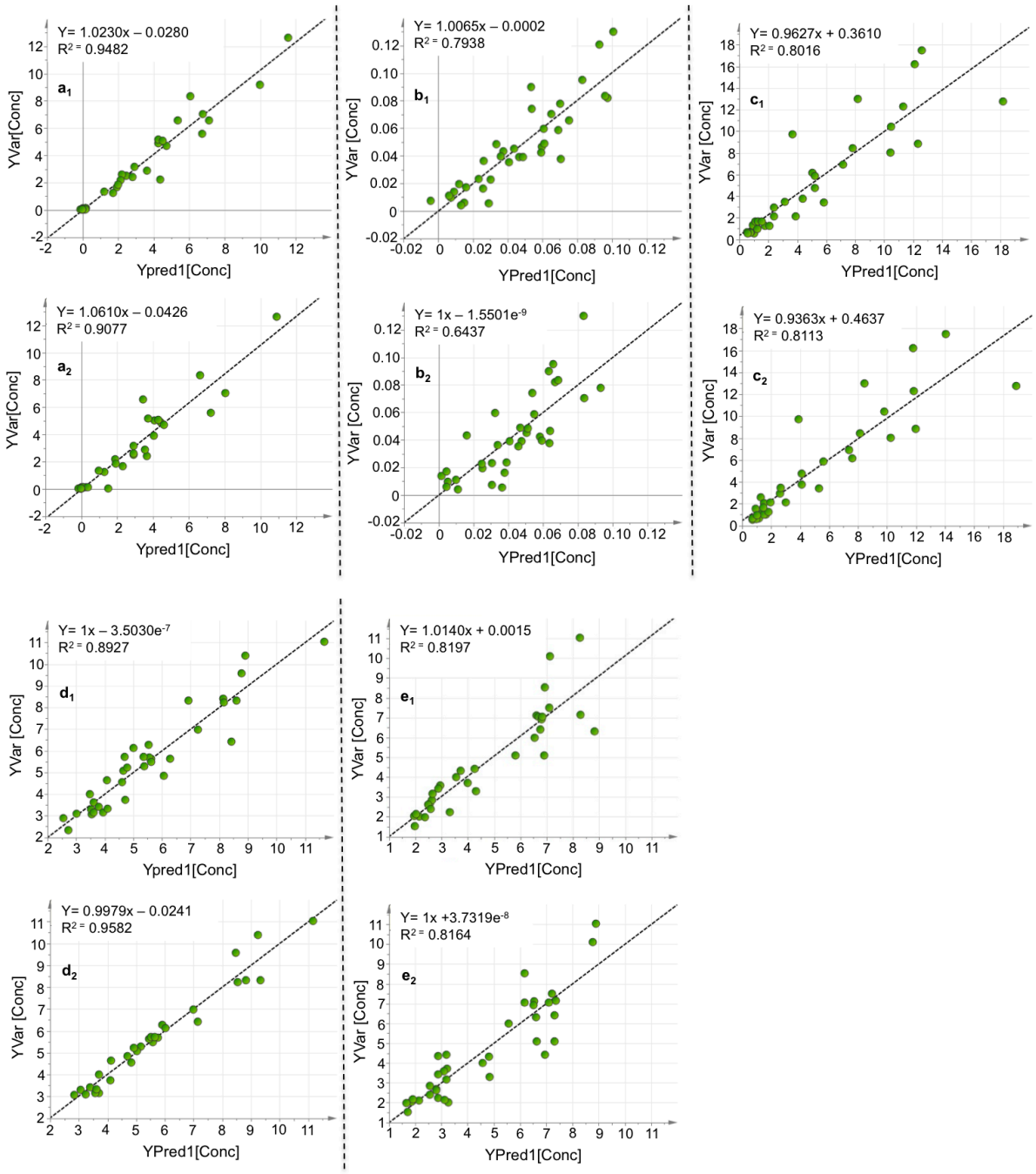


**FIGURE 5.3.** Graphical representation of the output after model implementation, relating the observed and predicted Y-variables (i.e. carotenoid concentrations). For each of the carotenoids, i.e. lycopene (a), α-carotene (b), zeaxanthin (c), β-carotene (d), and lutein (e), two separate figures were obtained, including the ions, obtained in the negative ($_1$) and positive ($_2$) ionization mode.

To reveal the significance of the various metabolite ions in carotenoid metabolism, S-plots were constructed in which each point represents a single metabolite ion (Figure 5.4, e.g. lycopene). The x-axis indicates the contribution (covariance (p)) of a metabolite ion to the variance of the observations, i.e. carotenoid concentrations. The further away a metabolite ion deviates from 0,

the higher it's contribution towards the observed variance. The y-axis refers to the correlation (p(corr)) between samples and the reliability of the results (31,42). For a relevant ion, both the contribution to the model expressed as p and the effect and reliability of this contribution expressed as p(corr) should be high. Therefore, cut-off values of $|p| \geq 0.03$ and $|p(corr)| \geq 0.5$ were introduced for exclusion of metabolite ions (31,43). For example, based on Figure 5.4 A, three ions, obtained in negative ionization mode, could be clearly distinguished from the bulk of ions and were therefore recognized as significantly contributing to the variance in lycopene concentration. Furthermore, these ions could be assigned an up-regulating (ion 960) or down-regulating (ions 12778 and 10770) correlation towards lycopene concentration. However, depending on the position in the S-plot and the associated p- and p(corr)-values, other ions could also be of significant importance towards the variance in lycopene concentration. As such, an exclusion of irrelevant ions could be made. For the ions (Figure 5.4 B), obtained in positive ionization mode, data could be interpreted in an identical way. Interpretation of the S-plot data was supported by the Variable Importance in Projection (VIP) scores. VIP-values reflect the relative importance of the individual X-variables (metabolite ions) in explaining the Y-variable (carotenoid concentration) and are classified as follows: VIP > 1 (highly influential), 0.8 < VIP < 1.0 (moderately influential) and VIP < 0.8 (less influential) (44). In this study, all metabolite ions with VIP-values lower than 0.8 were excluded.

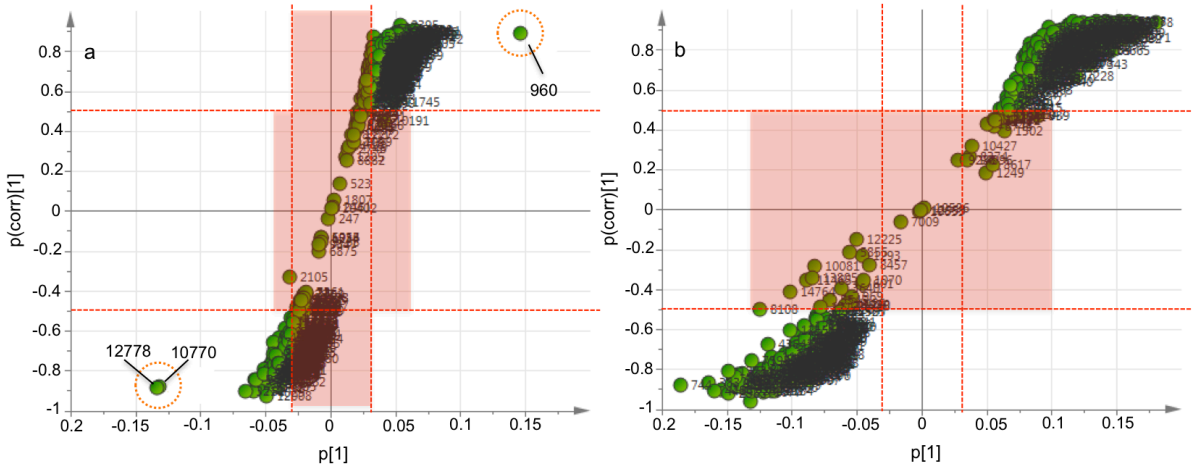


**FIGURE 5.4.** Loading S-plots representing the leading contributing ions, obtained in negative (A) and positive (B) ionization mode, towards the metabolism of lycopene. Cut-off values of $|p| \geq 0.03$ and $|p(corr)| \geq 0.5$ were applied. The areas shaded in red enclose the metabolite ions that were not compliant with the set cut-off values.

### 3.1.4 STEP 4: IDENTIFICATION OF RELEVANT METABOLITE IONS

By means of the predictive modelling, the relevance of metabolite ions towards carotenoid metabolism was mathematically defined. Subsequently, it was determined whether or not any of these relevant ions could be classified as phytohormones. For this purpose, a workflow as depicted in Figure 5.5 was implemented. A first step within this workflow was established by Sieve™ Database Lookup, using a database in which the molecular formulas of 275 different phytohormonal compounds were included. The accurate masses of the corresponding $[M+H]^+$ and $[M-H]^-$ ions were thereby matched against the *m/z*-values of the relevant metabolite ions. The maximally allowed mass deviation was set at 5 ppm. Additional confirmation of a metabolite's molecular formula was based on the mass spectrometric isotopic pattern. The $^{13}C$ isotopic ion was found suitable as a diagnostic ion when the calculated relative ion intensities complied with CD 2002/657/EC (36) requirements: for theoretical determined relative intensities of >20 to 50%, >10 to 20% and ≤10%, the maximum permitted tolerances were, respectively, ±25%, ±30% and ±50% (36). Moreover, the chromatographic performance of the peaks was evaluated in terms of peak shape (See Chapter II) ($A_s \leq 1.5$) (37), points over the peak (≥ 15) (38), and signal-to-noise ratio (≥ 3) (36). Based on all above-listed criteria, the total number of metabolite ions was reduced to 12 (Table 5.4). Herewith, a putative annotation (19) was established (Table 5.4), suggesting following candidate identities: 1-acetylindole-3-carboxaldehyde, 2-oxindole-3-acetic acid, 5-methoxyindole-2-carboxylic acid, 5-hydroxyindole-3-acetic acid, *trans*-zeatin-O-glucoside, *cis*-zeatin-O-glucoside, *trans*-zeatin-7-glucoside, *cis*-zeatin-7-glucoside, *trans*-zeatin-9-glucoside, *cis*-zeatin-9-glucoside, cucurbic acid, *cis*-12-oxo-phytodienoic acid, *trans*-12-oxo-phytodienoic acid, 3-oxo-2-(2-(Z)-pentenyl)cyclopentane-1-octanoic acid, jasmonic acid methyl ester, tuberonic acid, tuberonic acid methyl ester and 9,10-dihydrojasmonic acid. Additional identity confirmation of the putative annotated metabolites was performed by matching the experimental collision-induced dissociation fragmentation spectra with computational fragmentation spectra (39). For this purpose, all candidate compound structures were processed by Mass Frontier 5.0 (Thermo Fisher Scientific, San José, USA), predicting fragmentation spectra in both the protonated and deprotonated molecular ion modes, using the general and library fragmentation mechanisms. The maximum number of reactions steps was 4. The candidate compounds, for which no agreement was found between the predicted and experimental fragmentation spectra, were excluded for further investigation. This

implied that a number of metabolites (i.e. metabolites 5-11, Table 5.4) were of no further interest since they could no longer be classified as known phytohormones. The identity of the other metabolites was verified with authentic standards. In addition, concentration ranges were determined for the remaining metabolites, thereby considering the analysed tomato fruits. Concentration levels (on a dry weight basis) ranged from 0 (i.e. below LOD) to 0.052 ng $mg^{-1}$ for cucurbic acid; from 0 (i.e. below LOD) to 2.7 ng $mg^{-1}$ for *cis*-12-oxo-phytodienoic acid; from 0 (i.e. below LOD) to 38.9 ng $mg^{-1}$ for 1-acetylindole-3-carboxaldehyde; from 0.1 to 2.2 ng $mg^{-1}$ for 2-oxindole-3-acetic acid; from 44.3 to 136.4 ng $mg^{-1}$ for *cis*-zeatin-O-glucoside.

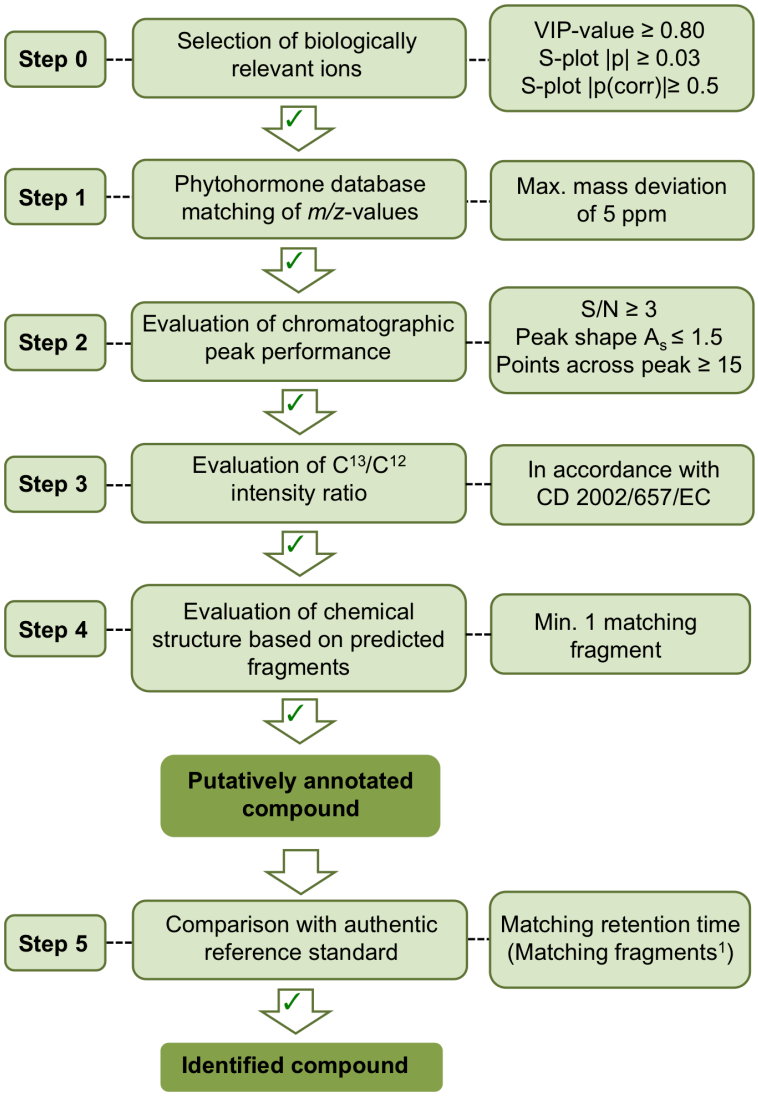


**FIGURE 5.5.** Schematic representation of the workflow that was applied for identification of biologically relevant metabolite ions. For each of the considered identification steps, decision criteria and their associated threshold-values are provided. [1]Comparison of the fragmentation profiles between the sample metabolites and authentic reference standards was only to support our findings but had no decisive power since fragmentation profiles were obtained with different analytical instruments.

**TABLE 5.4.** Metabolite ions with their respective identification number (ID), mass over charge ratio (*m*/*z*), retention time (RT), elemental composition of the associated molecule, molecular weight (MW), mass deviation (Δm), and candidate identities. These ions were revealed to be involved in carotenoid metabolism (cfr. associated carotenoids) and could be classified as hormonally active compounds. Their significance with respect to the metabolism of a certain carotenoid is described by the VIP-score (Variable Importance in Projection). The actual involvement of the individual metabolites is described by their correlation coefficient (Pearson r, Spearman ρ, or Kendall τ). Only the metabolites with IDs 1, 2, 3, 4, and 12 could be effectively matched with one of the candidate identities.

| ID | *m*/*z* | RT (min) | Elemental formula | MW | Δm (ppm) | Associated carotenoid(s) | VIP | r, ρ,τ | Candidate identities |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 188.0701 | 3.95 | $C_{11}H_9NO_2$ | 187.0633 | 3.47 | α-Carotene | 6.19 | +0.403 | 1-Acetylindole-3-carboxaldehyde |
| | | | | | | β-Carotene | 1.14 | +0.544 | |
| 2 | 380.1572 | 4.45 | $C_{16}H_{23}N_5O_6$ | 381.1648 | 1.02 | β-Carotene | 0.89 | +0.529 | *Cis*/*trans*-zeatin-O-glucoside; *cis*/*trans*-zeatin-9-glucoside; *cis*/*trans*-zeatin-7-glucoside |
| | | | | | | Zeaxanthin | 0.80 | -0.563 | |
| 3 | 293.2104 | 6.96 | $C_{18}H_{28}O_3$ | 292.2038 | 2.60 | β-Carotene | 1.04 | +0.494 | *Cis*/*trans*-12-oxo-phytodienoic acid |
| 4 | 211.1338 | 6.01 | $C_{12}H_{20}O_3$ | 212.1412 | 0.82 | β-Carotene | 0.92 | -0.673 | Cucurbic acid; 9,10-dihydrojasmonic acid |
| | | | | | | Lutein | 1.36 | +0.846 | |
| 5 | 293.2126 | 7.68 | $C_{18}H_{30}O_3$ | 294.2195 | 1.37 | β-Carotene | 0.81 | -0.636 | 3-Oxo-2-(2-(Z)-pentenyl)-cyclopentane-1-octanoic acid |
| 6 | 225.1478 | 4.92 | $C_{13}H_{20}O_3$ | 224.1412 | 3.35 | Lycopene | 0.98 | +0.817 | Jasmonic acid methyl ester |
| 7 | 241.1423 | 6.40 | $C_{13}H_{20}O_4$ | 240.1362 | 4.84 | Lycopene | 0.99 | -0.805 | Tuberonic acid methyl ester |
| 8 | 241.1426 | 5.25 | $C_{13}H_{20}O_4$ | 240.1362 | 3.32 | Lycopene | 1.00 | +0.726 | Tuberonic acid methyl ester |
| | | | | | | Lutein | 1.14 | -0.548 | |
| 9 | 241.1429 | 4.54 | $C_{13}H_{20}O_4$ | 240.1362 | 2.30 | Lycopene | 1.35 | +0.847 | Tuberonic acid methyl ester |
| 10 | 225.1133 | 5.31 | $C_{12}H_{18}O_4$ | 226.1205 | 0.34 | Zeaxanthin | 0.86 | -0.553 | Tuberonic acid |
| 11 | 225.1132 | 5.15 | $C_{12}H_{18}O_4$ | 226.1205 | 0.06 | β-Carotene | 1.06 | -0.651 | Tuberonic acid |
| | | | | | | Lycopene | 0.87 | -0.707 | |
| 12 | 190.0506 | 5.10 | $C_{10}H_9NO_3$ | 191.0582 | 1.99 | Zeaxanthin | 0.89 | -0.537 | 2-Oxindole-3-acetic acid; 5-methoxyindole-2-carboxylic acid; 5-hydroxyindole-3-acetic acid |

In conclusion, on the basis of relative retention time, accurate mass, and isotopic pattern (Figure 5.6), metabolites 1, 2, 3, 4, and 12 were unambiguously identified as 1-acetylindole-3-carboxaldehyde (PubChem Compound Identifier (CID) 89915), *cis*-phytodienoic acid (CID 5280441), *cis*-zeatin-O-glucoside (CID 5280589/5461146/25244165), cucurbic acid (CID 5281159/5282268) and 2-oxindole-3-acetic acid (CID 3080590), respectively. In addition, comparison between the fragmentation spectra of authentic standards and targeted metabolites strengthened identity assignment (data not shown). Although a high number of corresponding fragments was observed, the relative ion intensities did sometimes deviate. This finding may originate from the different analytical platforms that were used to generate fragmentation profiles from authentic standards and metabolites, respectively (45,46). Taking into account the classification, defined by Sumner *et al.* (2007) (19), compounds were identified up to 'level 1' ('identified compounds'). It is indeed stated that a minimum of two orthogonal and independent data sources is required to reach this particular level of identification (47). In this study, additional orthogonal data (i.e. more than two) were used, as such providing additional confidence and unambiguous identification.

The involvement of these particular phytohormones in carotenoid metabolism was up to now described by their individual VIP-score and position in the S-plot. However, these descriptors are only indicative for the relative importance of a metabolite within the (O)PLS model (48). To verify the actual involvement of the revealed phytohormones, individual correlation plots were established (49). Each of these plots represented the relationship between a specific carotenoid and an associated relevant phytohormone in terms of abundance (data not shown). During statistical analysis, various regression models were tested; i.e. linear, logarithmic, exponential, inverse, power, cubic and quadratic models. It has indeed been demonstrated that plant physiological processes, mediated by phytohormones, are often not linearly responding towards altered phytohormone levels (50-54). Normality of the datasets was verified by the Kolmogorov-Smirnov test (P-value $\leq 0.05$). The regression curve that best fitted the data was determined by SPSS™ 22.0 and was evaluated by the P-value ($\leq 0.05$) correspondingly the goodness of fit ($R^2$). For all correlation plots, significant $R^2$-values, varying between 0.280 and 0.760, were obtained, indicating that the identified phytohormones are effectively involved in the metabolism of one or more carotenoids. In addition, depending on the best-fitting regression model type, Pearson (r), Spearman ($\rho$), or Kendall ($\tau$) correlation coefficients (Table 5.4) were determined and evaluated by

the P-value (≤ 0.01). As such, the specific influence of each phytohormone, resulting in either an increased or a decreased carotenoid concentration, could be ascertained. It should be noted that the stimulating or inhibiting influence from the phytohormones might be reflected at the level of carotenoid biosynthesis (55,56) or degradation (1). Calculated correlation coefficients were all significant since P-values were all ≤ 0.01.

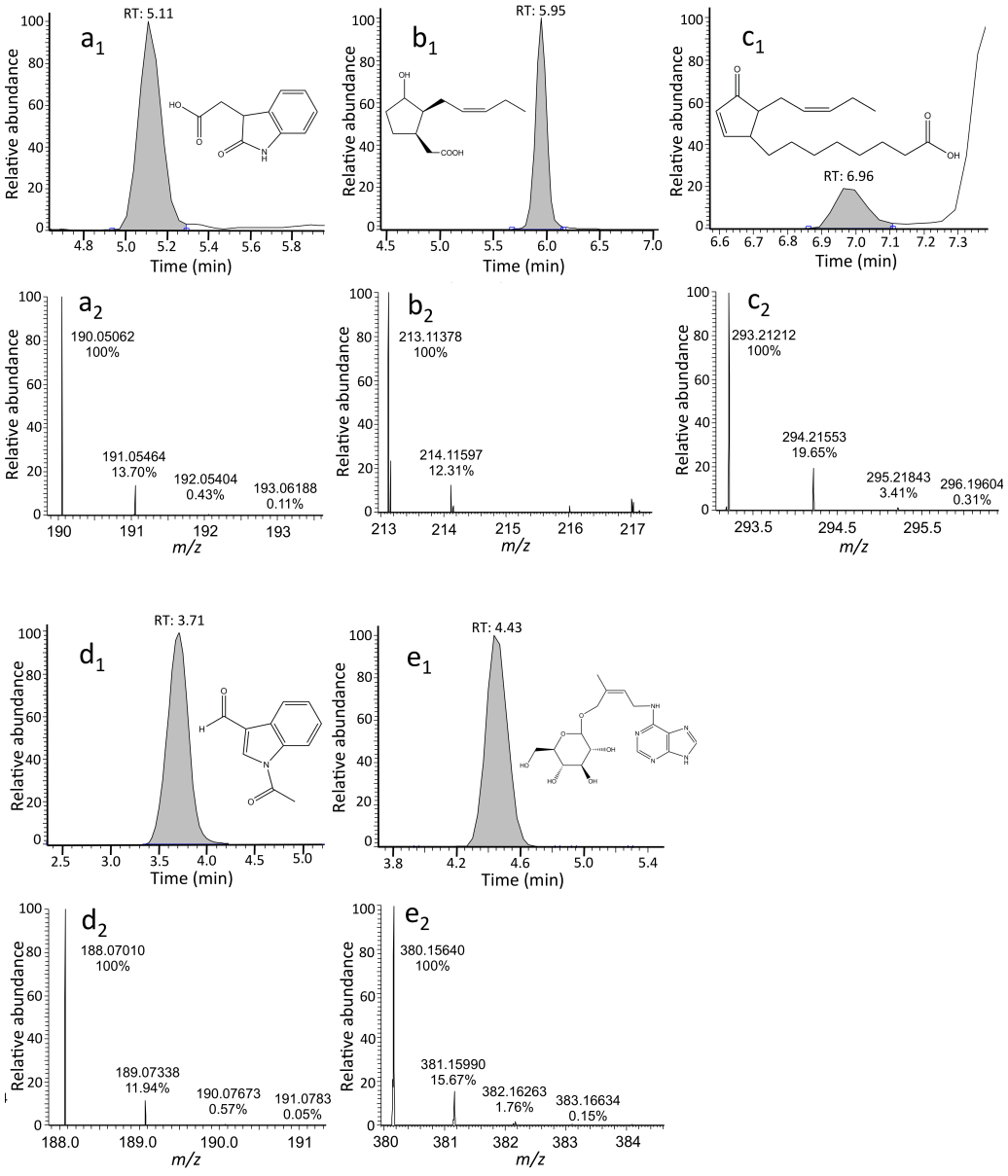


**FIGURE 5.6.** Chromatograms ($_1$) and isotopic patterns ($_2$) of the phytohormonal metabolites that were found involved in carotenoid metabolism (i.e. 2-oxindole-3-acetic acid (a), cucurbic acid (b), *cis*-12-oxo-phytodienoic acid (c), 1-acetylindole-3-acetic acid (d), *cis*-zeatin-O-glucoside (e)). These data were obtained from analyzed tomato fruit samples. Theoretically calculated $^{13}C/^{12}C$ ratios were, respectively, 10.82% (a), 12.98% (b), 19.47% (c), 11.90% (d), and 17.31% (e).

## 3.2 AN INTEGRATED CHEMOMETRIC STRATEGY AS PART OF (PLANT) METABOLOMICS

Although a metabolomic approach has the intrinsic quality to expand our knowledge on biochemical pathways and their regulation in biological systems, optimization of an integrated chemometric strategy is indispensable to deal with complicated, multidimensional datasets. The ability to mine the generated LC-HRMS data and perform reliable differential analysis fulfils indeed a pivotal role in the success of plant metabolomics (57). Within this context, data pre-processing and data mining constitute essential parts of the required chemometrics strategy. Whereas data pre-processing relates to chromatographic matching, mass profiling and peak listing, data mining focuses on data differential comparisons (57). Within metabolomic-oriented papers, unsupervised PCA is predominantly used for achieving the natural interrelationship, including grouping, clustering, and detection of outliers, among observations without *a priori* knowledge of the data set. Additionally, sophisticated supervised (O)PLS-DA (discriminant analysis) methods are applied to visualize variations between sample groups (species, treatments, phenotypes, etc.) and to define the discriminating performance of variables. With (O)PLS-DA, classification and discrimination problems are addressed by pre-defining the Y-variable as specific descriptor (e.g. 0/1) (58). As a consequence, such an approach is frequently applied for biomarker identification in genotyping and phenotyping, population screening, determining authenticity, plant response towards biotic or abiotic stress, etc. (57). However, the objectives of this study point towards a slightly different multivariate data analysis strategy, namely (O)PLS instead of (O)PLS-DA. Indeed, discriminant analysis by classification of the samples in distinct groups based on e.g. colometric values (*a*lb**) (59), days after anthesis or actual carotenoid concentration would only indicate markers for fruit development or ripening. No information would be obtained about their direct involvement in carotenoid metabolism and relation to carotenoid concentration. Therefore, an (O)PLS-based approach, whereby the Y-variable was described as a quantitative variable was selected. Such (O)PLS-models were also used in other studies (60-62), in which the applied metabolomics and associated metabolite marker identification were addressed in a similar way as in this study. Although supervised models have been recognized as valuable statistical tools, a number of limitations have been identified as well. One of the major drawbacks relates to the risk of model over-fitting. Therefore, model validation is crucial to ensure robustness, significance, and proper model fit (32,63). Ideally, the availability of a second, independent data set would be ideal

to verify model validity. Alternatively, there are a number of methods to perform cross-validation based on the data that are used for modelling (64). Here, 7-fold cross-validation was performed to validate the generated OPLS-models. Using cross-validated ANOVA the results of the cross-validation are converted into a familiar and easily understood 'standard ANOVA format' (34). The significance of an OPLS model can also be estimated through response permutation testing (34,64), during which the predictive measures are interpreted. It may be clear that a thoughtful validation strategy is needed to acquire reliable supervised models.

## 3.3 PHYSIOLOGICAL RELEVANCE OF IDENTIFIED METABOLITE MARKERS

*Cucurbic acid and cis-12-oxo-phytodienoic acid (jasmonates)*

In this study, it has been demonstrated that both cucurbic acid and *cis*-12-oxo-phytodienoic acid are involved in the regulation of β-carotene metabolism. However, opposite effects from these phytohormones were observed, i.e. down-regulation in the case of cucurbic acid ($\rho$ = -0.673) and up-regulation in the case of *cis*-12-oxo-phytodienoic acid ($\rho$ = +0.494). In addition, based on our findings, cucurbic acid was attributed an up-regulating function ($\rho$ = +0.846) in the metabolism of lutein. As described by Piotrowska and Bajguz (2011) (65), both metabolites have been recognized as major members of the jasmonate hormonal class, which is typically involved in biotic and abiotic stress responses (66). However, jasmonates are assigned a regulating role in various other plant physiological and developmental processes, including ripening and carotenoid metabolism (55,67). In this context, their regulatory functions relate to, amongst others, degradation of chlorophyll, synthesis of carotenes, production of ethylene, accumulation of anthocyanins, modification of cell walls, and the promotion of ripening-related compounds (55,68-71). When considering individual jasmonate class members, it appears that especially jasmonic acid and jasmonic acid methyl ester have frequently been studied (70,72,73). Therefore, the progress that has been made in understanding the signalling and functioning of jasmonates primarily relates to these two compounds. The knowledge about other jasmonates, including 12-oxo-phytodienoic acid and cucurbic acid, is just recently emerging (74). To indicate the interrelationship between these phytohormones and their link with jasmonic acid, the biosynthesis pathway is presented in Figure 5.7. It should be noted that this pathway focuses on the main route for jasmonic acid formation. Alternative pathways are described by Schaller *et al*. (2008) (75) and Wasternack (2007) (76).

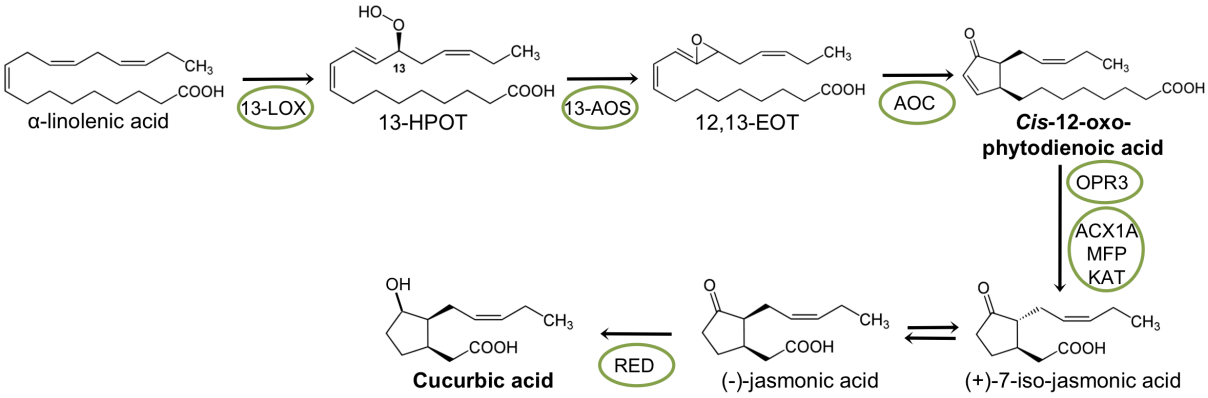


**FIGURE 5.7.** Pathway for biosynthesis of *cis*-oxo-phytodienoic acid and cucurbic acid. Synthesis of these jasmonates is initiated by lipoxygenases (3-LOX), which catalyze the dioxygenation of polyunsaturated fatty acids such as α-linolenic acid (18:3) into 13(*S*)-hydroxyperoxy-octadecaditrienoic acid (13-HPOT). The following step is performed by allene oxide synthase and includes the dehydration of 13-HPOT to form an unstable allene oxide, i.e. 12,13(*S*)-epoxy-octadecatrienoic acid (12,13-EOT). This compound undergoes cyclization, which may result in the synthesis of cis-12-oxo-phytodienoic acid (OPDA), being the precursor for biologically active jasmonic acid. The final steps towards the formation of jasmonic acids encompass reduction (by 12-oxophytodienoate reductase, OPR3) and three consecutive phases of β-oxidation (by acyl-CoA oxidase (ACX), multifunctional protein (MFP), and 3-ketoacyl-CoA thiolase (KAT)) (75). The formation of cucurbic acid is established by reduction (RED) of the keto group from jasmonic acid (77,78) (Adjusted from Wasternack, 2007 (76)).

12-Oxo-phytodienoic acid is an early intermediate in the octadecanoid pathway, whereby α-linolenic acid is converted to jasmonic acid through a number of enzymatic steps (79). For a long time, this particular jasmonate was therefore only ascribed significance as a precursor for other jasmonates. Only recently, 12-oxo-phytodienoic acid has been demonstrated as a biologically active molecule, fulfilling a role in the induction of defence response genes. It is suggested by Stintzi *et al*. (2001) (80) that this jasmonate may function cooperatively with jasmonic acid or even independently in inducing gene expression. Although the main functioning of 12-oxo-phytodienoic acid refers to environmental stress responses, other functions have been described as well (66,74,80). It is, however, generally stated that little is known about the induction of gene expression or other functions, associated with 12-oxo-phytodienoic acid (66). Therefore, this study makes an important contribution in expanding the knowledge about the functions of this particular phytohormone. The recognized hormonal activity of 12-oxo-phytodienoic acid together with the involvement of other closely related jasmonates in fruit ripening, support our finding of *cis*-12-oxo-phytodienoic acid being involved in β-carotene metabolism. As this phytohormone has a positive

regulating influence on β-carotene synthesis, it may be assumed that the enzyme lycopene β-cyclase (LCYB) is up-regulated by 12-oxo-phytodienoic acid. However, since this particular enzyme is also involved in α-carotene synthesis and no effects have been noted for 12-oxo-phytodienoic acid on α-carotene metabolism, it is hypothesized that the enzyme, responsible for formation of the ε-ionone end-group (i.e. lycopene ε-cyclase) is the rate-determining factor in the conversion of lycopene into α-carotene.

Cucurbic acid is a phytohormone that is closely related to and derived from jasmonic acid and 7-*iso*-jasmonic acid (81). As stated by Miersch *et al*. (2007) (82), this metabolic derivative exhibits a number of biological activities, which may vary from the activities of jasmonic acid. However, the current knowledge about the physiological functions of this phytohormone is rather limited (83). The functions that are ascribed to cucurbic acid are up to date limited to growth inhibition and induction of defence genes (84-86). In this study, a down- and up-regulating influence from cucurbic acid towards, respectively, β-carotene and lutein concentration were determined. Based on the specific carotenoid alterations during tomato fruit development (20), it can be stated that cucurbic acid therefore acts as an inhibitor of fruit ripening. Since β-carotene and lutein are synthesized via two separate pathways, both starting from lycopene (87), it is hypothesized that cucurbic acid has a stimulating action towards the expression of the enzymes (i.e. lycopene ε-cyclase and carotene ε-hydroxylase) that are specifically involved in the lutein pathway. Inhibition of enzymes that are responsible for converting lycopene into β-carotene as an alternative hypothesis is refuted since these particular enzymes (i.e. lycopene β-cyclase) are also involved in the formation of lutein. The same is true with respect to carotene β-hydroxylase. As such, it can be stated that the abundance of cucurbic acid determines the degree of lycopene consumption by each pathway. As such, the inhibiting effect of cucurbic acid towards β-carotene is considered to be indirect. This finding could be of particular interest for targeted manipulation of the tomato fruit carotenoid composition.

*Oxindole-3-acetic acid and 1-acetylindole-3-carboxaldhyde (auxins)*

Auxins are generally suggested to function as ripening inhibitors, which have to be inactivated to advance fruit maturation and ripening (88-92). However, the specific influence and role of auxins is controversial (93). For example, Trainotto *et al.* (2007) (94) strengthened the alternative hypothesis of auxins having a stimulating and autonomous role in climacteric fruit ripening. As a consequence, there is a continuous aim to better understand the plant control mechanisms towards auxin metabolism in regulating developmental events (93). This is particularly important in the case of indole-3-acetic acid, the major natural auxin, for which the levels seem to be very strictly regulated, as has been described by Kawaguchi and Syõno (1996) (95). Biosynthesis, transport and inactivation pathways are likely to be an integral part of the homeostatic control of indole-3-acetic acid levels (93). A major route for irreversible inactivation of free indole-3-acetic acid proceeds through oxidation, resulting in 2-oxindole-3-acetic acid (Figure 5.8) (96). In this study, increased levels of 2-oxindole-3-acetic acid were associated with reduced zeaxanthin concentration levels ($\rho$ = -0.537). Since zeaxanthin levels decrease as fruit maturation progresses (20), it is concluded that 2-oxindole-3-acetic acid has a promoting influence on tomato fruit ripening. This influence may originate from either the active involvement of 2-oxindole-3-acetic acid or the inactivation of free indole-3-acetic acid into 2-oxindole-3-acetic acid. However, our study indicates no positive influence from indole-3-acetic acid towards zeaxanthin concentration, which would be required for validity of the latter option. Therefore, it may be concluded that the active involvement of 2-oxindole-3-acetic acid is responsible for alterations in zeaxanthin concentration, typically associated with tomato fruit development and ripening. As no effects on precursors of zeaxanthin have been observed, the main possible targets of 2-oxindole-3-acetic acid are the enzymes, which are involved in the conversion of $\beta$-carotene into zeaxanthin, i.e. carotene $\beta$-hydroxylases.

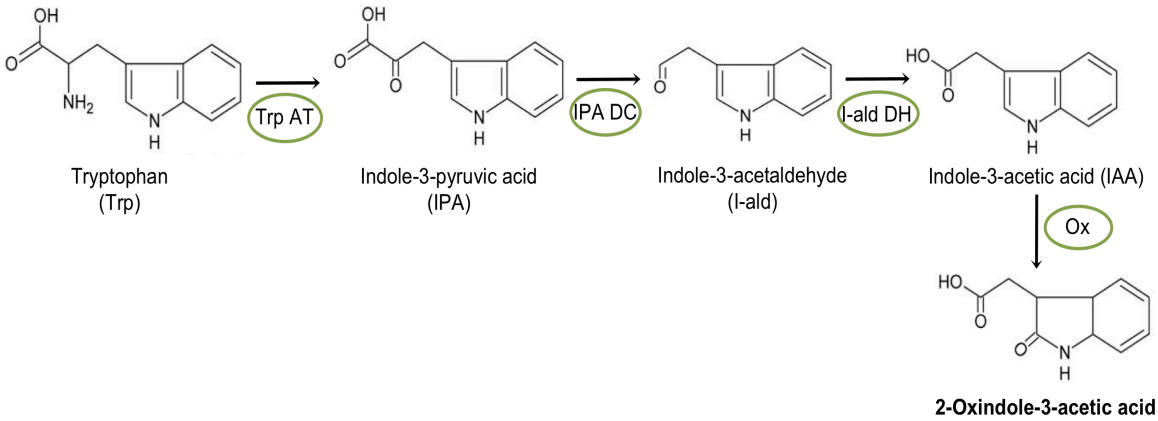


**FIGURE 5.8.** Biosynthesis of IAA through the tryptophan-dependent pathway, using tryptophan as a precursor. The first step is catalysed by aminotransferase (Trp-AT), which transfers the amino group from tryptophan to generate indol-3-pyruvic acid. The second step is an oxygen and NADPH-dependent reaction, catalysed by a decarboxylase (IPA DC) and rendering indole-3-acetaldehyde. The third step includes the action of dehydrogenase (I-ald DH) with formation of IAA (97). Inactivation of IAA to 2-oxindole-3-acetic acid through oxidation (Ox) has been defined as an important pathway for IAA degradation. However, no genes or enzymes have been identified so far (98). Alternatively, IAA may be synthesized via the tryptophan-independent pathways, using most likely indole-3-glycerol phosphate or indole as a precursor. However, little is known about this biochemical pathway (99) (Adjusted from Zhao *et al*. 2012 (97)).

The knowledge about the role and significance of 1-acetylindole-3-carboxaldehyde in plant developmental and physiological processes is very restricted. Only in the study of Wardrop & Polya (1980) (100) this particular metabolite was investigated, indicating an inhibiting action towards indole-3-acetic acid by hindering the binding to its receptors. It is clear that further research on 1-acetylindole-3-carboxaldehyde is required to reveal its potential in the regulation of other plant processes. In this study, it was demonstrated that 1-acetylindole-3-carboxaldehyde is strongly involved in the metabolism of both $\alpha$- and $\beta$-carotene ($\tau$ = +0.403 and $\rho$ = +0.544, respectively). Especially the high VIP-scores (6.19 and 1.14, respectively), determined during the predictive modelling, indicate significant value from this auxin towards carotene metabolism. The regulating influence of this phytohormone is assumed to manifest at the level of the lycopene $\beta$-cyclase enzymes, involved in conversion of lycopene in both $\alpha$- and $\beta$-carotene. 1-acetylindole-3-carboxaldehyde may also have a regulating influence on $\varepsilon$-cyclase, as this was earlier hypothesized as the rate-determining factor (see above, *cis*-12-oxophytodienoic acid).

*Cis-zeatin-O-glucoside (cytokinins)*

Many physiological effects of cytokinins are well established and these phytohormones are therefore known to be involved in various aspects of the plant life cycle. However, as described by Roisch and Ehneß (2000) (101), their main function relates to the promotion of cell division and associated control of growth and development (101). With respect to tomato fruit development, cytokinins are strongly represented during the early stages of development, reaching their lowest levels at the red-ripe stage. It has indeed been suggested by Srivastava and Handa (2005) (13) that cytokinins are generally less involved in ripening processes or may block various cellular differentiation and gene expression pathways, which are associated with fruit ripening. With the latter option, it is assumed that as ripening progresses, cytokinin concentrations decrease and the blocking actions gradually disappear (13).

Zeatin-glucosides have frequently been associated with storage functions in plants, hereby regulating the levels of biologically active zeatin by means of reversible sequestration (Figure 5.9) (102). However, although zeatin-7-glucoside and zeatin-9-glucoside are mostly considered as inactive derivatives, zeatin-O-glucoside has been recognized as extremely active in the studies of Rodo *et al*. (2008) (103) and van Staden *et al*. (1977) (104). In our study, *cis*-zeatin-O-glucoside was attributed an up-regulating ($r = +0.529$) and down-regulating ($\rho = -0.563$) influence on, respectively, β-carotene and zeaxanthin concentration (Table 5.4). Since β-carotene acts as a precursor in zeaxanthin synthesis (87), the hypothesis arises that *cis*-zeatin-O-glucoside has a negatively regulating influence on β-carotene hydroxylase. This enzyme is indeed responsible for formation of zeaxanthin by introducing hydroxyl moieties on the cyclic β-ionone end-groups of β-carotene. Based on the specific influence of *cis*-zeatin-O-glucoside towards the metabolism of these carotenoids and the typical carotenoid profiles during ripening (20), a positive impact from this phytohormone on fruit ripening is concluded. However, since the opposite is generally claimed for cytokinins, zeatin-O-glucoside might also effectively act as a storage form. In our study no other cytokinins were however found involved in zeaxanthin metabolism, which points towards active regulating actions from zeatin-O-glucoside. This finding confirms earlier research (103,104), recognizing this cytokinin as extremely active.

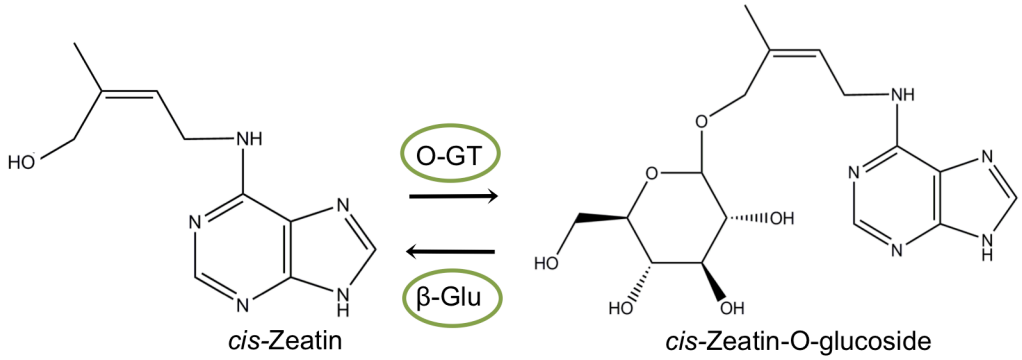


**FIGURE 5.9.** Conversion of zeatin into zeatin-O-glucoside by O-glucosyltransferases. This particular process may be reversed by β-glucosidase, thereby generating zeatin (103).

## 4. CONCLUSIONS

This research aimed at revealing phytohormonal metabolites that are involved in the metabolism of the carotenoids α-carotene, β-carotene, lycopene, lutein, and zeaxanthin. For this purpose, the phytohormone profiles of 50 tomato fruits were acquired with Orbitrap-MS and interpreted towards the associated carotenoid concentration levels. The applied analytical platform yielded high-quality accurate mass LC-MS data and enclosed as such an attractive approach for metabolite profiling of tomato fruits. Using chemometrics data analysis, 5 phytohormones could be recognized as significantly contributing towards carotenoid metabolism. These phytohormonal markers were unambiguously identified as *cis*-12-oxo-phytodienoic acid, cucurbic acid, 2-oxindole-3-acetic acid, 1-acetyl-3-carboxaldehyde, and *cis*-zeatin-O-glucoside. This study proved the metabolomics approach, combining HRMS and multivariate statistical analysis, as a powerful tool to profile and differentiate metabolite compositions among different samples. As such, phytohormonal markers, which are accountable for the carotenoid profile changes during tomato fruit development, could be identified and assigned a prominent role in carotenoid metabolism. As a result, the improved knowledge about phytohormonal regulation of carotenoid metabolism encloses the potential for improving tomato fruit nutritional quality by targeted control of agronomic conditions, exogenous use of plant bioregulators, or genetic engineering. Future research should evidently focus on the practical implementation of such strategies, thereby exploiting the potential of these new insights.

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# CHAPTER VI

## METABOLIC FINGERPRINTING TO STUDY THE IMPACT OF SALINITY ON CAROTENOID CONTENT IN DEVELOPING TOMATO FRUITS

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**Adapted from:**

Van Meulebroek L., Hanssens J., Steppe K., Vanhaecke L. (2014)

To be submitted.

# ABSTRACT

The presence of health-promoting substances has become a significant aspect of tomato fruit quality and of growing interest for consumers. This has prompted research into the identification of means, which have the ability to promote tomato fruit nutritional quality. However, the outcome of an agronomic practice is often uncertain due to the interaction with the plant's genetic background and environment. In this study, the potential of altered nutrient solution salinity to enhance carotenoid accumulation in cherry tomato fruit (*Solanum lycopersicum* L. cv. Juanita) was evaluated by focusing on the underlying mechanisms of carotenoid metabolism. A greenhouse experiment with five salinity treatments (ranging from 2.0 to 5.0 dS $m^{-1}$) was carried out, whereby fruits at varying stages of development were harvested. A metabolomics approach was performed and revealed 46 compounds (of which 1 phytohormone and 18 putatively annotated metabolites), involved in carotenoid metabolism and influenced by salinity. The specific effect of altered salinity on these metabolites was verified and related to the regulation of carotenoid metabolism. This strategy indicated however little or no potential of this agronomic practice to promote the accumulation of lutein, zeaxanthin, lycopene, and β-carotene in tomato fruit. This finding was confirmed by evaluating the carotenoid concentration levels on a dry weight basis, which were not significantly affected by the imposed salinity treatments. The presented strategy encloses an attractive methodology to determine and exploit the potential of altered agronomic practices in promoting tomato fruit carotenoid content. Although the interactive effects with the environment and the cultivar may still exert their influence, this approach facilitates the assessment of optimal growth conditions. In addition, the acquired knowledge may be of substantial value in modeling tomato fruit quality.

# 1. INTRODUCTION

Tomato (*Solanum lycopersicum* L.) is one of the most important horticultural crops worldwide and assigned a key role in the human diet. As a major component of daily meals in many countries, tomato constitutes a valuable source of diverse health-beneficial constituents. Especially vitamins, minerals, carotenoids, and phenolic compounds have been allocated beneficial effects on human health (1,2). As such, regular consumption of fresh tomatoes or tomato-based foods has been associated with a reduced incidence of various chronic degenerative diseases including certain types of cancer and cardiovascular disorders (3-5). With this rationale, the presence of health-promoting substances has become a significant aspect of tomato fruit quality appreciation and of growing interest for consumers (6). Improvement of tomato fruit nutritional quality has thus emerged as an urgent issue for growers who want to meet the ever-increasing demands of consumers in a highly competitive fresh market (1,7). This has evidently prompted research that aims at the identification of means, which have the ability to enhance the accumulation of bioactive compounds in tomato fruit (8). Herewith, especially carotenoids such as lycopene and β-carotene are designated as the target compounds because of their predominant contribution towards overall tomato fruit nutritional quality (2,8,9).

In this context, management of the nutrient solution electrical conductivity (EC) has intensively been assessed as a strategy to enhance carotenoid concentration levels in tomato fruit (1,6,10-13). It has indeed been postulated that incrementing the EC-level may be beneficial to crops by improving their nutritional value (13,14). Initially, any improvements were exclusively attributed to a concentration effect of compounds in tomato fruit, which is the result of osmotic stress and related impaired water uptake by the fruits. However, physiological plant responses may be activated as well. More specifically, increased salinity may induce the up-regulation of gene-encoding enzymes, involved in the key steps of carotenoid biosynthesis (15,16). For example, in the study of Wu *et al.* (2008) (7), enhanced lycopene concentration levels were observed when tomato plants were grown hydroponically using a nutrient solution with an EC-level of 4.8 dS $m^{-1}$ compared to standard EC-conditions of 2.4 dS $m^{-1}$. Moreover, the increase in lycopene concentration (34 to 85%) for the four cultivars tested was significantly higher than the increase in total soluble solids (12 to 22%). These findings indeed suggest an improved nutritional quality

because of specific plant stress responses, rather than being the result of reduced water content of the tomato fruits. However, although various other studies have indicated the potential of altered EC-levels to improve tomato fruit nutritional quality (6,11,13,17), conflicting or less conclusive data regarding the effects of this agronomic practice have been reported as well (10,12,18,19). The final outcome with respect to tomato fruit nutritional quality is strongly influenced by the synergistic and antagonistic effects between environment, genetic background, and agronomic setting (1). As such, defining optimum conditions to maximize the biosynthesis of carotenoids is rather difficult or even impossible (20). In this regard, predictive and mechanistic fruit quality models are particularly interesting since they may assist in defining the conditions that are appropriate to promote high nutritional tomato fruit quality (21). However, little progress has been achieved in modeling fruit quality because of the system's complexity and the underlying mechanisms of metabolism, which are only partially understood (21). A deepened physiological and biochemical knowledge on the metabolism of carotenoids in relation to their regulation and growth environment is thus essential in the pursuit of improved tomato fruit nutritional quality (1). Phytohormones are of particular interest in this context since their diverse regulating actions may enfold the missing link between growth environment and carotenoid metabolism. Phytohormones have indeed been assigned a crucial role in the regulation of the plant response towards environmental stimuli such as salinity and water availability (22,23). In addition, these signaling biomolecules are involved in the complex processes of fruit development and ripening, which are accompanied by both qualitative and quantitative changes of the carotenoid profile (4,24).

In this study, alteration of the nutrient solution EC-level was assessed as a potential strategy to enhance carotenoid levels in tomato fruit and improve nutritional quality. To fully exploit the potential of this agronomic practice, it was opted to focus on metabolic changes in response to altered EC-levels and towards carotenoid metabolism. Since the functioning of phytohormones is regulated through a complex signaling network of additive, synergistic and antagonistic actions between multiple phytohormones (25), a metabolomics approach was followed. This approach was founded on ultra-high performance liquid chromatography (UHPLC) hyphenated with full-scan high-resolution mass spectrometry (MS) and multivariate data analysis. Broadening the fundamental knowledge about the complex interplay between environment and tomato fruit metabolism is regarded as indispensable for further progress in tomato fruit quality optimization.

# 2. MATERIALS AND METHODS

## 2.1 PLANT MATERIAL AND EXPERIMENTAL SETUP

The experiment was carried out in a 60-$m^2$ greenhouse compartment of the Institute for Agricultural and Fisheries Research (ILVO, Melle, Belgium). Cherry tomato plants (*Solanum lycopersicum* L. cv. Juanita) were obtained from Hollandplant (Bergschenhoek, the Netherlands) and introduced into the greenhouse compartment on November 28, 2013. Tomato seedlings were at that moment about four weeks old and immediately transplanted to 15-L rock wool slabs (Grodan Master, Hedehusene, Denmark) whereby a final density of about 1.9 plants $m^{-2}$ was achieved. Temperature was regulated with heating set points of 17 °C at night and 22 °C during the day (Figure 6.1 A), and supplementary light was provided at a photon flux density of 50 μmol PAR $m^{-2}$ $s^{-1}$ when natural solar radiation reached values below 100 W $m^{-2}$. Daily global radiation is presented in Figure 6.1 B. Irrigation of tomato plants was conducted by means of a drip irrigation system with one emitter per plant and consisted of six irrigation sessions per day. The duration of a session was set in order to achieve a minimum drain of about 20% and to prevent as such the accumulation of salts in the substrate.

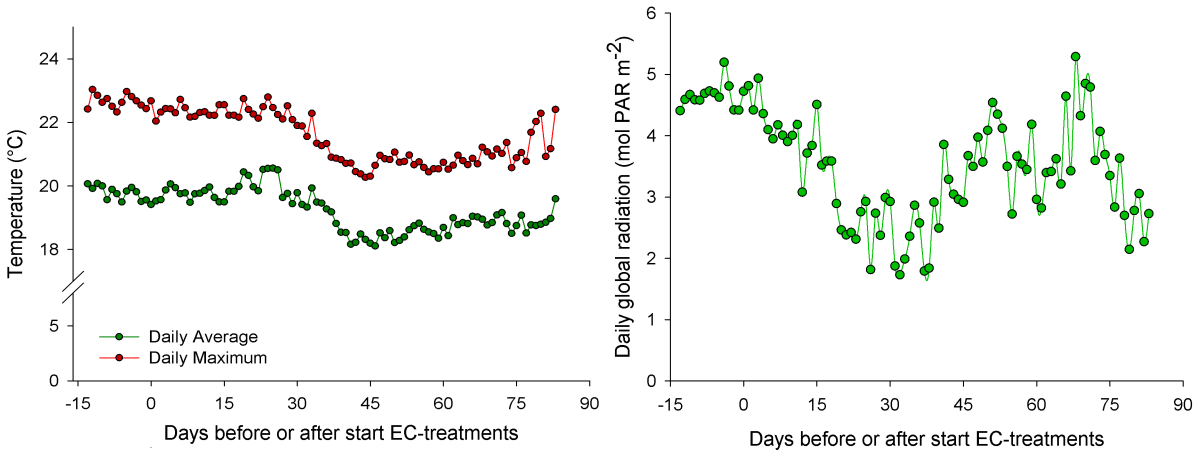


**FIGURE 6.1.** Average and maximum daily air temperatures (A), recorded inside the greenhouse compartment during the experiment. The daily global sum of radiation (B) was determined as well and comprised natural and artificially supplemented PAR-radiation. The microclimatic data are expressed in function of the number of days after or before the actual start of the EC-treatments, i.e. when nutrient solutions reached their final EC-levels (December 12, 2013).

The experimental set-up included five different treatments, which related to the salinity of the irrigated nutrient solutions. Each treatment comprised seven tomato plants (excl. border plants), whereby the considered nutrient solution EC-levels reached values of 2.0, 2.5, 3.0, 4.0, and 5.0 dS $m^{-1}$. The nutrient solution with an EC-level of 2.5 dS $m^{-1}$ consisted of (in mM or μM*) 13.6 $NO_3$, 4.7 Ca, 3.6 K, 3.5 $SO_4$, 2.3 Mg, 1.2 $H_2PO_4$, 1.0 $NH_4$, 0.2 Fe, 15.9* Na, 10.1* Mn, 7.5* B, 5.0* Zn, 0.8* Cu, and 0.5* Mo, which is common practice in commercial tomato production. On the basis of this standard nutrient solution, the solutions with variant EC-levels were prepared by either dilution or addition of appropriate amounts of $NaCl/CaCl_2$ (2:1, molar basis) (26). Initially, all tomato plants were irrigated with the standard nutrient solution for five days after which the final EC-levels were gradually established over a period of 10 days. As such, the final EC-levels were reached at December 12, 2013 and maintained till the end of the experiment, i.e. March 4, 2014.

Harvest of tomato fruits was performed as illustrated in Figure 6.2 A and started on January 7, 2014. Six different stages of fruit development and ripening were considered and theoretically defined according to the days after anthesis (DAA). More specifically, the targeted number of DAA for stage 1 (S1), stage 2 (S2), stage 3 (S3), stage 4 (S4), stage 5 (S5) and stage 6 (S6) was respectively set at 15, 25, 35, 45, 55 and 65. For each stage, a total of 18 tomato fruits was collected per treatment, whereby the fruits originated from 3 different trusses of 3 different plants. Harvesting the fruits that were first or last set within a truss was avoided. The six fruits from a single truss were pooled per two (based on similar DAA), which resulted in three sampling entities per truss (Figure 6.2 B).

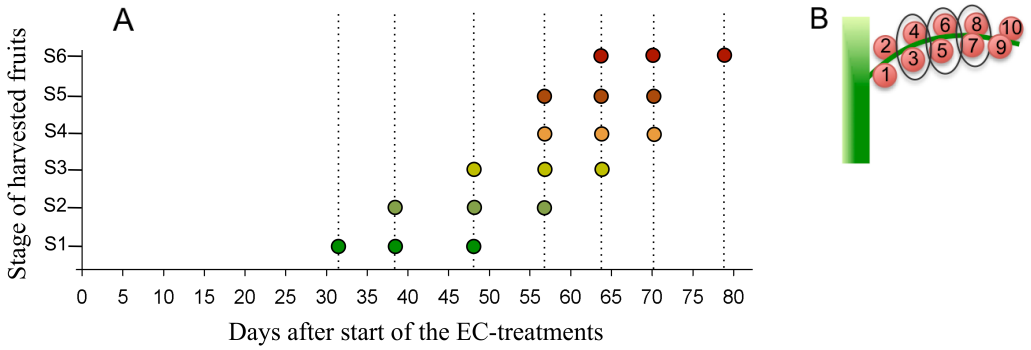


**FIGURE 6.2.** Schematic overview of the harvest strategy (A), which was executed for each individual EC-treatment. A total of seven harvest moments, represented by the dashed lines, were considered. At each harvest moment, specific stages of fruit development were contemplated as indicated on the Y-axis. Hereby, a total of 6 stages of fruit development and ripening were defined (i.e. from S1 to S6) based on DAA. Schematic representation of the sampling and pooling strategy (B).

The consecutive cutting, lyophilization, grinding and sieving of each individual entity resulted in a homogenous powder, allowing representative sampling. Plant material was kept cold (-20 °C) and shielded from light in order to prevent degradation of any fruit compound. A total of 180 samples, representing six different stages of development and five EC-treatments, was analyzed.

## 2.2 CHEMICALS AND REAGENTS

The carotenoid analytical standard all-*trans*-α-carotene was purchased from Wako Chemicals GmbH (Neuss, Germany), all-*trans*-β-carotene was from Sigma-Aldrich Co. (St. Louis, MO, USA), all-*trans*-lycopene was from Phytolab GmbH & Co. KG (Vestenbergsgreuth, Germany), all-*trans*-lutein was from Extrasynthese (Genay, France) and all-*trans*-zeaxanthin was from TRC Inc. (Eching, Germany). The internal standard β-apo-8'-carotenal was obtained from Sigma-Aldrich Co. All phytohormone analytical and deuterium-labeled internal standards were obtained from OlchemIm Ltd. (Olomouc, Czech Republic). The sugar standards D-glucose and D-fructose were from VWR International (Merck, Darmstadt, Germany). The standards for malic acid and citric acid were from VWR International and Sigma-Aldrich Co., respectively. Reagents were of analytical grade when used for extraction purposes and of LC-MS grade for (U)HPLC-Orbitrap-MS applications. They were respectively purchased from VWR International and Thermo Fisher Scientific Inc. (Loughborough, UK). Ammonium acetate and formic acid were obtained from VWR International, and magnesium carbonate was from Sigma-Aldrich Co. Ultrapure water was supplied by usage of a purified-water system (VWR International).

## 2.3 TARGETED ANALYSIS OF FIVE TOMATO FRUIT CAROTENOIDS

Targeted analysis of tomato fruit carotenoids was performed according to Van Meulebroek *et al.* (2014) (27) (Chapter II) and aimed at determining the accurate concentration levels of α-carotene, β-carotene, lycopene, lutein, and zeaxanthin. Carotenoid concentrations were thereby calculated using matrix-matched calibration curves whereby an internal standard (β-apo-8'-carotenal) was used to counteract potential fluctuations during analysis.

## 2.4 UNTARGETED ANALYSIS OF TOMATO FRUIT PHYTOHORMONES AND RELATED METABOLITES

Extraction and detection of tomato fruit phytohormones and related metabolites was performed as described by Van Meulebroek *et al.* (2012) (28) (Chapter III). The untargeted analysis was founded on the generic nature of the applied extraction procedure and the metabolomic screening possibilities of the employed Exactive™ single-stage Orbitrap mass spectrometer (Thermo Fisher Scientific, San José, USA). System stability during mass spectrometric analysis of samples was verified using three matrix-matched calibration curves (28), which were run at different time-points during analysis of the samples. These calibration curves included six concentration levels, considering at least one representative for each of the major hormonal classes. Coefficients of variance were each time calculated per concentration level and then averaged, resulting in 10.79% for $N^6$-benzyladenine, 8.94% for gibberellic acid, 7.90% for (±)-*cis, trans* abscisic acid, 7.64% for indol-3-acetic acid, 6.09% for epibrassinolide, 5.29% for salicylic acid, 4.74% for *trans*-zeatin, and 2.07% for jasmonic acid. Since these values were well below 15% (29), appropriate stability during analysis was concluded.

## 2.5 METABOLIC FINGERPRINTING OF TOMATO FRUITS

The acquired full-scan data from the untargeted phytohormone analysis were processed by Sieve™ (Thermo Fisher Scientific Inc., San Jose, USA), whereby non-differential single class analysis was implemented. This strategy solely aims at defining the metabolites that are present in the individual extracts in terms of *m*/*z*-value, retention time, and signal abundance. The main parameter settings for this metabolic fingerprinting included a scan range of 100-800 Da, a *m*/*z*-width of 10 ppm, a maximum peak width of 0.5 min, a minimum intensity threshold of 50,000 arbitrary units, and a maximum of 30,000 frames (i.e. unique combinations of retention time and *m*/*z*-value). The generated data matrix was normalized by the signal intensities of two deuterium-label internal standards (30), i.e. $d_6$-abscisic acid and $d_7$-$N^6$-benzyladenine, which were supplemented prior to extraction. Hereby, the abundances of the negatively ionized metabolites were normalized based on $d_6$-abscisic acid, presenting a same ionization polarity. The reverse was true for $d_7$-$N^6$-benzyladenine. These internal standards were specifically selected because of their complementary ability to appropriately counteract potential fluctuations during analysis for all major hormonal classes (28).

## 2.6 SCREENING FOR METABOLITES INVOLVED IN THE PLANT STRESS RESPONSE

Multivariate data analysis was performed by means of SIMCA$^{TM}$ 13 software (Umetrics, Malmö, Sweden) and aimed at elucidating those metabolites that are involved in the plant stress response towards altered EC-levels. For this purpose, data were reorganized into six data clusters, whereby each individual cluster contained the data about a well-defined stage of fruit development (i.e. S1, S2, S3, S4, S5, or S6). Within each cluster and thus for each stage of fruit development, potential effects of the imposed EC-treatments on the metabolomic fingerprints were investigated. Orthogonal partial least squares to latent structures discriminant analysis (OPLS-DA) was applied to model these multiple classes, i.e. to identify a relationship between the LC-MS data (X-variables) and the EC-treatments, which were described by discrete values (Y-variables). The advantage of OPLS compared to PLS relates to the applied model rotation whereby class separation is found in the first predictive component (tp, correlated variation) and variation that is not associated with class separation is presented in orthogonal components (to, uncorrelated variation) (31). Before OPLS-DA was applied, pareto-scaling (1/√SD, where SD is the standard deviation) and automatic transformation of the data were performed in order to standardize the range of independent X-variables and induce normality, respectively (30). Validity of the model was verified by CV-ANOVA (P-values < 0.05), permutation testing (n = 100), and three model characteristics (i.e. $R^2(X)$, $R^2(Y)$ and $Q^2(Y)$, calculated by 7-fold cross-validation) (32-34). With respect to the latter, a $Q^2(Y)$ of 0.5 indicates good model predictability.

## 2.7 SCREENING FOR METABOLITES INVOLVED IN CAROTENOID METABOLISM

Deepening the knowledge about carotenoid metabolism was founded on modeling the interrelationship between the tomato fruit fingerprints and their inherent carotenoid concentrations. Multivariate data analysis was performed by SIMCA$^{TM}$ 13 software whereby OPLS-models were specifically constructed for each of the targeted carotenoids. As such, these models intend to explain and predict one quantitative Y-variable (i.e. carotenoid concentration) from the X-matrix (i.e. the tomato metabolic fingerprints). A same practice with respect to data preprocessing (scaling and transformation) and model validation as earlier described was applied.

## 2.8 THE POTENTIAL OF ALTERED SALINITY TO IMPROVE NUTRITIONAL TOMATO QUALITY

To investigate the potential of altered nutrient solution salinity to enhance tomato fruit carotenoid content, a comparative evaluation of the metabolites that were retained by both untargeted screening approaches was conducted. To define any actual effects of the salt treatments, stem xylem water potential and fruit total water potential were determined on March 12$^{th}$ 2014. Xylem water potential of the stem was measured by using the pressure chamber (PMS Instruments Co., Corvallis, USA). Total fruit water potential was calculated as the sum of the turgor pressure and the osmotic potential. Fruit turgor pressure was determined using a custom-made pressure sensor. This pressure sensor consists of a sharpened microcapillary, a control rod and a pressure transducer and is filled with oil. For a detailed description of the pressure sensor and methodology, we refer to Mattheyses *et al.* (2014). Osmotic potential of the fruits was measured with the thermocouple psychrometer. The setup consisted of three chambers (C-52, Wescor, Logan, USA), a switch box (PS-10, Wescor, Logan, USA), a dewpoint microvoltmeter (HR-33T, Wescor, Logan, USA) and a recorder for the output signal.

In addition, the overall effect of altered salinity on tomato fruit was verified by means of some secondary parameters, i.e. fruit yield, sugar and organic acid concentration, and dry matter percentage. These traits were determined on the same tomato fruits that were used for carotenoid profiling and metabolic fingerprinting.

Extraction of sugars was realized on 50 mg tomato fruit tissue with 2 mL ethanol, at 70 °C for 10 min and 45 °C for 3 h. Subsequently, extracts were filtrated through a 0.45 μm PP syringe filter (Sigma-Aldrich Co., St. Louis, MO, USA). Detection and quantification of the main sugars, i.e. glucose and fructose, was achieved by HPLC coupled to Evaporative Light Scattering Detection (Grace Davison Discovery Sciences, Deerfield, IL, USA) (35).

Organic acids were extracted from 50 mg tomato fruit tissue with 2 mL ultrapure water at 100 °C for 15 min. Next, obtained extracts were centrifuged (5000 x g, 10 min) and filtrated through a 0.45 μm PP syringe filter (Sigma-Aldrich Co., St. Louis, MO, USA). Analysis of the targeted organic acids, i.e. malic and citric acid was achieved by HPLC coupled to LTQ$^{TM}$ XL linear ion trap mass spectrometry (Thermo Fisher Scientific Inc., San Jose, CA, USA). Chromatographic separation was achieved on a GraceSmart C18 column (5 μm, 150 x 2.1 mm) (Grace Davison Discovery Sciences,

Deerfield, IL, USA) whereby the mobile phase was adopted from Khan *et al.* (2012) (36). Mobile phase flow rate and column oven temperature were set at 250 μL $min^{-1}$ and 25 °C, respectively. The mass spectrometer was equipped with a HESI source, operating in negative ionization mode. The main instrumental parameters included a heater temperature of 350 °C, a capillary temperature of 250 °C, a tube voltage of -120 V, a capillary voltage of -50 V, a sweep gas flow rate of 1 au, an auxiliary gas flow rate of 15 au, and a sheath gas flow rate of 45 au. Fragmentation of malic and citric acid was established using a collision energy of 30 kV. Identification and quantification was based on the two most abundant *m/z* transitions, i.e. 110.84 and 172.89 for citric acid, and 86.84 and 114.80 for malic acid.

The results with respect to these parameters were interpreted for the final stage of fruit development, i.e. the harvest-ready tomato fruits. Furthermore, the statistical analysis was focused on the comparison of the conventional nutrient solution EC-level of 2.5 dS $m^{-1}$ versus the increased EC-levels of 3.0, 4.0, and 5.0 dS $m^{-1}$.

# 3. RESULTS AND DISCUSSION

## 3.1 METABOLIC FINGERPRINTING OF TOMATO FRUITS

Metabolic fingerprinting of the developing tomato fruits resulted in the detection of in total 21,698 monoisotopic ions of which the majority (69%) was obtained in the positive ionization mode. The corresponding, normalized data matrix was used to screen for metabolites that are involved in the plant stress response and carotenoid metabolism. Since these data enclose the ion abundances on a dry weight basis, possible concentration effects solely caused by osmotic stress and related impaired water uptake were excluded.

## 3.2 SCREENING FOR METABOLITES INVOLVED IN THE PLANT STRESS RESPONSE

In order to anticipate any potential influence of fruit age on the plant metabolic response, individual OPLS-DA models were generated for each of the defined stages of fruit development. This strategy was justified since no significant differences (one-way ANOVA, P-value > 0.05) were observed in DAA between treatments for each of the developmental stages. OPLS-DA models that were compliant with the suggested validation criteria are presented in Table 6.1.

**TABLE 6.1** OPLS-DA models that were generated to discriminate between EC-treatments and their associated metabolic fingerprints, thereby taking into account the various stages of fruit development. For each model the number of predictive (tp) and orthogonal (to) components is presented. For clarity reasons, following labeling of EC-treatments was introduced: A 2.0 dS $m^{-1}$; B 2.5 dS $m^{-1}$; C 3.0 dS $m^{-1}$; D 4.0 dS $m^{-1}$; E 5.0 dS $m^{-1}$.

| Model description | | Components (tp + to) | $R^2(X)$ | $R^2(Y)$ | $Q^2(Y)$ |
|---|---|---|---|---|---|
| ***S1*** | A versus C | 1+6 | 0.852 | 1.000 | 0.824 |
| | A versus D | 1+1 | 0.419 | 0.990 | 0.900 |
| | A versus E | 1+2 | 0.694 | 0.996 | 0.952 |
| ***S2*** | A versus D | 1+4 | 0.785 | 1.000 | 0.940 |
| | A versus E | 1+2 | 0.658 | 0.998 | 0.953 |
| ***S3*** | A versus E | 1+3 | 0.700 | 0.999 | 0.890 |
| ***S4*** | A versus D | 1+3 | 0.721 | 0.999 | 0.965 |
| | A versus E | 1+3 | 0.696 | 1.000 | 0.891 |
| ***S5*** | A versus D | 1+2 | 0.551 | 0.996 | 0.865 |
| | A versus E | 1+2 | 0.562 | 0.999 | 0.861 |
| ***S6*** | A versus C | 1+2 | 0.626 | 0.992 | 0.884 |
| | A versus D | 1+2 | 0.528 | 0.999 | 0.935 |
| | A versus E | 1+2 | 0.585 | 0.938 | 0.938 |

These two-class models had the ability to discriminate between two EC-treatments, whereby the treatment of 2.0 dS $m^{-1}$ was denoted as the common reference.

Appropriate visualization tools and related descriptors were employed for interpreting the data and selecting biochemical interesting ions, respectively. An overview of the implemented selection strategy is depicted in Figure 6.3.

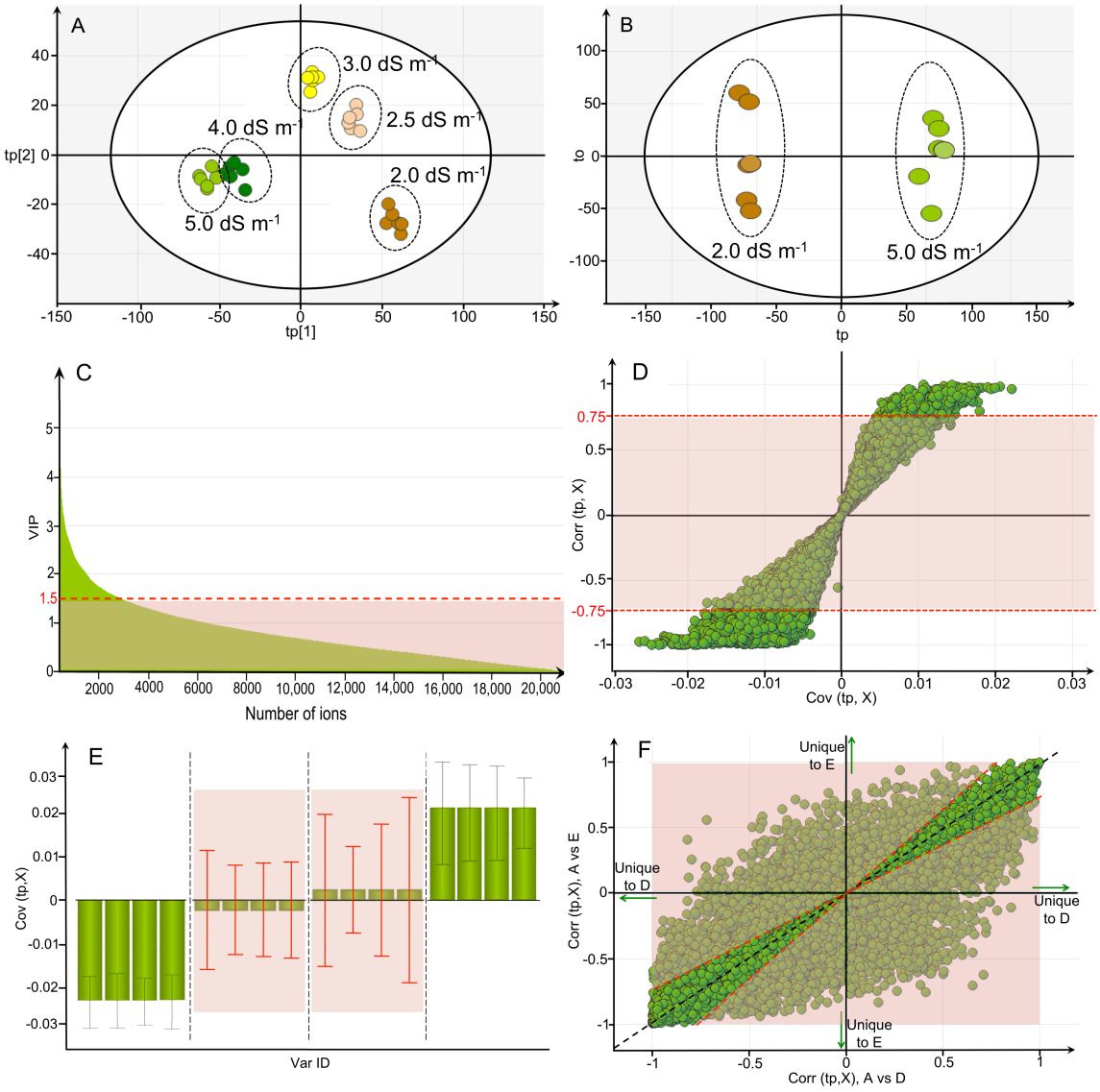


**FIGURE 6.3.** OPLS-DA model that was generated for the initial stage of fruit development (S1), whereby discrimination between the various EC-treatments was achieved on the basis of the metabolic tomato fruit fingerprints (A). Selection of relevant ions was performed for each of the successfully validated two-class models (e.g. 2.0 versus 5.0 dS $m^{-1}$, B) by using VIP-scores (C), S-plot (D), and loading-plot with jack-knifed confidence intervals (E). In addition, the SUS-plot was used to visualize the shared information for multiple two-class OPLS-DA models (F). Shaded areas represent the ions that were considered irrelevant and excluded. For clarity of figures, following labeling of treatments was introduced: A 2.0 dS $m^{-1}$; B 2.5 dS $m^{-1}$; C 3.0 dS $m^{-1}$; D 4.0 dS $m^{-1}$; E 5.0 dS $m^{-1}$.

A first selection criterion related to the Variable Importance in Projection (VIP) score, which reflects the importance of an ion towards the OPLS-DA model predictive component. Hereby, a VIP-score > 1 is generally associated with a significant ion (37). In this study, based on the large number of metabolites and the structure of the generated VIP-plots, a more stringent threshold VIP-score of 1.50 was adopted. The second selection step focused on the reliability (correlation) of the ions with respect to the model predictive component (30). This feature is visualized by the S-plot and referred to as corr(tp, X). A correlation coefficient of 0.75 (or -0.75) was set as a cutoff value. In addition, the loading plot (cov(tp, X)) with corresponding jack-knifed confidence intervals ($CIJF_{JK}$) represents a complementary tool, providing information about the metabolite variability. A small confidence interval renders more creditability on the selected ion (38). According to the study of Wager *et al.* (2014) (38), ions with a $CIJF_{JK}$ across zero were not retained in our study. The above-mentioned steps were implemented for each individual OPLS-DA model. However, in the case that multiple OPLS-DA models could be generated for a particular stage of development, it was expected that retained ions are corresponding between these models. The assumption was made that metabolites, which are biochemically involved in the plant salinity response, are not qualitatively differing as a function of salinity severity. Therefore, ions that were assigned opposite effects or not retained by each of the OPLS-DA models (associated with a particular developmental stage) had to be excluded. In this context, the shared and unique structure (SUS)-plot represented an interesting tool since information from multiple two-class models with a same reference is combined. Such a plot (Figure 6.3 F) is constructed based on the corr(tp, X) profiles from two or more models, whereby the position of an ion relative to the diagonal is indicative for its importance towards each of the various classes (30). An ion was recognized as a shared structure and thus retained if the corr(tp, X) ratio was between 0.75 and 1.25. A strong variation was noted in the number of ions that was retained for each developmental stage; i.e. 277 for S1, 942 for S2, 1200 for S3, 779 for S4, 742 for S5, and 84 for S6. This ion number was found strongly related to the number of validated OPLS-DA models (Table 6.1). The availability of multiple models indeed offers the possibility to effectuate an additional ion selection step (SUS-plot), as such increasing the reliability of the retained ions. The variation in the number of OPLS-DA models may be attributed to the prevailing climatic conditions at the time of harvest, which may antagonize

the salinity effects, or to varying climatic conditions at consecutive harvest moments, which may enlarge variation between samples within a treatment.

### 3.3 SCREENING FOR METABOLITES INVOLVED IN CAROTENOID METABOLISM

To deepen our knowledge about the underlying mechanisms of carotenoid metabolism, OPLS-models were specifically designed for each of the targeted carotenoids. These models intend to predict the tomato fruit carotenoid concentrations based on the associated metabolic fingerprints. Since both tomato fruit carotenoid concentrations and metabolite abundances were determined on a dry weight basis, possible concentration effects due to osmotic stress and impaired water uptake were precluded. Data coming from all fruits were used without any modifications or restructuring. OPLS-models that were used for the interpretation of metabolic fingerprints and selection of biochemically relevant metabolites are presented in Table 6.2.

**TABLE 6.2.** OPLS-models that were designed for the prediction of tomato fruit carotenoid concentration levels on the basis of associated metabolic fingerprints. Validation of these models was performed based on CV-ANOVA (P-value < 0.05), permutation testing and three model characteristics (($R^2(X)$, $R^2(Y)$, and $Q^2(Y)$). All of the presented models performed well for CV-ANOVA and permutation testing.

| Model description | Components (tp + to) | $R^2(X)$ | $R^2(Y)$ | $Q^2(Y)$ |
|---|---|---|---|---|
| Lutein | 1 + 4 | 0.564 | 0.957 | 0.915 |
| Zeaxanthin | 1 + 6 | 0.660 | 0.906 | 0.698 |
| β-carotene | 1 + 4 | 0.592 | 0.647 | 0.308 |
| Lycopene | 1 + 5 | 0.672 | 0.988 | 0.929 |

These models were compliant with the adopted validation criteria with the exception of the β-carotene model for which $Q^2(Y)$ was below the set 0.5 threshold value. It was, however, opted to further use this model since CV-ANOVA and permutation testing performed well. The model that was constructed for α-carotene was not compliant with a single validation criterion and therefore disqualified for further use. The main reason for the inadequate model performance may relate to the tomato cultivar's low α-carotene concentration levels near the limit of detection were observed. Model outputs, reflecting the predicted versus observed carotenoid concentrations, are illustrated in Figure 6.4.

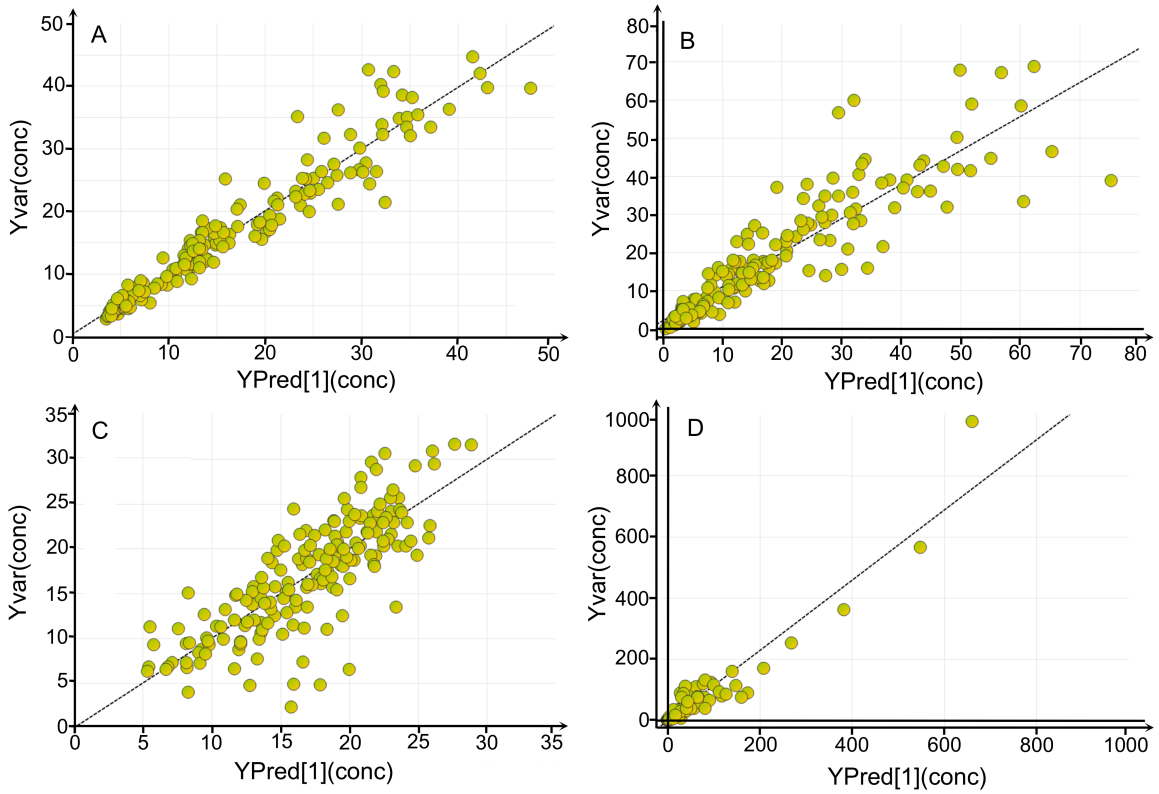


**FIGURE 6.4.** Graphical representation of the OPLS-models' output, relating the observed and predicted Y-variables (i.e. carotenoid concentrations). Individual models were generated for lutein (A), zeaxanthin (B), β-carotene (C), and lycopene (D).

Selection of relevant metabolites was performed as earlier described in section 3.2. Based on this extensive strategy of selection, the number of retained ions was 194 for lycopene, 408 for β-carotene, 1110 for zeaxanthin, and 1200 for lutein. These ions were assigned potential contributing value towards the metabolism of the target carotenoids.

## 3.4 THE POTENTIAL OF ALTERED SALINITY TO IMPROVE NUTRITIONAL TOMATO QUALITY

To determine the metabolites that were involved in the plant response to salinity and in carotenoid metabolism, the results of both screening approaches were comparatively evaluated. This revealed a total of 46 metabolites, which were assigned a key role in the plant response to salinity and carotenoid metabolism (Table 6.3). Hereby, ions that were unjustly assigned during Sieve™ based metabolic fingerprinting were excluded based on the chromatographic peak performance (peak shape). Taking into account that some metabolites were involved in the metabolism of multiple carotenoids, the following results were obtained: 34 metabolites for lutein, 4 for zeaxanthin, 8 for β-carotene, and 32 for lycopene.

Putative annotation of these metabolites was performed according to the identification strategy, presented in Chapter IV. An integrated approach of chemical formula prediction by MZmine (39) and structure evaluation by MetFrag (40) was realized, whereby the results from both software tools were simultaneously interpreted to determine a best identity match. The MS/MS dataset, used for this identification process, was obtained from the tomato fruits, analyzed by high-resolution hybrid quadrupole Exactive™ Orbitrap-MS and characterized in Chapter IV. This dataset (acquired by data dependent MS/MS) allowed the putative annotation of 19 metabolites, which are specified by the PubChem database compound identifier (CID) (Table 6.3). Moreover, one phytohormone was unambiguously identified by means of the associated reference standard; i.e. 1-acetyl-indole-3-carboxaldehyde.

**TABLE 6.3.** Chromatographic and mass spectrometric information about metabolite ions that were assigned a potential role in both carotenoid metabolism and salinity stress response. These metabolite ions were labeled with an identification number (i.e. Metabolite ID) for further graphical representations. Some of these metabolites were putatively annotated, which yielded a best identity match (PubChem CID). The fragmentation ratio was calculated as the ratio between the number of matching fragments of the CID-compound versus the highest number of matching fragments among all candidate structures.

| Metabolite ID | Ion. mode | *m/z*-value | RT (min) | CID | Fragmentation ratio (%) | Associated chemical formula |
|---|---|---|---|---|---|---|
| 1 | + | 476.2991 | 7.28 | | | |
| 2 | + | 269.2096 | 7.13 | | | |
| 3 | + | 409.1486 | 5.39 | 44144278 | 81.3 | $C_{20}H_{24}O_9$ |
| 4 | + | 711.2440 | 4.63 | | | |
| 5 | + | 576.2689 | 3.89 | | | |
| 6 | + | 785.8416 | 4.73 | | | |
| 7 | + | 572.4492 | 8.36 | | | |
| 8 | + | 311.2166 | 6.35 | | | |
| 9 | + | 337.2015 | 2.99 | | | |
| 10 | + | 602.4972 | 6.26 | | | |
| 11 | + | 355.1776 | 4.61 | | | |
| 12 | + | 275.0906 | 4.70 | | | |
| 13 | + | 596.4120 | 4.63 | | | |
| 14 | + | 531.2604 | 3.49 | | | |
| 15 | + | 528.3285 | 5.94 | | | |
| 16 | + | 530.2776 | 6.70 | | | |
| 17 | + | 485.1479 | 5.26 | 314405 | 100.0 | $C_{22}H_{28}O_{10}S$ |
| 18 | + | 221.1138 | 5.98 | 18519108 | 100.0 | $C_8H_{16}N_2O_5$ |
| 19 | + | 348.2155 | 6.20 | 22997503 | 76.4 | $C_{20}H_{29}NO_4$ |
| 20 | + | 390.1299 | 4.58 | | | |
| 21 | + | 462.1048 | 2.85 | 1190411 | 100.0 | $C_{14}H_{19}N_7O_9S$ |
| 22 | + | 410.1552 | 4.19 | | | |
| 23 | + | 377.1055 | 3.60 | | | |
| 24 | + | 775.3393 | 5.67 | 21630904 | 66.7 | $C_{36}H_{54}O_{18}$ |
| 25 | + | 188.0636 | 4.14 | 1-acetylindole-3-carboxaldehyde | | |
| 26 | - | 163.0394 | 3.26 | 691 | 100.0 | $C_9H_8O_3$ |

| | | | | | | |
|---|---|---|---|---|---|---|
| 27 | - | 565.0488 | 5.52 | | | |
| 28 | - | 773.3309 | 5.64 | 11767898 | 76.9 | $C_{43}H_{46}N_6O_8$ |
| 29 | - | 736.3310 | 3.86 | | | |
| 30 | - | 115.0393 | 3.61 | | | |
| 31 | - | 431.2446 | 7.37 | 12133739 | 85.8 | $C_{25}H_{36}O_6$ |
| 32 | - | 378.1348 | 2.94 | 25232866 | 83.2 | $C_{22}H_{21}NO_5$ |
| 33 | - | 425.1469 | 5.38 | 36942227 | 75.0 | $C_{21}H_{22}N_4O_6$ |
| 34 | - | 569.0984 | 6.55 | | | |
| 35 | - | 603.2794 | 8.08 | | | |
| 36 | - | 189.0764 | 4.58 | 26339616 | 85.6 | $C_8H_{14}O_5$ |
| 37 | - | 249.1246 | 2.86 | 45809090 | 100.0 | $C_{13}H_{18}N_2O_3$ |
| | | | | 18108416 | | |
| | | | | 43403806 | | |
| | | | | 43421263 | | |
| | | | | 43392898 | | |
| 38 | - | 286.1661 | 4.33 | | | |
| 39 | - | 378.1351 | 2.65 | 45898135 | 93.3 | $C_{22}H_{21}NO_5$ |
| 40 | - | 461.0163 | 4.36 | 17931268 | 42.9 | $C_{12}H_{21}NO_4$ |
| 41 | - | 242.1400 | 5.52 | | | |
| 42 | - | 361.0696 | 3.29 | 1014968 | 100 | $C_{17}H_{18}N_2O_3S_2$ |
| 43 | - | 463.2711 | 7.37 | 10457172 | 66.7 | $C_{26}H_{40}O_7$ |
| | | | | 9825709 | | |
| | | | | 9847284 | | |
| | | | | 21363440 | | |
| 44 | - | 486.0865 | 5.67 | | | |
| 45 | - | 777.2091 | 5.12 | | | |
| 46 | - | 189.0764 | 4.83 | 23399204 | 70.0 | $C_8H_{14}O_5$ |

The integrated evaluation of the metabolites' changes towards altered salinity and their effects on the carotenoid concentration levels may refute or prove the potential of this agronomic strategy as a potential tool to promote tomato fruit nutritional quality. In this regard the covariance (tp, X) is an interesting descriptor for both the direction and magnitude of these metabolite changes and effects. Therefore, individual covariance plots were established for each carotenoid (Figure 6.5). With respect to the metabolites' covariance regarding altered salinity, it was opted to use the data that were acquired from the OPLS-DA models, generated to discriminate between the EC-treatment of 2.0 dS $m^{-1}$ and 5.0 dS $m^{-1}$. As such, all developmental stages were represented (Table 6.1). Moreover, usage of the SUS-plot guaranteed that the change (up or downward) in concentration level of a retained ion was identical for all OPLS-DA models under consideration (i.e. for a particular fruit developmental stage). Furthermore, it was verified that the changes for the retained ions were also identical across the various developmental stages. If a metabolite was found significantly influenced by altered salinity in various stages of fruit development, the average covariance was used.

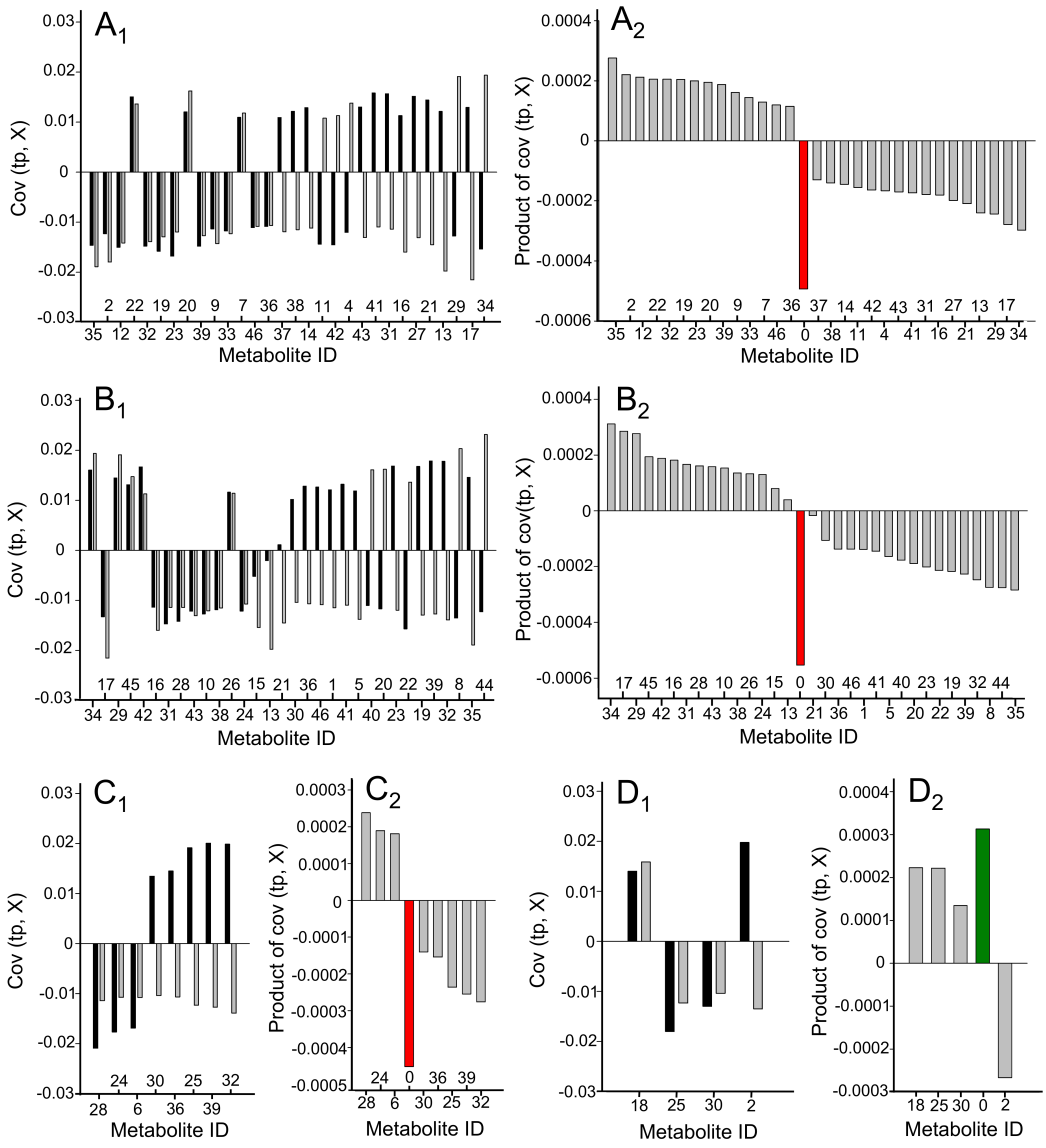


**FIGURE 6.5.** Individual covariance plots for lutein ($A_1$), lycopene ($B_1$), β-carotene ($C_1$) and zeaxanthin ($D_1$). The covariance (effect) of a metabolite with respect to the carotenoid concentration is indicated in black. The covariance of a metabolite towards altered salinity is indicated in grey and was acquired from the OPLS-DA models, discriminating between the EC-treatments of 2.0 dS $m^{-1}$ and 5.0 dS $m^{-1}$. Individual plots of covariance products for lutein ($A_2$), lycopene ($B_2$), β-carotene ($C_2$) and zeaxanthin ($D_2$). The metabolite with ID zero represents the sum of all covariance product functions. Red color indicates a total negative outcome and green color a total positive outcome.

With respect to these covariance plots, it can be stated that a metabolite with concurring covariances (i.e. with identical signs) may positively contribute towards the potential of altered salinity to promote tomato fruit carotenoid content. This particularly refers to metabolites, which are up-regulated under an increased nutrient solution EC-level and have a positive influence on the

accumulation of carotenoids, and vice versa. For the various carotenoids, the constructed covariance plots indicated equilibrium in terms of numbers of metabolites that would have an overall positive (identical covariance signs) and negative (opposite covariance signs) influence, respectively, on carotenoid accumulation in response to increased salinity. Based on these findings, it may be concluded that altered salinity has no or little potential to improve tomato fruit carotenoid content. In addition, the magnitude of the covariances is attributed significant value as well. Therefore, the final effect of a metabolite was calculated as the product of both covariances, which resulted in a new covariance function (41). The cumulative of these new covariance functions indicated an overall relatively small negative effect of altered salinity on the accumulation of lutein, β-carotene, and lycopene in tomato fruit. For zeaxanthin, a relatively small positive effect was revealed. These results endorse the previous made conclusions that altered salinity has no or little potential to improve tomato fruit carotenoid content. This finding was confirmed by evaluating the carotenoid concentration levels of the final stage of fruit development, for which no statistical differences (One-way ANOVA, P-value > 0.05) were observed between treatments (Figure 6.6).

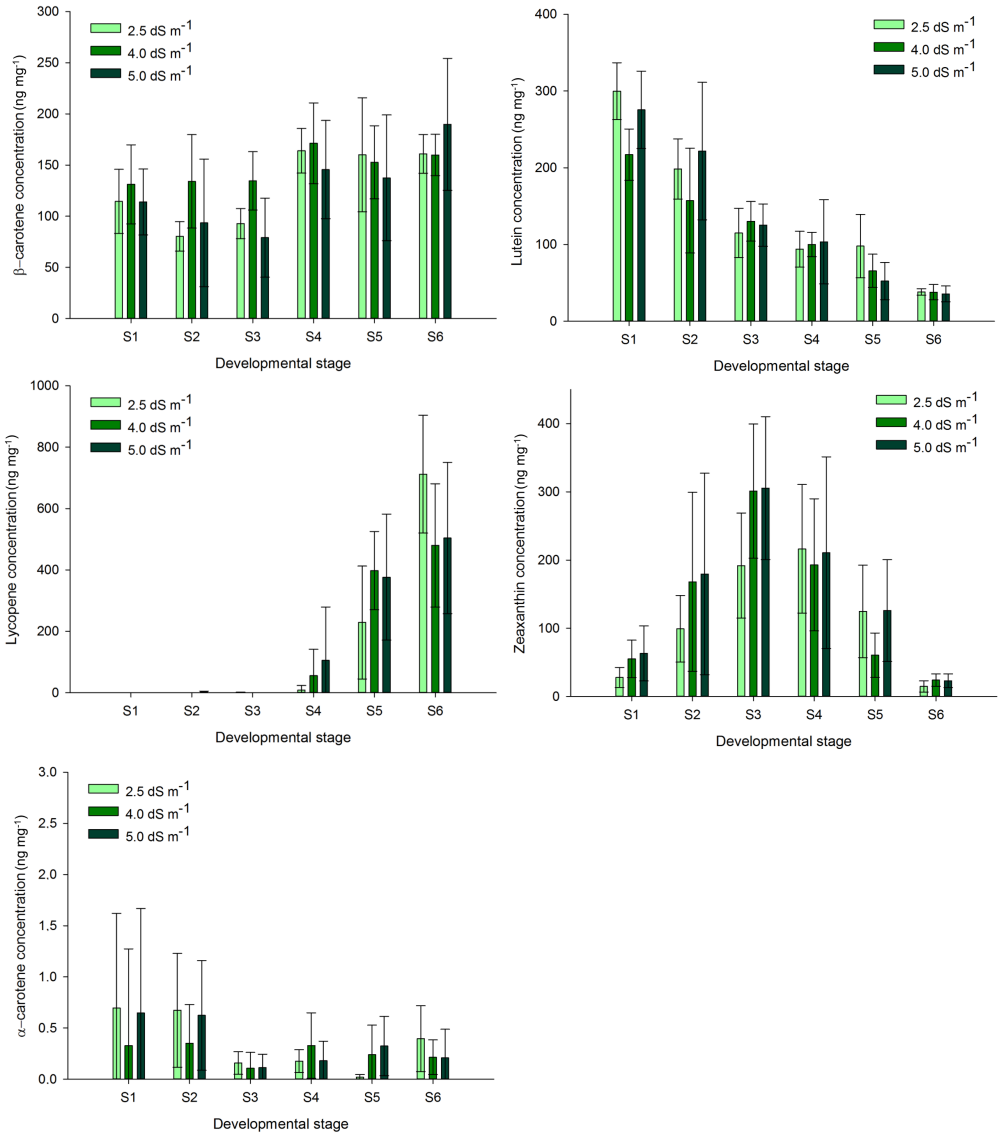


**FIGURE 6.6.** Carotenoid concentration levels (on a dry weight basis) for the various developmental stages and the treatments of 2.5, 4.0, and 5.0 dS $m^{-1}$ (n = 6, standard deviation).

Although this conclusion may concur with the findings of various other studies (18, 10,42-45), it cannot be renounced that some studies (1,7,13,17,46) clearly indicated the potential of altered salinity to increase carotenoid concentration levels in tomato fruit (up to 40%). Therefore, it was interesting to verify if tomato plants in this study effectively experienced any effects of imposed salinity treatments by considering the stem and fruit xylem water potential, which has been indicated by Choné *et al.* (2001) (47) to be a good indicator of water status. It may be deduced from Figure 6.7 that tomato plants effectively reacted towards the imposed stress conditions by lowered water potentials.

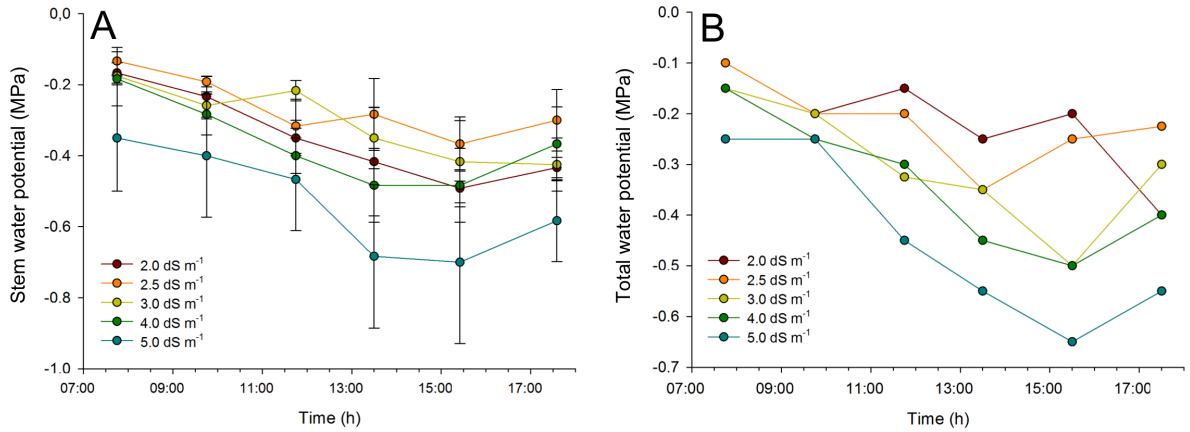


**FIGURE 6.7.** Xylem water potential measured for stem (n = 3) and total water potential measured for fruit, and this for the various salinity treatments.

One of the main reasons for these conflicting data may relate to the genetic background of the selected tomato cultivars, which may be associated with varying salt tolerances (22,48). Various mechanisms with respect to plant salt stress response have been recognized to be universal, but their relative importance may vary from species to species depending on the metabolic background (48). This finding has clearly been demonstrated in the study of Caro *et al.* (1991) (49) in which normal-fruited (*Solanum lycopersicum* L.) tomato cultivars were found to be less salt tolerant compared to cherry (*Solanum lycopersicum* L. var. cerasiforme) tomatoes. As such, under identical salinity conditions, a less pronounced stress state may be expected in cherry tomato cultivars. This could explain why in our study, which involved a cherry tomato cultivar, no effects of altered salinity on carotenoid accumulation were observed. Taking into account these statements, a further increase of nutrient solution EC-level may be appropriate. However, the structure of the covariance plots with a near-equilibrium between the number of metabolites with an overall negative and positive effect on carotenoid accumulation indicate little chance on success. This would imply that a relatively high percentage of those metabolites with a positive product of covariances are up-regulated and those with a negative product of covariances are down-regulated.

A second reason may relate to the prevailing environmental conditions during the experiment, which could interact with and influence the salinity effects (21). It has indeed been indicated by Cuartero and Fernández-Muñoz (1998) (50) that high temperatures, radiation, and low relative humidity enhance osmotic stress, associated with increased salinity. In our study no such microclimatic conditions were monitored and were considered rather antagonistic towards possible salinity effects.

A final cause of the conflicting data may relate to the compositional changes that were made to establish increased nutrient solution EC-levels. In the studies of Taber *et al.* (2008) (8) and Fanasca *et al.* (2006) (6), the selected nutrients used to raise the salinity were indeed indicated to affect components' concentration levels in tomato fruit. However, Ehret and Ho (1986) (53) stated that selected nutrients have only little influence on tomato fruit composition.

Although the underlying reasons for the observed results may be rather unclear, the presented methodology is believed to be of significant value in elucidating and exploiting the potential of set agronomic measures. Indeed, focusing on the regulatory role of phytohormones or related metabolites offers the advantage that interactive effects between environmental conditions, genetic background and agronomic settings can be evaluated on a more short-term basis. These key-regulating metabolites fluctuate more dynamically in response to prevailing conditions compared to the tomato fruit carotenoid content, as the concentration levels of these fruit components can be regarded as the cumulative outcome of all possible (interactive) effects that occurred during fruit development and ripening.

In addition, the overall effect of altered salinity on tomato fruit performance was verified by considering some secondary parameters, related to fruit yield and organoleptic quality.

Fresh tomato fruit weights (g $fruit^{-1}$), determined immediately after harvest, were not significantly different between treatments and thus not influenced by increased salinity. The average fresh weights for the treatments ranged from 9.46 ± 1.09 (2.5 dS $m^{-1}$) to 8.65 ± 1.16 (5.0 dS $m^{-1}$) g $fruit^{-1}$, whereby a continuous decline in average fresh weight was observed with increasing salinity. Dry matter percentages were calculated on the basis of fresh and lyophilized tomato fruit weights, and were significantly affected by altered salinity. More specifically, higher dry matter percentages

were obtained under the EC-level of 5.0 dS $m^{-1}$ compared to the EC-treatments of 2.5 and 3.0 dS $m^{-1}$. In addition, dry matter percentage was also significantly higher when the EC-level was increased from 2.5 to 4.0 dS $m^{-1}$. These results indicate that application of moderate salinity has no adverse effect on tomato fruit fresh yield and may even improve dry matter content, which has been reported earlier (7).

Tomato fruit taste and organoleptic quality is primarily determined by the presence of glucose, fructose, malic acid, and citric acid. Hereby, in particular the balance between these reducing sugars and organic acids have been suggested to be a decisive factor for appreciated tomato fruit taste (54). In our study, the ratio between the target sugars and organic acids (both on dry and fresh weight basis) were not significantly affected by altered salinity, indicating a preserved tomato fruit taste.

# 4. Conclusions

In this study, the potential of altered salinity to improve cherry tomato carotenoid content was investigated by focusing on the underlying mechanisms of metabolism. More specifically, a metabolomic approach was implemented to reveal phytohormones and/or related metabolites, which are involved in carotenoid metabolism and influenced by salinity. This strategy revealed 46 metabolites, which overall indicated no or little potential of altered salinity to enhance the accumulation of lycopene, β-carotene, lutein and zeaxanthin. The proposed strategy has the ability to both elucidate and exploit the potential of altered salinity in promoting tomato fruit carotenoid content. Indeed, the dynamic behavior of the metabolites allows a more rapid unraveling of the interactive effects between genetic background, agronomic settings and environmental conditions. In addition, this novel approach may enclose significant value in modeling tomato fruit nutritional quality.

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# CHAPTER VII

## GENERAL DISCUSSION AND FUTURE PERSPECTIVES

# 1. RESEARCH POSITIONING AND RELEVANCE

## 1.1 OVERALL IMPORTANCE OF TOMATO AS A RESEARCH OBJECT

Tomato (*Solanum Lycopersicum L.)* has become an important source of nourishment for the world's population and of substantial significance because of the abundantly present nutritional constituents. Especially carotenoids have been associated with diverse health-promoting properties (1). In this regard, the growing popularity of tomato fruits is reflected by the global production, which has increased over 300% in the last four decades and continues to rise. This finding relates to a current estimated tomato production of about 162 million tonnes per year (2). Moreover, in terms of absolute quantities, tomato enfolds about 15% of total vegetable production and is hereby ranked second after potato (3,4). In addition, based on the steady growth rhythm of global tomato production and the emergence of new markets, the success story of tomato is expected to continue (5).

In Belgium, tomato is the most important horticultural crop and predominantly produced for fresh market purpose. Herewith, high standards are imposed on tomato fruit quality, usually with regard to both internal and external traits. As such, the Belgian production system is typified by an almost year-round production in sophisticated glasshouses, which allow a strict control of growth conditions. In this regard, extremely high yields of ~60 kg $m^{-2}$ have been achieved (6-8). Total annual production in Belgium has been estimated to be 230,000 tonnes, which is produced on a total area of only 440 ha (2).

With respect to tomato fruit consumption, a worldwide increase has been verified during the last few years. More specifically, an annual growth rhythm close to 5% has been determined. Annual global consumption of both fresh and processed tomatoes is set on about 20.5 kg per capita, with relatively wide differences between regions. In Belgium, tomato fruit consumption is about 27.5 kg per capita whereby tomato is ranked first among all consumed vegetables (9,10).

Besides its explicit economical and cultural impact, tomatoes are commonly used as a model crop for diverse physiological, cellular, biochemical and genetic studies because they are quite easily grown, have a short life cycle and are easy to manipulate. Moreover, tomato plants are highly responsive to the environment, which is interesting for elucidating elementary mechanisms (7).

## 1.2 THE TOMATO QUALITY AIMS: RELEVANCE OF THIS RESEARCH

During the green agriculture revolution, breeding programs aimed at the development of hybrid cultivars with a high yield potential per hectare, a uniform and pleasing appearance, disease tolerance, and a long shelf life. These particular aims narrowed the variety among tomato cultivars that were available for large-scale markets. Moreover, little attention was given to those attributes, which are associated with tomato fruit flavor and nutritional value (3).

In recent years, however, the interest of consumers has been focused on the organoleptic and nutritional quality traits of tomato fruit (11). Indeed, a major complaint by consumers regarding fresh tomatoes concerns the lack of a characteristic taste and flavor. Even more, consumers are willing to pay a premium price for full-flavored tomatoes that meet their expectations (12). A similar finding was ascertained with respect to tomato fruit nutritional value and the inherent presence of health-promoting substances such as lycopene. Interest for healthy and balanced diet has incited consumers to actively search for food that holds health-beneficial properties (1,11).

As such, growers and breeders were evidently encouraged to redirect their aims and focus on improving tomato fruit quality traits. Their efforts have yielded some new cultivars, which specifically responded to the above consumer demands. For instance, in 2007, Flandria Ministar LycoPlus tomatoes were introduced into the fresh market and presented as very healthy because of the high lycopene content (13). In addition, some new cultivars (e.g. Tommies, Tasty Toms, Honey Tomatoes), characterized by an excellent flavor and appreciated sweet-sour taste, were introduced as well (14). Accompanied by an appropriate marketing strategy, these added values were shown to effectively increase sales (11).

The examples listed above are indicative for the potential of genetic improvements through breeding. Nevertheless, agronomic practices and environmental settings have been contemplated as an equivalent interesting context to promote tomato fruit quality. Unfortunately, the interactive effects between the imposed agronomic conditions, environment and genetic background hinder an efficient and rapid progress in terms of optimal cultivation conditions (3,15). Moreover, final tomato quality represents the cumulative outcome of all those effects that were encountered during the various stages of fruit development and ripening. In this regard, a more direct view on the influence of prevailing conditions on carotenoid metabolism and accumulation

would be extremely helpful in exploring the potential of controlled agronomic and environmental settings. The underlying mechanisms and involved biomolecules (e.g. genes, enzymes, secondary messengers, regulating metabolites) with respect to carotenoid metabolism may compose the fundamentals towards a more efficient assessment and optimization of imposed conditions (16).

Various phytohormones have been recognized to fulfill a regulating role in the metabolism or accumulation of tomato fruit carotenoids. However, the complex crosstalk between multiple phytohormones with antagonistic, synergistic and additive effects has been regarded as a considerable hindrance to efficiently deepen the knowledge about phytohormone regulation. As such, a multi-targeted analytical approach is designated significant relevance, but considered extremely challenging because of the large number of phytohormones, their wide chemical diversity, and their low concentration levels in complex plant matrices (17,18). This is certainly the case if one is using the established techniques for phytohormone analysis such as immunoassay and tandem mass spectrometry. Indeed, a number of shortcoming have been defined for these analytical platforms such as the impossibility of post-acquisition re-interrogation of acquired data, the limited number of compounds that can be measured, the impossibility to screen for unknown compounds, and the sometimes inadequate specificity (19,20). The ongoing evolution in chemical screening techniques, associated with continuous instrumental improvements, has responded towards these shortcomings. Indeed, the capabilities of full scan high-resolution mass spectrometers (e.g. TOF and Orbitrap-MS) and their hybrid versions are now offering the opportunity to accomplish the cited analytical and associated biochemical aims.

## 2. MAIN RESEARCH FINDINGS AND SCIENTIFIC CONTRIBUTIONS

Within the conceptual framework of this thesis, a number of objectives have been defined. The accomplishment of these objectives has been extensively described in the various research chapters and is reviewed in Figure 7.1. A detailed discussion about the eliciting findings and conclusions will be presented for each chapter and an integrated vision is provided on the entire study.

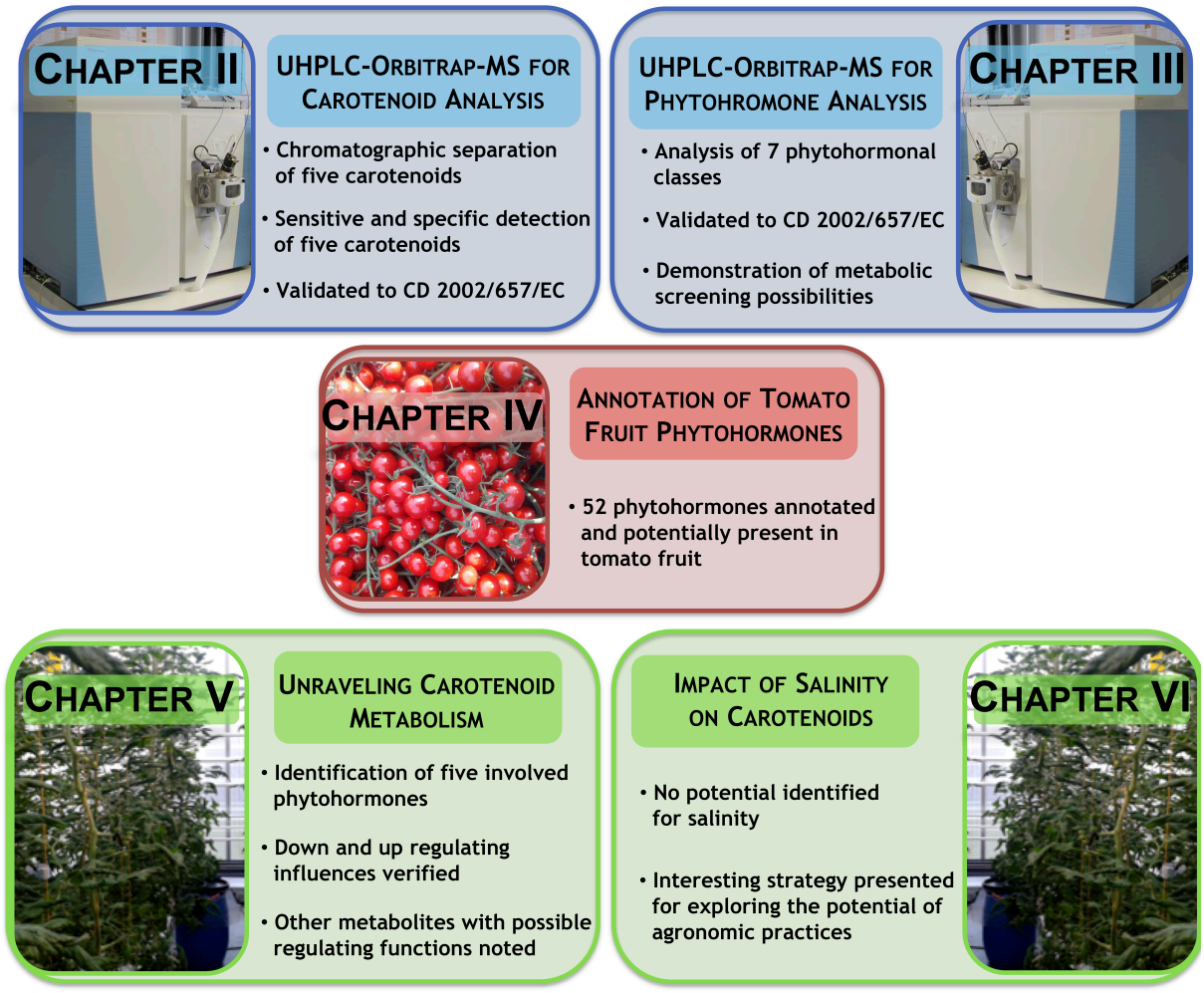


**FIGURE 7.1.** Schematic overview of the main accomplishments of this thesis.

## 2.1 TOMATO FRUIT CAROTENOID EXTRACTION AND HIGH-RESOLUTION ORBITRAP-MS ANALYSIS

There is no standard procedure for carotenoid extraction because of the wide variety of biological matrices containing these compounds and the wide range of carotenoid concentration levels that can be found in these matrices (21). However, carotenoid extraction from tomato and other fruit tissue is usually performed by liquid-liquid extraction whereby diverse mixtures of polar and non-polar solvents have been suggested (21,22). Most of these extraction procedures utilize large volumes of extraction solvent (~15 to 750 mL) and require relatively high amounts of plant material (~1 to 10 g) (23-28). The high solvent consumption evidently reacts against the objectives of green chemistry and constitutes a significant economic cost. Moreover, the high amounts of plant material that are needed strongly confine possible experimental set-ups in terms of number of executable analyses (e.g. biological replicates) and choice of biological entity (e.g. early-stage low-weight tomato fruits). Because of these enumerated shortcomings and the particular research objectives, the optimization of a rescaled extraction procedure was regarded as critically important. In this context, the use of an experimental design (i.e. D-optimal design) proved an expedient strategy to increase optimization efficiency by strongly reducing the number of experimental runs. Eventually, the rescaled extraction procedure resulted in an advanced sample throughput and a shortened exposure time of carotenoids to light and oxygen.

Initially, chromatographic separation of the selected carotenoids was intended by UHPLC and proved a challenging task due to the co-eluting behavior of the carotenoid stereoisomers. Although a number of studies have reported UHPLC based methods, no satisfactory results in terms of chromatographic resolution could be obtained for all carotenoids by adopting these protocols (29-32). This was certainly true for the more polar carotenoids, i.e. lutein and zeaxanthin ($C_{40}H_{56}O_2$), for which the functional hydroxyl groups gave rise to very short elution times. Therefore, it was opted to use a $C_{30}$ HPLC-column, which has been specifically designed to provide high shape selectivity for separation of hydrophobic and structurally related isomers (33). The optimized chromatographic method was able to separate the target carotenoids but was characterized by a prolonged analytical run time of 55 min. As such, the availability of $C_{30}$ UHPLC-columns would be most welcome to reduce analytical run time and solvent consumption.

Detection of carotenoids was achieved by high-resolution Exactive™ Orbitrap mass spectrometry. The inherent full scan operating principle of this apparatus enables post-acquisition re-interrogation of data and thus metabolic screening (20). Such a strategy is considered highly relevant since more than 700 different carotenoids have already been isolated and identified from various natural sources (21). As some of these carotenoids have been ascribed higher interest in terms of relative abundance or functionality, a partially targeted use of the presented methodology would be evident. Therefore, the quantitative performance of the concerned analytical platform was thoroughly validated. In this context, it must be stated that no official guidelines exist that systematically cover the various performance criteria, which meant that various validation strategies for carotenoid analysis have been implemented (34-38). A major part of the noted variation relates to the choice of reference material; being either pure standard solution or enriched plant material. In our validation study, it was opted to use enriched tomato fruit material to allow the evaluation of potential matrix effects on the assessed performance parameters. This implied that during calculations the endogenous carotenoid levels had to be taken into account. Validation of the analytical method was performed according to the criteria specified in CD 2002/657/EC (39) and included linearity, sensitivity, precision, accuracy, and selectivity/specificity. For all five carotenoids, highly satisfactory validation results were obtained using Orbitrap-MS. In addition, the analytical performance of the Orbitrap-MS was compared to well-established detection techniques such as tandem mass spectrometry (MS/MS) and UV-VIS. Although literature may provide a first indication about the performance of the developed method, the usage of different reference material might have a strong influence on the validation results. Therefore, the cited analytical platforms were validated as well to allow a most truthful comparative evaluation. Hereby, a better selectivity/specificity and sensitivity were observed for the Orbitrap-MS. The study of Garrido Frenich *et al.* (2005) (40) has reported that selectivity with UV-VIS is indeed inadequate for carotenoid analysis of complex food samples. In addition, Kaufmann *et al.* (2011) (41) reported similar findings when comparing Orbitrap-MS towards tandem mass spectrometry. The improved mass resolution between the analyte and the matrix-related isobaric interferences upon Orbitrap-MS is indeed able to reduce interferences and permits a more sensitive detection of analytes in complex matrices.

Around the same time that our method was published, Byttebier *et al.* (2013) (42) reported on the ionization and fragmentation pathways of carotenoids, using Orbitrap mass spectrometry including HCD-fragmentation. This study indicated the potential of fragmentation in generating valuable information about the type of functional groups, the polyene backbone and the location of double bonds in the ring structure of carotenoids. As such, HCD-fragmentation could provide a first step towards identification of unknowns. These and our findings proved Orbitrap mass spectrometry as an attractive analytical strategy for both quantitative and metabolic screening of carotenoids.

In addition to the use of the developed Orbitrap-MS method for carotenoid analysis of fresh tomatoes (Chapter V and Chapter VI), processed tomatoes have been successfully analyzed as well. More specifically, various processing techniques were considered whereby a distinction between various fruit parts was made. In this context, the method demonstrated great versatility since only small adjustments of the extraction procedure were required, i.e. a different amount of starting material. It should be noted that in this particular study and our own research a targeted approach was performed, aiming at a correct quantification of major carotenoids. As the importance of a limited number of carotenoids exceeds that of the other carotenoids, it may be assumed that this method is likely to be primarily used for targeted and thus quantitative purposes. In this regard, some potential hindrances towards correct quantification should be noted. Although validation of our method demonstrated high precision, the use of multiple internal standards would further increase this performance criterion. This is certainly of interest given the long duration of one analytical run and the matrix interferences, which may widely vary between samples. However, the availability of deuterium-labeled internal standards for carotenoids is limited and prices are very high. In addition, it is not always easy to establish matrix-matched calibration curves that cover a concentration range, which is adequate for all sample types that are analyzed within a single experiment. This mainly relates to the diverse endogenous carotenoid concentration levels. Therefore, it would be opportune to increase the number of calibration points and concentration range of the calibration curves, and to decide after analysis which calibration points should be included.

## 2.2 GENERIC EXTRACTION OF PHYTOHORMONES AND HIGH-RESOLUTION ORBITRAP-MS ANALYSIS

The specific requirements that were set to the analysis of phytohormones from tomato were based on the fundamentals of phytohormonal regulation. Indeed, the complex crosstalk and interactive effects between multiple phytohormones in regulating a plant's response endorsed the need for a generic extraction and multi-compound targeted detection method. Even more, a true metabolomics-oriented method was intended because of the large number of phytohormones, the importance of inactive conjugates and degradation products, and the incomplete knowledge about metabolite phytohormonal functionality (43-45).

The most critical steps during the development of the analytical method were encountered during optimization of the generic extraction protocol. Hereby, a first critical phase was related to the collection of plant material and the subsequent sample preparation, during which qualitative and quantitative changes of the metabolic fingerprint had to be avoided. In this regard, the use of liquid nitrogen for preserving the metabolic state at collection and homogenization was a rather evident choice for tomato leaf material, as had been reported by Pan *et al.* (2008) (46). In contrast, the use of liquid nitrogen was practically not that suited for the relatively voluminous tomato fruits and involved various freeze-thaw cycles. Alternatively, lyophilization proved much more convenient and was incorporated as part of the sample preparation. Although no detrimental effects were verified for the target phytohormones upon lyophilization, this process has been argued to have unanticipated and significant effects on botanical constituent profiles (47,48). In contrast, the review of Abascal *et al.* (2005) (49) states that insufficient information about lyophilization is available to assign this process a negative effect. As such, it would indeed be valuable to investigate the effects of lyophilization on the tomato fruit metabolic fingerprints, thereby using appropriate visualization tools such as PCA. A second crucial phase emerged as purification of the crude plant extract appeared inevitable for sensitive and selective detection of all selected phytohormones. Indeed, every additional purification step could result in a loss of compounds and negatively affect the generic character of the extraction protocol. However, indol-3-acetic acid and jasmonic acid were strongly hindered by matrix interferences or inadequately high detection limits were obtained. As reviewed by Fu *et al.* (2011) (44), the preferred strategy used for the purification of crude plant phytohormone extracts involves SPE. In our study, various

types of SPE cartridges were evaluated but none of these proved beneficial for all phytohormones. For example, usage of the Isolute C18 (EC) (50) cartridge resulted in an almost complete loss of *cis*-zeatin. The observed phenomenon, i.e. extreme differences in recovery between phytohormones in function of the applied SPE cartridge, was not consistent with the generic extraction and general metabolomic objectives. On the other hand, using a 'simple' Amicon® Ultra centrifugal unit resulted in an overall beneficial effect on the recovery and selectivity of all phytohormones. As such this extraction method excels in its simplicity, which allowed a high sample throughput (~50 samples a day).

The analytical method for the detection of phytohormones and related compounds was based on Exactive™ Orbitrap mass spectrometry, preceded by UHPLC. To the best of our knowledge, this is currently the only published method in which this analytical platform is used for the analysis of phytohormones. The main relevance of this novel method relates to its ability of post-acquisition re-interrogation of data, allowing metabolic screening experiments. In addition, validation of the analytical performance (with sensitivity and selectivity/specificity as main descriptors) proved this methodology expedient for quantitative phytohormone analysis of various plant matrices. Indeed, in comparison with other studies, the developed method yielded better (51-54) or comparable (43,55,56) LOD and LOQ values. Only in the study of Pan *et al.* (2008) (46), sensitivity outperformed our results. However, although not clearly stated, it may be deduced that sensitivity was determined based on pure standard solutions in this paper. Validation was performed according to CD 2002/657/EC and employed plant matrix as a reference material. As with carotenoid analysis, the lack of blank reference material is regarded as a significant hindrance for a well-founded comparison with other methodologies reported in literature.

Development and optimization of the analytical method was evidently based on the characteristics of the used tomato plant material. More specifically, the endogenous phytohormone concentration levels and present matrix interferences were considered as the main determinants towards the final established method. Although diverse types of plant material (i.e. tomato fruit and leaf) were included in our validation study, it was not proved whether the method would be able to perform well if other plant species are used. However, since its development, the Orbitrap-MS method has been used for phytohormone analysis of rice and wheat. This allowed to evaluate its versatility and

define some shortcomings. In general, lower phytohormone concentration levels were noticed for the measured plant material, which pointed towards some modifications of the extraction procedure. Adjustments with respect to the initial amount of needed plant material and/or solvent volumes were concluded to suffice in order to reach appropriate limits of detection. As such, satisfying results could be obtained in a very short period of time. In addition, analyses were performed by Exactive™ Orbitrap-MS, which shows somewhat inferior sensitivity in comparison with the more recent Orbitrap mass spectrometers. For example, it has empirically been ascertained that LODs were about ten times better on Q-Exactive™ Orbitrap-MS. Moreover, next-generation Orbirap mass spectrometers (e.g. Orbitrap Fusion Tribrid-MS) are attributed with even higher sensitivities, which may contribute to a lesser dependency on the plant material used and its inherent phytohormone concentration levels. With respect to the presence of matrix interferences, differences between plant species have effectively been observed. For few of the targeted phytohormones, quantification was somewhat impeded by these matrix compounds, insufficiently resolved by the implemented chromatographic method and set mass resolution. As such, the 'unpredictability' of present matrix interferences may indeed constitute a significant obstacle towards the implementation of the method along different plant species. In this regard, ultra-high field mass spectrometers (with resolution in excess of 450,000 FWHM) may overcome this problem. In addition, tribryd mass spectrometers enfold a third dimension of separation by utilizing ion mobility mass spectrometry (57).

## 2.3 ANNOTATION OF PHYTOHORMONES FROM TOMATO FRUIT

Currently, the process of metabolite identification is recognized as a significant bottleneck in deriving biological knowledge from untargeted metabolomic studies. Indeed, identification of biologically interesting metabolites is a labor-intensive step, which has to be addressed before any biological interpretation is possible (58,59). Without knowledge of exact chemical structures, it is difficult to evidence biological significance or make mechanistic connections to biochemical pathways that have been associated with specific plant processes. In this context, various computational strategies and sophisticated software tools have been suggested in supporting metabolite identification (e.g. 60-69).

In this work, a number of software tools were employed for annotation of tomato fruit phytohormones and obtaining a first indication about the phytohormone profiles of developing tomato fruits. More specifically, a suspect screening strategy was implemented and performed according to an exclusion-based workflow. Based on the criteria that were set within each identification step, an ion was whether or not transferred to the following step and associated next level of identity assessment. Such an approach is in contrast with untargeted screening strategies in which the diverse criteria are used to retain the best possible identity match. Our strategy was rather cautious and strived for the exclusion of those metabolites that were almost certainly no phytohormones. This implicated that the generated profiles were composed of metabolites that were not decisively stated to be phytohormones but had a realistic expectance of being so. The phytohormonal profiles, including both chromatographic and mass spectrometric data, enabled a more rapid screening for phytohormones in the performed metabolomic experiments (i.e. Chapter V and Chapter VI).

The initial step of phytohormone profiling was conducted by TraceFinder™, which proved a very effective tool for peak detection and database matching. The elaborated system of adjustable algorithm settings strongly advanced reliable peak detection and integration. As an alternative, Sieve™ software might be used for targeted screening as well, but yielded a relatively high percentage (~60%) of 'noise' peaks, which is attributed to the lack of an elaborated system of adjustable settings. After initial screening, prediction and ranking of candidate chemical formulas for detected *m/z*-values was performed by MZmine software. The study of Pluskal *et al.* (2012) (61) was hereby used as guidance, suggesting the isotope pattern score as main ranking criterion and a minimum MS/MS score of 100%. However, such high MS/MS values were never reached in our study and were usually ranging between 75 and 95%. These differences are presumed to predominantly relate to the matrices, i.e. tomato fruit in this study and yeast cell extracts in the study of Pluskal *et al.* (2012) (61). Therefore, no discriminating value to the MS/MS score was assigned. Furthermore, it was noticed that the top rank positions were very often occupied by candidate chemical formulas that contained one or more phosphorus atoms. Although no exact information is available about the prevalence of phosphorus containing plant metabolites, this outcome was strongly questioned. This finding is exemplified in Table 7.1, presenting the MZmine results for gibberellic acid.

**TABLE 7.1.** Top 10 ranked candidate chemical formulas for gibberellic acid, spiked to tomato fruit extract. The correct formula for gibberellic acid is highlighted.

| Candidate Formula | Molecular mass | Mass difference | Isotope pattern score (%) | MS/MS score (%) |
|---|---|---|---|---|
| $C_{16}H_{29}O_2P_3$ | 346.1380 | 0.0036 | 98.41 | 83.33 |
| $C_{16}H_{28}O_4P_2$ | 346.1463 | 0.0047 | 97.59 | 88.89 |
| $C_{12}H_{24}N_6O_2P_2$ | 346.1436 | 0.0020 | 96.93 | 88.89 |
| $C_{18}H_{23}N_2O_3P$ | 346.1446 | 0.0030 | 94.95 | 86.11 |
| $C_9H_{18}N_{10}O_5$ | 346.1462 | 0.0046 | 94.91 | 84.72 |
| $C_{15}H_{18}N_6O_4$ | 346.1390 | 0.0026 | 94.59 | 80.56 |
| $C_{14}H_{22}N_2O_8$ | 346.1376 | 0.0040 | 94.31 | 80.56 |
| $C_{16}H_{14}N_{10}$ | 346.1403 | 0.0013 | 94.22 | 70.83 |
| $C_{19}H_{22}O_6$ | 346.1416 | 0.0000 | 93.81 | 75.00 |
| $C_{14}H_{19}N_8OP$ | 346.1419 | 0.0003 | 93.79 | 83.33 |

It can indeed be noted that the correct formula was only ranked 9$^{th}$ whereas the top positions were occupied by formulas containing phosphorus. A possible explanation is founded on the measured $C^{13}/C^{12}$ isotope ratios, which were verified to be consistently lower than the theoretically expected ratios. For this reason, formulas with a carbon number that is lower than the true number are higher ranked. Hereby, the 'loss' of carbon weight is compensated by higher amounts of the other elements including phosphorus. It is also noteworthy that strongly diverging structures (e.g. $C_9H_{18}N_{10}O_5$, Table 7.1) were sometimes present in the generated candidate lists. Therefore, although this tool may be useful for suspects screening, a more critical assessment seems designated for untargeted identification purposes. In this regard, other strategies for chemical formula prediction may be explored; e.g. SigmaFit (Bruker Daltonics), Masterview (Absciex), and *SIRIUS* (63).

The final step within the presented workflow involved chemical structure evaluation by MetFrag software, which is a combinatorial fragmentation tool. However, the lack of a criterion for evaluating the overall match value is a current limitation to these and other fragmentation tools (64). Indeed, it has been stated by Hill *et al.* (2008) (64) that it is not possible to decide on the basis of number of matching fragments or ranking whether a compound should be regarded as a possible candidate or not. As such, identification of unknown metabolites is still regarded, unfortunately, as a significant hindrance. However, software programs with novel algorithms for fragment prediction and chemical structure ranking are regularly launched, contributing towards better identification performances. For example, such a new strategy of identity ranking has

recently been reported by Allen *et al.* (2014) (65), thereby introducing competitive fragmentation modeling. Within this concept, the formation likelihood of various latent fragments that possibly precede the final observed fragment are determined and used for identity ranking. The presented strategy was demonstrated to outperform the identity ranking results, obtained by MetFrag and FingerID. An alternative approach for compound identification is based on the various online databases such as METLIN, HMDB, MassBank, and NIST. These databases enclose compound fragmentation spectra and may thus assist in identifying unknowns as well (66,67). In this context, analytical scientists should be incited to share their data. A mass spectral repository, i.e. a central open source mass spectral database of CID spectra acquired with various instrument in different laboratories, would undoubtedly contribute to a more efficient identification of unknown (known) metabolites. Information such as the biological matrix, instrument and analytical conditions should be precisely documented, however, to correctly interpret the available information (68).

## 2.4 UNRAVELING THE REGULATING ROLE OF PHYTOHORMONES IN CAROTENOID METABOLISM

Positioning of this research indicated a strong interest for improving tomato fruit carotenoid content by agronomic measures and/or environmental control. In this context, a better understanding of the underlying mechanisms of carotenoid metabolism was defined as a critical aspect for a more efficient interpretation of the influential factors on tomato fruit carotenoid content. One of the great challenges of understanding these metabolic processes concerns the identification of those mechanisms by which carotenoid biosynthesis is regulated (70). A first level of regulation with respect to carotenoid biosynthesis and accumulation relates to the control of plastid development. Indeed, the size and number of chloroplast plastids strongly influence the capacity for carotenoid biosynthesis and deposition (71,72). A second level concerns the transcriptional regulation of upstream carotenogenic genes and expression of genes downstream of the accumulating carotenoids. Post-transcriptional regulation at enzymatic levels have also been indicated a significant regulating role (73). The interpretation of our results was directed towards this second level as these mechanisms are considered most important (70). In the past few years, genes and cDNA encondig nearly all enzymes, involved in carotenoid biosynthesis, have been identified and sequenced (Figure 7.2) (74).

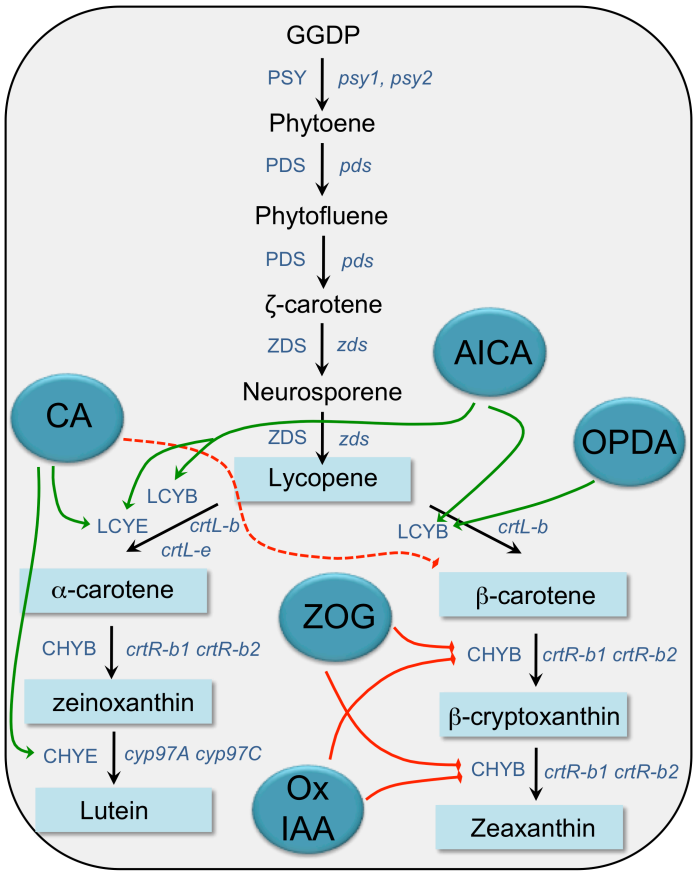


**FIGURE 7.2.** Schematic overview of the pathway of carotenoid biosynthesis with indication of the various involved enzymes (left side of the black arrows) and genes (right side of the black arrows). Following enzymes are involved in the synthesis of the various carotenoids: PSY, phytoene synthase; PDS, phytoene desaturase; ZDS, ζ-carotene desaturase; LCYB, lycopene β-cyclase; LCYE, lycopene ε-cyclase; CHYE, carotene ε-hydroxylase ; CHYB, carotene β-hydroxyase. The various phytohormones, found to be involved in carotenoid metabolism are indicated as well (CA, cucurbic acid; OPDA, *cis*-2-oxo-phytodienoic acid; OxIAA, 2-oxindole-3-acetic acid; ZOG, *cis*-zeatin-O-glucoside; AICA, 1-acetylindole-3-carboxaldehyde). Hereby, positive and negative influences on specific biosynthetic steps, either by post-transcriptional enzyme regulation or regulation of gene expression, are indicated by green and red arrows, respectively.

With respect to the phytoene synthase enzyme (PSY), responsible for the first step in carotenoid biosynthesis, two genes have been identified, i.e. *psy1* and *psy2* (75). This enzyme is normally associated with chloroplast or chromoplast membranes because of the need to deliver the lipid-soluble phytoene to the membranes where phytoene and subsequent intermediates and end products of the pathway are localized (73). The desaturation reactions, following phytoene synthesis, are catalyzed by two related enzymes, i.e. phytoene desaturase (PDS) and ζ-carotene

desaturase (ZDS), and yield consecutively phytofluene, ζ-carotene, neurosporene, and lycopene. These enzymes are located in both the plastid membranes and stroma. Associated genes have been identified and are described by Wong *et al.* (2003) (76) and Lopez *et al.* (2008) (77). The cyclization of lycopene, thereby introducing β- or ε-rings, is catalyzed by β-cyclase (LCYB) and ε-cyclase (LCYE). The cDNAs, which encode for these lycopene cyclases, are *CrtL*-b and CrtL-e (78). The biosynthesis of xanthophylls, derived from β-carotene, involves carotenoid β-hydroxylases belonging to the non-heme di-iron group. These enzymes are encoded by genes *CrtR-b1* and *CrtR-b2*. The biosynthesis of lutein also requires the combined and sequential action of two distinct hydroxylases belonging to the subfamilies A and C of the Cyt P450 family 97, encoded by the genes *CYP97A29* and *CYP97C11* (79).

Knowledge about the gene sequences, encoding for specific enzymes that are involved in carotenoid metabolism, enclose the potential to validate our results. Indeed, a regulating influence on gene expression and thus altered enzyme production may be verified by real-time quantitative PCR (Polymerase Chain Reaction). This approach allows to quantify the amounts of mRNA (messenger RNA), reflecting gene expression, in the samples under consideration (80). Various studies have already applied qPRC to investigate carotenoid metabolism (81-83).

The main pillar of this study involved the determination of the relationship between the metabolic tomato fruit fingerprints and the inherent carotenoid concentration levels. For this purpose, OPLS-models were generated since these allow to predict a quantitative Y-variable (carotenoid concentration) based on the X-matrix (metabolite abundances) (84). This OPLS-modeling strategy (and not OPLS-DA) was regarded as crucial to maximize the chance that relevant metabolites were effectively involved in carotenoid metabolism and not solely in general fruit ripening. Among the defined biochemically relevant ions, a total of 12 ions were putatively assigned a phytohormonal identity. Eventually, the usage of reference standards revealed 5 phytohormones that were highly or moderately influencing carotenoid metabolism. As these particular phytohormones are rarely cited in literature, little information for biochemical interpretation was available. As such, their presumed role in carotenoid metabolism is therefore not completely certain. Indeed, notwithstanding the applied strategy of OPLS-modeling, the established correlation or relationship between phytohormone and carotenoid may provide insufficient power to assume a regulating role in the metabolism of that carotenoid. For example, there is still the possibility that the revealed

phytohormone is involved in other ripening processes and solely has a concentration profile, which is concurrent with that of a certain carotenoid. As a consequence, the presented approach would benefit from an assessment of the expression of specific genes, which are hypothesized to be involved in the phytohormonal regulation of carotenoid metabolism. In this context, an integrated approach of proteomics and genomics, focusing on the effects of these phytohormones on the translation of genes into those enzymes that are involved in the biosynthetic pathway of carotenoids (Figure 7.2) would be highly useful (73). For this purpose, the above-described RT q-PCR approach encloses potential for validation of our results and to elucidate a possible causal relationship. In addition, since post-transcriptional regulation of enzymes has also been identified as a controlling element, assessing enzyme-activity would also be eligible.

Overall, the number of identified phytohormones was relatively small and their impact was considered reasonably limited. Therefore, it would be interesting to focus on those metabolites that were considered highly contributing to the predictive power of the OPLS-models but that could not be assigned a phytohormone identity on the basis of the used phytohormone database. However, an elaborated process of identification would be needed to determine the chemical structure of these metabolites. The original strategy was chosen because the phytohormonal character of the retained metabolites strengthened the assumption of an actual regulating role in the metabolism of carotenoids.

## 2.5 IMPACT OF SALINITY ON CAROTENOID METABOLISM

In the previous research chapter, a number of phytohormones was revealed, which was indicated to fulfill a regulating role in the metabolism of one or more carotenoids. Although the obtained results proved the applicability of the targeted strategy, this strict focus on known phytohormones may hamper the true potential of such metabolomic experiments. Therefore, in this research chapter, it was opted to abandon this targeted approach and perform a fully untargeted search for what was referred to as ‘phytohormones and related metabolites’. As such, a more effective assessment of imposed agronomic conditions in terms of altered tomato fruit metabolic fingerprints and associated carotenoid accumulation could be effectuated. More specifically, altering the electrical conductivity of the irrigated nutrient solution was assessed and this for two principal reasons. Firstly, the ability of this agronomic measure to enhance tomato fruit nutritional

quality has been reported and suggested to be equivalent to genetic improvements (85-88). Secondly, controlling nutrient solution EC-levels as a function of prevailing environmental conditions is regarded as a practically manageable measure, which allows to maximally exploit the available potential in promoting nutritional quality.

Increased salinity as a mean to promote tomato fruit carotenoid content was assessed by evaluating the influence of those metabolites that were found significantly involved in both the salinity response and carotenoid metabolism. To this extent, a strategy of OPLS and OPLS-DA modeling was capable of assigning 46 metabolites a key role in both processes. In the context of multivariate data analysis, some considerations should be taken into account with respect to the applied modeling approaches. Hereby, OPLS-DA is regarded as a rather straightforward technique, which has the capacity to reveal those metabolites that are able to discriminate between defined treatments. Such a strategy was applied to evaluate the effect of the imposed EC-treatments and elucidate those metabolites that are significantly involved in the salt stress response. However, with respect to OPLS-modeling, the underlying mechanisms of the investigated processes may strongly influence the obtained results and should be taken into consideration during interpretation. This kind of modeling was used to determine the metabolites that are involved in carotenoid metabolism. In our opinion, two main hypotheses of regulation mechanism may be suggested. A first possibility of regulation relates to an increased or decreased carotenoid concentration due to an increased or decreased phytohormone concentration. With this hypothesis, an additional increase of the carotenoid concentration would need for example an additional increase in phytohormone concentration. As such, a linear relationship is obtained. It may be assumed that this kind of mechanism is nicely modeled by OPLS. However, validation of the results would need, as earlier described, evaluation of gene expression or enzyme activity. A second approach encompasses the need for a one-time increase of phytohormone concentrations to effectuate an increased biosynthesis of a carotenoid, which transcends the catabolism of the carotenoid, resulting in a continuous increase in carotenoid concentration. This process is less likely to be captured by the model principal components without over-fitting the model. In this case, it would be interesting to shift towards discriminant analysis. For instance, focusing on the metabolic fingerprints that are associated with the fruits before and after reaching the ‘breaker’-stage. In this way the chance is minimized that screened out metabolites are just involved in fruit

development and not carotenoid metabolism. However, this scenario includes a loss of information that is contained in the quantitative data regarding the carotenoid concentrations. There is indeed an increased chance that screened out metabolites are not correlated with carotenoid metabolism and solely with other fruit developmental processes.

The overall influence of the screened out metabolites towards carotenoid accumulation was determined based on the metabolite covariances, which were characterized by both magnitude and sign. An integrated evaluation of these descriptors indicated no or little potential for altered salinity in enhancing the carotenoid content of the selected cherry tomato cultivar. As very high increases in carotenoid accumulation (up to 40%) (87,89) have been reported, it is postulated that the genetic background and selected cultivar are principally determining the inherent potential of agronomic conditions. Therefore, it would be interesting to itinerate this experiment with a different cultivar for which such potential has been demonstrated, enabling us to validate and explore the proposed strategy. In addition, information about the identity of all biochemically interesting metabolites would allow a profound biological interpretation of the results. By using the constructed phytohormone database and reference standards, only one metabolite could be assigned a phytohormonal identity, i.e. 1-acetylindole-3-acetic acid. However, the applied database is not claimed to completely cover all possible phytohormones that have been reported in literature so far. Therefore, additional identification acts towards the unknown metabolites were undertaken. In contrast to the strategy that has previously been discussed (Chapter IV), a more integrated approach with a simultaneous evaluation of the various identification phases was performed. As such, 19 metabolites could be assigned an overall best-matching identity. In this context, a significant hindrance was encountered, which relates to the methodology by which fragmentation spectra were acquired, i.e. dd-$MS^2$ using Q-Exactive Orbitrap-MS. Indeed, since a truly untargeted metabolomics strategy was performed, the fragmentation spectra that were generated according to the used inclusion list were of no value. As such, useful structural information was exclusively provided through TopN dd-$MS^2$ whereby for each single scan the N most abundant ions were fragmented. This implied that only a fraction of all metabolites, present in the tomato extracts, was fragmented. Based on these fragmentation spectra, only 19 of the 46 biochemically relevant metabolites could be annotated. As a consequence, such untargeted strategies always require additional analyses to establish fragmentation patterns for all relevant

metabolites, thereby using suited inclusion lists. This of course is associated with extra labor, time, and instrument occupation. In addition, there is the need for new biological samples, which are not always immediately at dispose. Therefore, progress towards even more faster scanning analytical instruments would be highly beneficial in addressing this problem.

Although the suggested identification strategy may provide a strong first indication about the chemical structure of an unknown metabolite, additional efforts would be required to reach the highest level of metabolite identification as defined by Sumner *et al*. 2007 (90). In this report, it is stated that at least two independent and orthogonal parameters should be verified, relative to an authentic compound that is analyzed under identical experimental conditions. However, since the number of acceptable candidate structures is often considerably large and diverse, additional filter options could be taken into consideration; e.g. LogP prediction (91), retention time prediction (92), isotopic abundance, and ionization mode (64).

It should be remarked that in preceding studies the truss tomato cultivar Moneymaker has been used whereas in the salinity experiment cherry tomato cultivar Juanita was considered. For this particular cultivar, no information about its salt tolerance has been described in literature. Moreover, it is generally stated that cherry tomatoes are less susceptible to increased salinity. For these reasons, the selected cultivar may not have been the best choice to validate the presented methodology. However, two salinity experiments with Moneymaker tomatoes were effectively initiated but prematurely discontinued due to a too severe prevalence of whiteflies (*Bemisia tabaci*). As the leaf damage, caused by these insects, may affect the hormonal status of the tomato plants, the salinity experiment had to be executed again. To regain some of the time lost, it was opted to acquire seedlings of a few weeks old. Unfortunately, at the moment of purchase, the contacted tomato cultivation company could provide us with a cherry tomato cultivar. Although this tomato type was advantageous with respect to sampling (more fruits, so more possibilities to harvest), a positive outcome of the experiment was uncertain. Indeed, no significant differences in carotenoid content between treatments were observed. In this context, although intended, another cultivar, known to strongly react to altered salinity would have been more appropriate. On the other hand, a slight modification of the experimental set-up could also be more effective; thereby considering cultivars that are considered and not considered to be tolerant to saline conditions. Herewith, only two salinity treatments (e.g. 2.5 and 5.0 dS $m^{-1}$)

would be appropriate so that it would remain practicable. Another strategy to detect those metabolites that are involved in the salinity stress response would enclose sampling and analysis of tomato fruits in a period during which EC-levels were continuously increased to reach the defined treatment EC-levels. In addition, this would allow investigating (e.g. by PCA) whether or not a salinity stress response was provoked. In this experiment, a plant response, reflecting the imposed salinity conditions, was effectively established as demonstrated by the stem and fruit water potential. In this regard, monitoring the EC-value of the nutrient solution drain would also have been interesting to define the degree of salinity, that was really encountered by the tomato plants.

## 2.6 SOME ADDITIONAL NOTES ON OUR RESEARCH

Although the various objectives and the paths to their accomplishment have extensively been discussed in the previous paragraphs, some additional thoughts are shared.

The main principle of the proposed metabolomic framework relates to the used analytical platform and its inherent capabilities. Indeed, the usage of full-scan high-resolution mass spectrometry enabled various metabolomic strategies and proved very useful in accomplishing the research objectives. However, one shortcoming is certainly to be noted: there was no possibility of measuring the gaseous phytohormone ethylene, which has nevertheless frequently been reported with respect to fruit ripening and carotenoid accumulation (93). As such, it would be highly designated to incorporate, as a part of the established metabolomic framework, an analytical strategy that allows the determination of ethylene. In this regard, both gas chromatography for ethylene (94) and liquid chromatography for its precursor aminocyclopropane-1-carboxylic acid (ACC) (i.e. as a conjugate following derivatization) (95) would hold possible strategies. Furthermore, a specific methodology of sample preparation and extraction would also be required.

Identification of the biochemically relevant metabolites is considered as a critical step towards biological interpretation and validation of the obtained results. Although the process of identification is probably a challenging task in many research areas, this is certainly the case for developmental plant processes such as ripening. These processes are generally characterized by continuous and gradual changes of the metabolic fingerprints, representing subtle changes that are difficult to capture and often masked by varying environmental conditions. Moreover, a large

number of metabolites is involved in the regulation of these processes, all with their specific significance. Contrastingly, when searching for physiological biomarkers in for example human diseases, it generally suffices to only define a limited number of metabolites for diagnostic purposes (96,97). It is usually not required to identify these metabolites as they are solely used to predict or affirm a disease state. This is generally true for all kinds of marker applications, which is evidently in contrast with approaches that strive for gaining new insights into fundamental processes and the underlying mechanisms. However, identification of metabolites in marker applications might be interesting as well for understanding disease development and discovering remedies. Moreover, considering for example insect attacks in plants or a disease condition of animals, distinct control and treatment groups can be defined, each characterized by their inherent metabolic fingerprints (98,99). As such, unraveling the underlying mechanisms of tomato fruit carotenoid metabolism is regarded as an extremely difficult task because of the subtlety of the process and the large number of metabolites that seems to be involved. Furthermore, the lack of available reference standards for the definite identification of interesting secondary plant metabolites is an additional limitation within metabolomics experiments (66). Quantification of absolute metabolite concentrations will be useful, in particular for comparison between studies, and point towards the need of authentic standards (100). However, in this context, saturated *in vivo* labeling of reference samples, using stable isotopes such as $^{1}H$ and $^{13}C$ is being used as an aid to relative component quantification in comparable samples (66).

# 3. FUTURE PERSPECTIVES

## 3.1 STEP 1: FURTHER EXPLORING THE UNDERLYING MECHANISMS OF CAROTENOID METABOLISM

Although in Chapter V some phytohormones have been revealed to be involved in the metabolism of diverse carotenoids, the OPLS-modeling rendered some additional metabolites with a high influence on the accumulation of the target fruit constituents. Identification of these metabolites is needed to maximize the output of the performed metabolomic experiment towards better understanding the underlying mechanisms of carotenoid metabolism. This will need an integrated approach in which the inclusion of various filter options should allow to achieve an acceptable number of candidate structures for final identity confirmation by authentic standards (64,90). Within this context, the increasing number and size of online available database may greatly contribute towards advanced structural evaluation and the associated quest for the best identity match. Hereby, species-specific database enclose additional value as the suggested candidate structures are confirmed to be present in the species under consideration. For instance, Moco *et al.* (2006) (101) introduced the MoTo Database, which is a metabolite database that is dedicated to LC-MS based metabolomics of tomato (*Solanum lycopersicum* L.). A subsequent step within the research area would relate to the validation of the hypothesized metabolite regulation actions and their specific action mechanisms. Indeed, various regulation routes have been defined including transcriptional regulation of genes involved in carotenogenesis, post-transcriptional regulation of enzymes, and control of plastid development (56-58). An interesting example in this regard concerns the study that was performed by Cookson *et al.* (2003) (110). The underlying stimulators for the increased pigmentation of the *high pigment*-1 (*hp-1*) tomato mutant phenotype were determined by studying plastid development, expression of carotenogenic genes, and phytoene synthase (PSY) enzyme activity. This particular enzyme, encoded by *pTom5* cDNA (108), is associated with the rate-limiting step of carotenogenesis and therefore of significant interest (70). Within this setting, it is noteworthy to mention that enormous progress has been established with regard to the amino sequence signatures of the various enzymes involved in carotenogenesis (Figure 7.2) and their associated genetic codes (106). In addition, to specifically focus on the previously identified metabolites, a target alteration of tomato fruit metabolic fingerprints is required. To this end, exogenous applications, incubation strategies, and *in vitro* culturing of tomato fruit pericarp disks or complete tomato fruits have been frequently applied (102-104).

## 3.2 STEP 2: MECHANISTIC MODELING OF TOMATO FRUIT QUALITY

The specific research aspects that have been highlighted in the previous paragraph would enclose a confirmation of the involvement of metabolites in the regulation of carotenoid metabolism. The knowledge about the confirmed metabolites and their dynamic responses towards varying cultivation conditions allow an efficient gathering of data regarding the interactive effects between the concentration levels of these metabolites and the prevailing conditions. As these interactive effects might be very complex, an integrated strategy of mechanistic plant modeling would enclose an interesting approach to handle these data and allow a precise management towards improved nutritional quality (111). This strategy involves the establishment of model equations that are able to simulate the relationship between tomato fruit accumulation and environment. However, a tomato fruit quality model is only useful if it takes into account several other quality traits, the underlying processes and their interrelations (112). Therefore, monitoring diverse parameters that are related to tomato fruit quality (sugars and organic acids), ecophysiological plant performance (sap flow, diameter variation, and water potential), and environment (radiation, temperature, and relative humidity) would be necessary (111-114). The respective data sets may be used for constructing a mechanistic plant model, whereby various interrelated submodels are typically defined (Figure 8.3) (112).

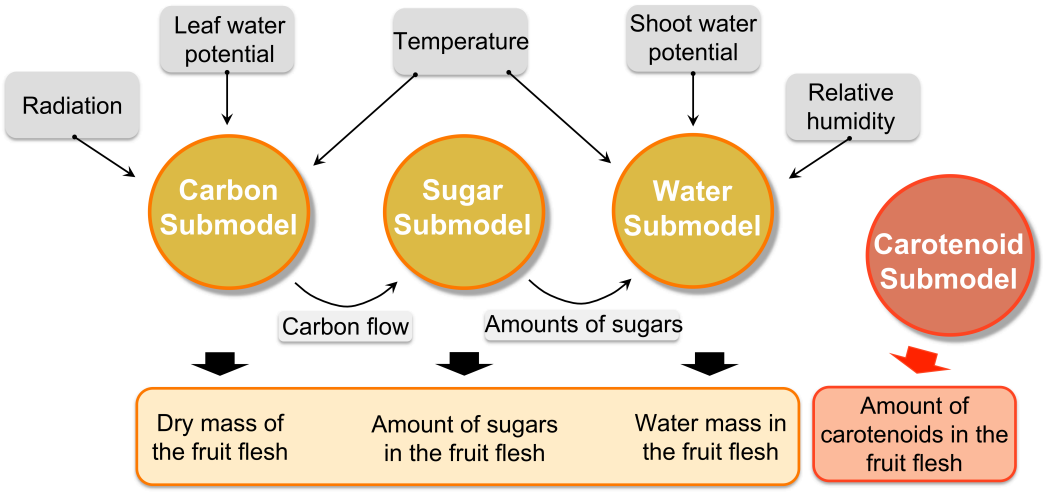


**FIGURE 7.3.** Simplified representation of a tomato fruit quality model, including various submodels (scheme based on Lescourret and Génard (2005) (112)). Integration of a carotenoid submodel, to be established by defining the interrelation with the other submodels, would yield a model for nutritional tomato fruit quality.

Currently, various models have already been established to simulate tomato fruit quality in terms of sugar concentrations, size, dry matter content, water content, size, and water content (112-117). These models and their underlying theories may constitute a valuable basis for modeling carotenoid content and thus nutritional quality.

## 3.3 STEP 3: USAGE OF THE PLANT MODEL

The final evident step to realize the ultimate aim of improved nutritional quality enfolds the usage and testing of the plant model in a real situation. Herewith, it is important to evaluate the effectiveness of the plant model on a whole growth season basis. Indeed, a possible improved accumulation may be expected to either enhance tomato fruit nutritional quality or expedite the arrival of the harvest-ready fruit stage (visually assessed). In addition, the selection of appropriate parameters that are quickly and easily to handle is crucial. A nice example of the usage of plant models is presented by Baert and Steppe (2013) (118), in which the water status of grape plants is predicted by using a mechanistic water transport and storage model. This strategy represents a promising tool within the context of an automatic plant-based system for water status monitoring and controlled irrigation. In this particular context, the focus is indeed placed on an agronomic measure (water gift) that is convenient to manage.

## 3.4 STEP 4: START OF A NEW METABOLOMICS CYCLE

Among the various tomato fruit constituents that have been assigned health-beneficial effects, carotenoids have been considered as most interesting. However, other substances such as vitamins and polyphenols have been attributed significant health-promoting properties as well and may therefore be a next focus in improving overall tomato fruit nutritional quality. In this regard, the presented metabolomic framework may constitute a valuable fundament.

In addition, the metabolomics cycle may be oriented towards other aspects of tomato plant production and performance. For example, it has been frequently described that phytohormones are involved in plant disease and defense against pathogens. However, until recently, most studies on the role of phytohormones in plant-pathogen interactions focused on salicylic acid, jasmonic acid, and ethylene. It is now clear that pathogen-induced modulation of signaling via other hormones contributes to virulence. A context of complex crosstalk and induced hormonal changes that modulate disease and resistance is emerging, with outcomes depending on pathogen lifestyle

and the genetic constitution of the host. In this setting, the established metabolomic framework encloses the potential to make a valuable contribution towards this important aspect of plant production (119). Another interesting and economic important research angle relates to the occurrence of physiological diseases such as blossom-end rot, which is characterized by a necrotic lesion at the underside of the tomato fruit. This disease is generally attributed to a deficiency of calcium (i.e. $Ca^{2+}$) in the fruit and associated disintegration of the cell membranes. However, evidence is emerging that phytohormones such as abscisic acid and gibberellins may fulfill a significant role in the mediation of blossom-end rot (120,121). The framework that has been established for metabolomic profiling of phytohormones and related metabolites in this work may be useful for this purpose as well.

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# SUMMARY

In recent years, the interest of consumers in health-promoting substances in their food has increased and in line with this the nutritional aspects of fresh tomato fruit as well. This trend has prompted research that aims at the identification of agronomic practices, which have the potential to promote tomato fruit nutritional quality. In this context, in particular carotenoids are envisaged because of their predominant contribution towards overall tomato fruit nutritional value. However, the deficient knowledge about the underlying regulating mechanisms of carotenoid metabolism envelops a major hindrance to efficiently study the imposed agronomic means. Therefore, the research within this doctoral thesis aimed at unraveling the regulating mechanisms of carotenoid metabolism by contemplating the regulating roles of phytohormones. To this end, a metabolomic framework was established, which was mainly founded on the screening possibilities of sophisticated analytical instruments and subsequent multivariate data mining.

**CHAPTER I** – In this chapter, a comprehensive overview is provided on the diverse health-promoting tomato fruit constituents, whereby chemical and health-promoting properties are defined. In this regard, especially carotenoids have been acknowledged as main descriptors of tomato fruit nutritional quality. In addition, the various phytohormonal classes are reviewed, indicating a large variation in terms of chemical structure and physiological functionality. As such the potential of a regulating function of phytohormones with respect to carotenoid metabolism in tomato fruit is highlighted. Moreover, a summary is given on the predominant analytical strategies that are used for extraction and detection of both compound groups. Herewith, the analytical platforms and their capabilities that are employed in this doctoral thesis are discussed in detail. As these analytical instruments are able to actualize various metabolomic experiments, the basis principles of metabolomics are defined as well. Finally, the conceptual framework and research objectives of this thesis are formulated.

**CHAPTER II** – This particular chapter describes the development and validation of a generic extraction protocol and full-scan high-resolution mass spectrometric method for the analysis of tomato fruit carotenoids. Herewith, representatives for both the class of carotenes ($\alpha$-carotene, $\beta$-carotene, lycopene) and xanthophylls (lutein, zeaxanthin) were considered. The extraction protocol was optimized by a D-optimal design and included liquid-liquid extraction, which was typified by very low volumes of extraction solvent and amounts of needed tomato fruit tissue.

Detection of carotenoids was achieved by HPLC-Exactive™ Orbitrap mass spectrometry and proved an attractive strategy for metabolomic screening strategies. Moreover, good quantitative performance was attained as demonstrated by validation. The validation protocol was performed according to CD 2002/657/EC and included linearity, precision, accuracy, specificity/selectivity and sensitivity. Comparison with well-established UV-VIS and MS/MS approaches for carotenoid analysis showed superior performance of Orbitrap-MS in terms of specificity/selectivity and sensitivity (ranging from 1 to 3.8 pg $\mu L^{-1}$)

**CHAPTER III** – In this chapter, a holistic analytical strategy is achieved for phytohormonal profiling of tomato fruit and leaf tissue. To this end, it was opted to consider various phytohormonal compounds, representing seven major hormonal classes and wide chemical diversity. Generic extraction was performed by solid-liquid extraction and followed by purification using Amicon® Ultra centrifugal units. Phytohormones were separated and detected by UHPLC hyphenated with Exactive™ Orbitrap-MS. Validation of the method was performed according to CD 2002/657/EC and generally yielded satisfactory results with respect to linearity (≥ 0.99), accuracy (between 80 and 110%), precision (≤ 15%), and specificity/selectivity. In addition, good sensitivity was obtained for all phytohormonal classes (ranging from 0.05 to 0.42 pg $\mu L^{-1}$). Besides the excellent quantitative performance, the metabolomic screening possibilities were demonstrated, rendering the developed method an interesting strategy for tomato phytohormonal profiling.

**CHAPTER IV** – The objective of this chapter relates to defining the phytohormonal profiles of tomato fruit, covering various developmental stages. For this particular purpose, UHPLC and high-resolution hybrid quadrupole Exactive™ Orbitrap-MS were employed. This analytical platform combines the capabilities of tandem mass spectrometry and the strengths of high-resolution mass measurements, as such offering great identification potential towards unknown metabolites. This potential was exploited by an integrated strategy of chemical formula prediction and *in silico* based chemical structure evaluation. A total of 52 metabolites were annotated a potential phytohormonal identity and recognized as typifying the tomato fruit hormonal profile. In this regard various phytohormonal classes were represented; i.e. gibberellins, auxins, cytokinins, jasmonates, abscisates, strigolactones, brassinosteroids, salicylates, and polyamines.

**CHAPTER V** – This chapter aimed at deepening the knowledge on the regulatory role of phytohormones in carotenoid metabolism. More specifically, it was envisaged to reveal those phytohormones that are involved in the metabolism of $\alpha$-carotene, $\beta$-carotene, lycopene, lutein, and zeaxanthin. The phytohormonal profiles of 50 tomato fruits were defined and evaluated towards the associated carotenoid concentration levels. For this purpose, a data mining strategy of differential expression and orthogonal partial least squares analysis was implemented. As such, five metabolites were revealed as strongly influencing carotenoid accumulation, i.e. *cis*-zeatin-O-glucoside, 1-acetylindole-3-carboxaldehyde, 2-oxindole-3-acetic acid, *cis*-12-oxo-phytodienoic acid and cucurbic acid.

**CHAPTER VI** – The research within this chapter focused on the potential of altered nutrient solution salinity to enhance the accumulation of carotenoids in cherry tomato fruit (*Solanum lycopersicum* L.). To this end, the regulating role of phytohormones towards carotenoid metabolism was assessed. A greenhouse experiment with five salinity treatments (ranging from 2.0 to 5.0 dS $m^{-1}$) was carried out whereby fruits at varying stages of development were harvested. Using the analytical and data mining strategies, earlier discussed (Chapter II, III, and IV), a total of 46 metabolites were revealed to be involved in carotenoid metabolism and strongly influenced by salinity. This approach eventually indicated little or no potential of altering the EC-levels of the irrigated nutrient solution to promote the accumulation of carotenoids in the tomato cultivar under consideration. The underlying reasons for these findings are discussed and the strengths of the applied metabolomic strategy for assessing and exploiting the potential of imposed agronomic measures towards improving nutritional quality are defined.

**CHAPTER VI** – In this chapter, general conclusions and future research perspectives are formulated. In short, this doctoral study provides an attractive metabolomic framework to investigate the regulating role of phytohormones in carotenoid metabolism and to efficiently examine the potential of agronomic measures to promote tomato fruit nutritional quality. In this context, the need for identification of unknown metabolites and its inherent challenges, have been pointed out to constitute the main obstacle for biological interpretation, validation, and application of the results obtained.

# SAMENVATTING

De laatste jaren tonen consumenten een verhoogde interesse voor de nutritionele waarde van voedingsmiddelen en de aanwezigheid van diverse gezonde bestanddelen. Deze evolutie heeft er toe geleid dat tomatentelers volop inzetten op de kwaliteit van hun producten en daarbij op zoek gaan naar agronomische middelen, die een verbeterde nutritionele kwaliteit kunnen realiseren. Hierbij wordt vooral een verhoogde concentratie aan carotenoïden nagestreefd gezien de belangrijke bijdrage tot de globale nutritionele kwaliteit van tomaten. Ondanks reeds heel wat vooruitgang werd geboekt, vormt de gebrekkige kennis omtrent het metabolisme van carotenoïden een belangrijk obstakel voor een efficiënte evaluatie en optimalisatie van agronomische maatregelen. Daarom werd in deze doctorale thesis getracht om deze kennis verder uit te diepen door de regulerende rol van fytohormonen in het metabolisme van carotenoïden nader te bekijken.

**HOOFDSTUK I** – In dit hoofdstuk wordt een overzicht gegeven van de verschillende componenten, die gezondheidsbevorderende effecten worden toegeschreven en aanwezig zijn in tomaat. Hierbij worden zowel hun chemische als fysiologische eigenschappen toegelicht. Anderzijds worden de verschillende fytohormonale klassen beschreven waarbij hun chemische en functionele diversiteit duidelijk tot uiting komt. Vervolgens omsluit dit hoofdstuk een overzicht van de verscheidene analytische technieken, die veelvuldig worden toegepast voor de extractie en detectie van zowel carotenoïden als fytohormonen. Hierbij wordt vooral ingegaan op het analytische instrumentarium dat gebruikt werd in deze doctorale thesis. Aangezien de metabolomische screeningsmogelijkheden van de apparatuur hier een belangrijk onderdeel van uitmaken, worden de basisprincipes van metabolomics gedefinieerd. Ten slotte wordt het conceptueel kader geschetst en de opbouw van de verschillende onderzoeksfasen aangegeven.

**HOOFDSTUK II** – Dit hoofdstuk beschrijft de ontwikkeling en validatie van een analytische methode voor de detectie en kwantificatie van carotenoïden in de tomatenvrucht. Hierbij werden carotenoïden beschouwd, die zowel behoren tot de klasse van de carotenen ($\alpha$-carotene, $\beta$-carotene en lycopene) als de klasse van de xanthofyllen (luteïne en zeaxanthine). Het generische extractieprotocol werd geoptimaliseerd door gebruik te maken van een D-optimaal design en baseerde zich op vloeistof-vloeistof extractie. De lage benodigde hoeveelheden extractiesolvent en plantmateriaal vormden hierbij belangrijke eigenschappen van het geoptimaliseerde protocol. Scheiding en detectie van de geselecteerde carotenoïden gebeurde aan de hand van HPLC,

gekoppeld aan hoge-resolutie Exactive™ Orbitrap massaspectrometrie, een combinatie die uitermate geschikt bleek voor metabolomische screeningsdoeleinden. Bovendien werd via validatie vastgesteld dat de ontwikkelde methode een correcte kwantificatie van carotenoïden toelaat. Deze validatie werd uitgevoerd volgens CD 2002/657/EC waarbij lineariteit, precisie, juistheid, gevoeligheid en specificiteit/selectiviteit werden geëvalueerd. Daarbij bleken voornamelijk de gevoleigheid (met detectielimieten van 1 tot 3.8 pg $\mu L^{-1}$) en de specificiteit/selectiviteit veel beter dan andere bestaande analytische methoden zoals UV-VIS en MS/MS.

**HOOFDSTUK III** – In dit hoofdstuk werd de ontwikkeling van een holistische strategie beoogd voor de analyse van fytohormonen in zowel vrucht- als bladmateriaal van tomaat. Hiertoe werden een aantal fytohormonen geselecteerd, die zeven hormonale klassen vertegenwoordigden en tevens een grote chemische diversiteit omsloten. Generische extractie werd verkregen via vaste fase-vloeistofextractie en werd gevolgd door een opzuiveringsfase waarbij gebruik werd gemaakt van Amicon® Ultra centrifugale filters. Scheiding en detectie van fytohormonen werd gerealiseerd door middel van UHPLC, gekoppeld aan Exactive™ Orbitrap-MS. Validatie van de ontwikkelde methode werd gerealiseerd volgens de CD 20O2/657/EC richtlijnen en leverde in het algemeen goede resultaten op voor de verschillende prestatiecriteria; lineariteit (≥ 0.99), juistheid (tussen 80 en 110%), precisie (≤ 15%) en specificiteit/selectiviteit. Daarenboven werd ook een goede gevoeligheid vastgesteld voor de verschillende doelcomponenten (detectielimieten tussen 0.05 en 0.42 pg $\mu L^{-1}$). Naast de goede kwantitatieve prestaties, maken ook de metabolomische screenings-mogelijkheden deze methodologie tot een zeer interessante strategie voor de fytohormonale profilering van tomatenvruchten en -bladeren.

**HOOFDSTUK IV** – Het voornaamste doel van dit hoofdstuk omvat de bepaling van de fytohormonale profielen van tomatenvruchten, dewelke verschillende ontwikkelingsstadia omvatten. Hiervoor werd gebruik gemaakt van UHPLC en hoge-resolutie hybride quadrupool Q-Exactive™ Orbitrap-MS. Dit analytische platform combineert de mogelijkheden van tandem-massaspectrometrie en de sterktes van hoge-resolutie accurate massabepaling, en biedt zo heel wat potentieel voor de identificatie van onbekende metabolieten. Dit potentieel werd ten volle benut tijdens de hormonale profilering waarbij een geïntegreerde strategie werd toepast voor bepaling van de chemische compositie en structuur. Op basis van deze strategie werden 52 metabolieten een

potentieel hormonale identiteit toegewezen. Hierbij waren verschillende klassen vertegenwoordigd door één of meerdere metabolieten: brassinosteroïden, strigolactonen, auxines, gibberellines, cytokinines, jasmonates, abscisaten en polyamines.

**HOOFDSTUK V** – In dit hoofdstuk werd getracht de kennis omtrent het metabolisme van carotenoïden verder uit te diepen door de regulerende rol van fytohormonen te bepalen. Meer bepaald werd de betrokkenheid van fytohormonen in het metabolisme van $\alpha$-caroteen, $\beta$-caroten, lycopeen, luteïne en zeaxanthine bestudeerd. Hiertoe werden de fytohormonale profielen van 50 tomatenvruchten onderzocht en geëvalueerd ten opzichte van de carotenoïde concentratieniveaus aanwezig in deze vruchten. Door daarbij gebruik te maken van diverse multivariate data-analyse strategieën werden vijf fytohormonen een belangrijke rol toegekend binnen het carotenoïde metabolisme: cucurbine, *cis*-zeatine-O-glucoside, 1-acetylindol-3-carboxaldehyde, 2-oxindol-3-azijnzuur, *cis*-12-oxo-phytodienoic zuur.

**HOOFDSTUK VI** – Het onderzoek binnen dit hoofdstuk richtte zich op het potentieel van toegepaste agronomische maatregelen voor een verbeterde nutritionele kwaliteit. Meer bepaald werd nagegaan of een gewijzigde saliniteit positieve effecten kon teweegbrengen met betrekking tot de carotenoïde concentratie in kerstomaat (*Solanum lycopersicum* L. cv. Juanita). Hiervoor werd een serre-experiment opgezet waarbij tomatenplanten werden geïrrigeerd met vijf verschillende voedingsoplossingen, getypeerd door hun saliniteitsniveau (variërend van 2.0 tot 5.0 dS $m^{-1}$). Door gebruik te maken van de analytische en data-interpretatie strategieën, eerder beschreven (Hoofdstuk II en III), konden 46 metabolieten worden geselecteerd, die een sterke invloed hebben op het metabolisme van carotenoïden en tevens sterk beïnvloed worden door de saliniteit van de voedingsoplossing. Evaluatie van de specifieke effecten toonde aan dat een gewijzigde saliniteit weinig of geen potentieel heeft om de accumulatie van carotenoïden in de beschouwde cultivar te verhogen. De onderliggende redenen voor deze resultaten worden uitvoerig besproken waarbij ook de tegenstrijdige resultaten van andere studies in acht worden genomen. Tenslotte wordt ook ingegaan op de sterktes en mogelijkheden van de uitgewerkte metabolomische strategie voor gelijkaardig onderzoek.

**HOOFDSTUK VI** – In dit hoofdstuk worden de algemene conclusies en toekomstperspectieven van dit doctoraatsonderzoek geformuleerd. Samenvattend kan gesteld worden dat binnen deze studie een interessant metabolomisch kader werd gerealiseerd waarbinnen de beschreven strategieën heel wat potentieel voor onderzoek bieden. Hier werd de regulerende rol van fytohormonen in het metabolisme van carotenoïden onderzocht en bepaald of een gewijzigde saliniteit van de voedingsoplossing een mogelijkheid biedt voor een verbeterde nutritionele kwaliteit. Binnen dit kader werd de identificatie van ongekende metabolieten als een moeilijke opdracht erkend, maar absoluut vereist voor de biologische interpretatie, validatie en toepassingen van de gerealiseerde kennis.

# CURRICULUM VITAE

# Curriculum Vitae

## PERSONALIA


| | |
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## EDUCATION

### PhD research (2011-2014):

- "Ecophysiological control of phytohormones to optimize tomato fruit quality: a chemical-analytical approach" (IWT 101188)
- Ghent University, Faculty of Veterinary Medicine, Department of Veterinary Public Health and Food Safety, Laboratorium of Chemical Analysis
  (Prof. dr. ir. Lynn Vanhaecke)
- Ghent University, Faculty of Bioscience Engineering, Departement of Applied Ecology and Environmental Biology, Laboratory of Plant Ecology
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### Basic training (October - December 2010):

- Basic training for scientific research
- Ghent University, Faculty of Veterinary Medicine, Department of Veterinary Public Health and Food Safety, Laboratorium of Chemical Analysis
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### Dehousse-scholarship (July - September 2010):

- "Profitable ecophysiological-energetic cultivation in an intelligent controlled greenhouse setting" (IWT 070570)
- Ghent University, Faculty of Bioscience Engineering, Departement of Applied Ecology and Environmental Biology, Laboratory of Plant Ecology (Prof. dr. ir. Kathy Steppe)

## Higher education (2005 - 2010):

- 1st and 2nd Master in Bioscience Engineering (Option agriculture)
  3rd Bachelor in Bioscience Engineering (Option agriculture)
  1st and 2nd Bachelor in Bioscience Engineering

- Masterthesis: “Optimization of the tomato fruit antioxidant content: a chemical-analytical and ecophysiological approach"

- Ghent University, Faculty of Bioscience Engineering

## Secondary education (1999 – 2005):

- Math-Science

- Koninklijk Atheneum, Zottegem

## SCIENTIFIC PUBLICATION

- **Van Meulebroek L**, De Swaef T, Steppe K, Bleyaert P, Vanhaecke L, 2010. Kan zoutstress bij tomaat bijdragen tot een betere nutritionele kwaliteit? *Proeftuinnieuws* 17:34-35.

- **Van Meulebroek L**, Vanhaecke L, De Swaef T, Steppe K, De Brabander H F, 2012. U-HPLC-MS/MS to quantify liposoluble antioxidants in red-ripe tomatoes, grown under different salt stress levels. *Journal of Agricultural and Food Chemistry* 60:566 - 573.

- **Van Meulebroek L**, Vanden Bussche J, Steppe K, Vanhaecke L, 2012. Ultra-high performance liquid chromatography coupled to high resolution Orbitrap mass spectrometry for metabolomic profiling of the endogenous phytohormonal status of the tomato plant. *Journal of Chromatography A* 1260: 67-80.

- De Swaef T, Driever S, **Van Meulebroek L**, Vanhaecke L, Marcelis L, Steppe K, 2013. Understanding the effect of carbon status on stem diameter variations in tomato. *Annuals of Botany* 111:31-46.

- Vanhaecke L, **Van Meulebroek L**, De Clercq N, Vanden Bussche J, 2013. High resolution orbitrap mas spectrometry in comparison with tandem mass spectrometry for confirmation of anabolic steroids in meat. *Analytica Chimica Acta* 767: 118-127.

- **Van Meulebroek L**, Vanden Bussche J, De Clercq N, Vanhaecke L. (2014). A Metabolomics Approach to Unravel the Regulating Role of Phytohormones Towards Carotenoid Metabolism in Tomato Fruit. *Analytical and Bioanalytical Chemistry* 406:2613-2626.

- Orellana G, Vanden Bussche J, **Van Meulebroek L,** Vandegehuchte M, Janssen C, Vanhaecke L, 2014. Validation of a confirmatory method for lipophilic marine toxins in shellfish using UHPLC-HR-Orbitrap MS. Analytical and Bioanalytical Chemistry 406:5303-5312.

- Vanden Bussche J, Decloedt A, **Van Meulebroek L**, De Clercq N, Lock S, Stahl-Zeng J, Vanhaecke L, 2014. A novel approach to the quantitative detection of anabolic steroids in bovine muscle tissue by means of a hybrid quadrupole time-of-flight-mass spectrometry instrument. *Journal of Chromatography A* 1360:229-239.

- Bloemen J, Agneessens L, **Van Meulebroek** L, Aubrey DP, McGuire MA, Teskey RO, Steppe K, 2014. Stem girdling affects the quantity of CO2 transported in xylem as well as CO2 efflux from soil. *New Phytologist* 201:897-907.

- **Van Meulebroek L**, Vanden Bussche J, De Clercq N, Steppe K, Vanhaecke L (2014). A Metabolomics Approach to Unravel the Regulating Role of Phytohormones Towards Carotenoid Metabolism in Tomato Fruit. *Metabolomics*, accepted.

## SCIENTIFIC TRAINING AND ACTIVITIES

- 2014: Poster presentation (no attendence) at HTC13/HTSP3 (Belgium)
  "Unravelling the phytohormonal status and carotenoid profile of tomato plants by using metabolomics, including extraction and UHPLC-Orbitrap-MS"

- 2013: Certificate of attendence Recent Advances in Food Analysis (Czech Republic)
  Oral presentation: "A better understanding of carotenoid metabolism in tomato fruit by using the metabolomics capabilities of full-scan Orbitrap MS"

- 2013: Certificate of attendence Trends in Food Analysis VII (Belgium)
  Oral presentation: "Unravelling the regulating role of phytohormones towards carotenoid metabolism in tomato fruit by using the metabolomic screening possibilities of Orbitrap-MS"

- 2013: Poster presentation (no attendence) at EuroFoodChemXII (Turkey)
  "The analytical performance of high resolution Orbitrap mass spectrometry for the detection and quantitation of carotenoids in tomato fruit"

- 2012: Certificate of attendence ExTech XIV (Italy)
  Oral presentation: "Metabolomic profiling of phytohormones in tomato: the crucial role of generic extraction and full-scan high resolution Orbitrap mass spectrometry"

- 2012: Certificate of attendance HTC12/HTSP2 (Belgium)
  Poster presentation: “Metabolomic profiling of phytohormones in tomato: exploiting the high resolution and full-scan characteristics of the Orbitrap mass spectrometer”

- 2011: Certificate of attendance EuroFoodChem XVI (Poland)
  Posterpresentation: “U-HPLC-MS/MS analysis to quantify the antioxidant content of tomatoes, subjected to different cultivation conditions”

- 2011: Certificate of attendence KVCV symposium (Belgium)
  Poster presentation: “U-HPLC-MS/MS analysis to quantify the antioxidant content of tomatoes, subjected to different cultivation conditions”

- 2010: Certificate of attendance Thermo Fisher training course (Belgium)
  Orbitrap Exactive™ Training Course

## AWARDS

- 2011: Poster award KVCV symposium (Belgium)

## DOCTORAL SCHOOLS

- 2014: qPCR course
- 2013: Q-Exactive Operations Training Course
- 2013: Basics of Biology for Engineers
- 2013: Mass Spectrometry Data Processing
- 2013: 6th From PhdD to Job Market
- 2012: Advanced Academic English:
  Conference Skills – Effective Slide Design
- 2012: Statistics – Introduction to SPSS
- 2012: Creative thinking

## TUTOR OF MASTER STUDENTS

- 2011-2014: One thesis student of Pharmaceutical Science
  Four thesis students of BioScience Engineering

# DANKWOORD

# CLUEDO The special thank you edition

**Speelduur:** 4 jaar

**Aantal spelers:** onbeperkt

Gelijkenissen met gebeurtenissen en personen berusten eerder niet op toeval

**Spelinhoud:**

- 1 pion: Gemotiveerde doctoraatstudent-speurder
- 2 pionnen: Enthousiaste speurderpromotoren
- Vele pionnen: Hulpspeurders
- Waardevolle pionnen: Familiespeurders
- 1 spelbord
- 1 dobbelsteen
- 3 geheime hulpmiddelen
- *1 geheim slachtoffer*

**Spelomschrijving**

- Moeilijkheidsgraad: ****
- Strategie: ***
- Geluk: **
- Spelplezier: ****
- Samenwerkingsfactor: ***

Gebaseerd op waargebeurde feiten, maar ook een heel klein beetje verzonnen

**De teerling is geworpen.**

Een sombere en kille winterdag werd plots opgeschrikt door een intense regenvlaag. Het hevige getokkel van de regen tegen de ramen bleek de voorbode van een onheilspellend en mysterieus bericht. Een groezelige envelop bereikte immers zijn bestemmeling en werd met trillende handen geopend. Een handgeschreven brief onthulde een ongelooflijk verhaal. Diverse individuen waren het slachtoffer geworden van een bijzonder genadeloze en harteloze moordenaar. De opdracht van het geheime IWT vennootschap was dan ook klaar en duidelijk; het onderzoek naar deze uiterst gruwelijke misdaad kreeg absolute prioriteit! Het takenpakket voor deze vierjarige missie was zwaar en uitdagend, maar een einde in mineur was geen optie voor deze speurtocht. De informatie over de feiten was uiterst karig, maar het doel meer dan duidelijk: de slachtoffers, dader(s), motief, moordwapen en plaats van delict moesten worden bepaald! De teerling was geworpen…

**De eerste stap: een goed onderzoeksteam**

Wie vertrouwd is met het betere speurwerk weet dat een dergelijke zoektocht een werk van lange adem is waarbij heel wat pistes dienen onderzocht te worden. Verschillende verdachten worden aan de leugendetector gelegd, diverse moordwapens worden nauwkeurig op vingerafdrukken gecontroleerd en voetafdrukken worden op diverse locaties onder de loep genomen. Een vakkundig onderzoeksteam was dan ook een absolute noodzaak…

**Mogelijk moordwapen: de Engelse sleutel**

De sleutel tot succes binnen deze uitdagende onderzoeksmissie leg ik zonder twijfel in de handen van prof. Lynn Vanhaecke. Als toegewijd onderzoeker leidt ze een goed draaiend detectivebureau waar de loep steeds op het juiste spoor wordt gericht. Een kleine aanwijzing ging nooit ongezien voorbij en leidde vaak tot een doorbraak in het onderzoek. Details zijn nu eenmaal belangrijk in een sector waar alles draait om het vinden van datgene dat niet wil of mag gevonden worden. Ze gunt haar speurders ook de nodige vrijheid om zelf onderzoeksacties op poten te zetten, iets wat ik ten zeerste apprecieerde. Ook wanneer de tijd genadeloos wegtikte en de (teelt)seizoenen voorbijraasden, kon ik meer dan ooit rekenen op haar onvoorwaardelijke hulp en inzet. Lynn gaat werkelijk door een vuur voor haar vele speurders! Procedurefouten maken hier nauwelijks een kans! Een welgemeende dankjewel voor de steun tijdens deze unieke ervaring…

**Mogelijk verdachte: dominee Groenewoud**

Groene vingers zijn een belangrijke troef voor een voltijds onderzoeker en halftijds ecofysiologische speurder. Het wroeten in de aarde en de verbeten strijd met tegendraadse planten werkt nu eenmaal heel inspirerend. Dit brengt me naadloos bij prof. Kathy Steppe, die via haar enthousiasme de ‘groene’ eco-speurder in ieder van ons volledig naar boven weet te brengen. Haar overvloed aan ideeën werkte inspirerend en vormde vaak de stimulans om nieuwe onderzoekpistes te verkennen. De ‘sprekend plant’ werd me hierbij als geheime informant voorgesteld. Een gouden zet zo bleek. Ook het tot stand brengen van geheime en minder geheime vennootschappen wierp zijn vruchten af en bracht het onderzoek in een stroomversnelling.

**Een eerste hulpmiddel: de klankentapper.**

Nog voor de start van mijn eigenlijke speurtocht werd ik tijdens een twee uur durend verhoor op de rooster gelegd en getest of ik wel klaar was voor deze onderzoeksmissie. Daarbij werd ik reeds uitgevraagd over mijn geheime informant, die zowaar vergeleken werd met de befaamde klankentapper van Suske & Wiske. Een terechte opmerking van prof. Hubert De Brabander, die me steeds is bijgebleven. Een man van anekdotes. Bedankt voor de ontvangst op jouw labo!

**Mogelijke plaats delict: het laboratorium**

Analyse van het bewijsmateriaal bleek een uiterst uitdagende en delicate zaak. Contaminatie en degradatie van het zorgvuldig verzamelde materiaal konden immers de mogelijke bewijslast volledig vernietigen. Gelukkig stond een heel analytisch team te trappelen om me met woord en daad bij te staan. Door hun instructies kon een serieuze bewijslast op tafel gelegd worden. Bedankt Lucie, Mieke, Joke, Dirk, Ine, Vicky en Beata. Hierbij ook een speciale vermelding voor 'superheld' (elk avontuur heeft er eentje nodig), die een ijzersterk dossier in elkaar wist te steken. Eentje zonder anonieme bronnen zowaar!

**Mogelijke plaats delict: de keuken**

Een goed detective wordt gekenmerkt door een niet te stillen honger naar informatie en houdt de oren dan ook steeds gespitst. De keuken mag dan misschien een alledaags plekje lijken waar boterhammetjes achteloos worden verorberd, het bleek een uiterst interessante plek om de laatste nieuwtjes en weetjes te vergaren. Een belangrijk moment dus voor een detective om die ene gouden tip te ontwaren. Uiteraard komt het er op aan om de vele nonsens en filosofische praatjes te kunnen onderscheiden van de waardevolle elementen. Daarbij bedankt aan iedereen van de verschillende vakgroepen, die hebben bijgedragen aan een overvloed van verhalen. Het onontbeerlijk notitieboekje van een detective is op die manier snel gevuld.

**Mogelijke plaats delict: de serre**

De kiem van deze missie is misschien wel terug te vinden in een Beitemse serre. Daar genoot ik mijn opleiding tot speurder onder de vleugels van Tom. Zijn strategische en tactische werkwijze om de grootste mysteries op te lossen heeft me heel wat bijgebracht en misschien wel aangezet om eenzelfde carrière op te starten. Bij deze ook een oprechte dankjewel voor de onvoorwaardelijke steun bij het opstarten van mijn eigen missie!

**Mogelijk moordwapen: gif**

Ik weet niet of het aan de giftige solventdampen lag, die mijn geest mogelijks bezoedelden, maar de avonden met "vzw De Kwisvrienden" waren telkens een geanimeerd schot in de roos. De spectaculaire bolo-challenges, de waardevolle prijzen (van badmatjes tot lintmeters), de voetjesvoor-uitspattingen en een gewelddadige indianendans staan voor eeuwig in mijn geheugen gegrift. Deze ontspannende momenten heeft een hardwerkende detective nu eenmaal nodig om zijn kijk op de feiten weer scherp te kunnen stellen. Verder droeg de duivelse en legendarische WK voetbalpronostiek ook duidelijk bij tot de ontwikkeling van mijn strategische vaardigheden, die helaas soms genadeloos en onterecht werden afgestraft! Ik ben mentaal alvast klaar voor een volgende campagne. Bedankt dus Nathalie, David, Ine, Kaat, en Annelies voor de vele leuke momenten (en een goede gezondheid).

**Een tweede hulpmiddel: de brommer**

Een kort dankwoordje aan mijn 'brommer' is absoluut vereist aangezien onze wegen binnenkort waarschijnlijk scheiden. Je stelde me in staat om de verplaatsingen tussen de verschillende plaatsen delict te realiseren en de bewijslast te vervoeren. Samen kwamen we tot rust na een hectische dag. Bedankt.

**Mogelijke plaats delict: de serre (bis)**

Het reeds geleverde onderzoek deed het vermoeden groeien dat deze locatie wel eens de ideale plaats kon zijn voor een koelbloedige misdaad. Aangezien steeds meer elementen in die richting wezen, werd de serre nogmaals onder de loep genomen. Daarbij werd ons onderzoek echter serieus belemmerd! Gelukkig kon ik rekenen op de hulp van Jochen om de weelderige en weerbarstige tomatenplanten te trotseren en terug te dringen. Hoewel onze observatiepost heel wat hard werk vergde, zorgden de roerende heksen en dansende planten voor verzachtende omstandigheden. Tot nader order werden hier geen giftige solventdampen waargenomen… Bij deze ook bedankt aan Geert en Philip voor het installeren van het benodigde spionagemateriaal.

**Mogelijk moordwapen: de kandelaar**

De kandelaar mag dan wel een bron van sfeer en verlichting betekenen, het blijft ook een veelvuldig geraadpleegd moordwapen. Waakzaamheid is dus constant vereist. Gelukkig waren mijn collega-speurders volledig te vertrouwen en zorgden ze voor een goede sfeer op de werkvloer. Een speurdersteam waar je op rekenen kan! Bedankt Julie VDB, Julie K, Anneleen, Kaat, Lieselot, Karen, Gabriel, Klaas, Nathalie en Jella! Allen droegen jullie bij tot het ontwikkelen van hoogtechnologische speurmethodes en dataverwerkingssystemen waardoor de misdaadacties steeds correct in kaart gebracht konden worden. Tevens dank ik jullie allen bij het opsporen van bewijsmateriaal in de vele in beslag genomen gebakjes. Helaas zonder resultaat…

**Een derde hulpmiddel: de hulpjes.**

Hoewel de hulpjes zich vaak verdekt opstellen en onder de radar werken, leveren ze zeer waardevol werk. Lies en Jolien, bedankt voor jullie bijdrage in dit onderzoek.

**Mogelijke verdachte: mevrouw Blaauw van Draet**

Even ter verduidelijking voor zij die niet zo goed zijn in het complexe speurwerk; de rode draad in dit verhaal is het spelletje cluedo. Een gezelschapsspel waar tactisch vernuft en inzicht absolute vereisten zijn om de moordenaar te ontmaskeren. Ideale vaardigheden dus voor een speurder! Bedankt dus Julie, Kaat, Lander en Bert om me te helpen bij het ontwikkelen van deze vaardigheden.

**Mogelijke plaats delict: de zitkamer**

Eens een dossier wordt afgesloten komt deze bij de jury terecht en volgt de zitting. Een raad van specialisten buigt zich dan over het werk en velt zijn oordeel. Bij deze een welgemeende dankjewel voor de waardevolle suggesties; prof. Robert Hall, prof. Kristof Demeestere, prof. Marie-Christine Van Labeke, prof. Els Prinsen, prof. Sara De Sager, en dr. Kris Van Audenaert.

**Mogelijk moordwapen: het touw**

De eindjes aan elkaar kunnen knopen en het beheren van de werkingsmiddelen is uiteraard een belangrijke factor voor een gerust gemoed. Het is één van de vele (administratieve) taakjes waar Soetkin zich mee bezighoudt. Een werkelijk manusje-van-alles en eeuwig optimist. Bedankt voor de vele hulp en leuke praatjes! (een Tiny-verhaal had ook gekund, maar was waarschijnlijk net iets minder spannend geworden…).

**Mogelijke verdachte: Rosa Roodhart**

Een goed detective heeft nood aan een goede thuis, een hartverwarmend nest. En deze detective heeft geluk en kent zo'n plaatsje. Aan mijn ouders, broer en zus (en Stijn), bedankt voor de onvoorwaardelijke steun en interesse. Tijdens de drukke tijden kon ik steeds op jullie rekenen! Gelukkig maar. Ook een warme knuffel aan de kleinste speurders van de familie, Lucas en Siebe. Het samen spelen doet een vermoeide detective deugd en zorgt voor een volledige herbronning. Daar kan zelfs een frisse 'appelsini' niet tegenop!

**Eureka: het euforisch moment**

De doorbraak en het euforisch moment. Het is soms lang wachten om de resultaten te boeken waarop werd gehoopt. De vele tegenslagen en hoge tijdsnood hebben een sterke poging ondernomen om deze detective te kraken, maar het mysterie is opgelost. De ontknoping is op komst…

Lieven.

## De ontknoping