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Targeted and non-targeted studies on *Alternaria alternata* metabolites

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Abstract

Fungi of the genus *Alternaria* are widely occurring plant pathogens infecting a multitude of crops, fruits, and vegetables in the field and during postharvest storage. Beside the immediate economic losses due to the deterioration of the agricultural goods, *Alternaria* fungi biosynthesize a multitude of secondary metabolites potentially harmful to animal and human health, the so-called "mycotoxins". However, *Alternaria* mycotoxins are not routinely analyzed and no maximum levels have been established by the European Food Safety Authority (EFSA). A first estimation conducted by the EFSA on the risk for public health related to the occurrence of *Alternaria* mycotoxins in food revealed the need for additional data on toxicity as well as on the occurrence of the secondary metabolites biosynthesized by *Alternaria* fungi. Besides, the majority of all the metabolites produced by *Alternaria* fungi, the so-called "mycobolome", has neither been identified nor characterized yet.

In the present thesis, a sensitive LC-MS/MS method for the accurate quantification of the *Alternaria* mycotoxins alternariol, alternariol monomethyl ether, tentoxin, altertoxin I, alterperylenol, and tenuazonic acid was developed. The mycotoxins were either quantified applying stable isotope dilution assays or by matrix-matched calibrations. The newly developed method was validated for starch, as a matrix for cereals, and for fresh tomato purée. The method validation resulted in low limits of detection and limits of quantification for all analytes in both matrices demonstrating the sensitivity of the method. Additionally, good recoveries were determined at three different spiking levels, respectively. High inter-injection, intra-day and inter-day precisions confirmed the robustness and stability of the method. Subsequently, several cereal- and tomato-based infant food products were analyzed for their *Alternaria* mycotoxin contamination. Scattered samples contained high

amounts of *Alternaria* mycotoxins possibly constituting a risk to the health of infants. However, the mean and median of the *Alternaria* mycotoxin contamination was low. Thus, the consumption of the infant foods is unlikely to pose a risk to the health of infants on average.

To obtain deeper insights into the mycobolome of *Alternaria* fungi, non-targeted metabolomics investigations were conducted by Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS) in the present thesis. *Alternaria alternata* and *Alternaria solani* isolates were cultivated in a chemically defined liquid medium and were analyzed by FT-ICR-MS. The ultra-high resolution of FT-ICR-MS allowed the unambiguous assignment of elemental compositions to the measured mass signals. Structural hints for the molecular formulae were obtained by performing database searches against the databases *Antibase* and *Kyoto Encyclopedia of Genes and Genomes*. Differences in the metabolite production of *Alternaria alternata* and *Alternaria solani* were identified statistically. Furthermore, variations in the mycobolome of the *Alternaria alternata* isolates were identified. Additional LC-MS/MS measurements of the *Alternaria* mycotoxins alternariol, alternariol monomethyl ether, tentoxin, altertoxin I, altertoxin II, alterperylenol, stemphyltoxin III, and tenuazonic acid also revealed differences in the mycotoxin production of the *Alternaria alternata* and *Alternaria solani* isolates.

Kurzzusammenfassung

Schimmelpilze der Gattung *Alternaria* sind weit verbreitete Pflanzenpathogene. Sie befallen eine Vielzahl an Getreide-, Obst- und Gemüsesorten, sowohl während deren Wachstum auf dem Feld als auch während deren Lagerung, wodurch erhebliche finanzielle Einbußen durch den Verderb von Lebens- und Futtermitteln auftreten. Darüber hinaus sind *Alternaria* Schimmelpilze für die Bildung toxischer Sekundärmetabolite, den sogenannten Mykotoxinen, in Lebens- und Futtermitteln verantwortlich. Diese Toxine stellen bei Verzehr zwar eine potenzielle Gesundheitsgefährdung für Mensch und Tier dar, unterliegen aber weder gesetzlichen Höchstmengenbeschränkungen noch werden sie in der Routineanalytik erfasst. Dies ist vor allem auf das Fehlen von maximal zulässigen Grenzwerten von Seiten der Europäischen Behörde für Lebensmittelsicherheit (EFSA) zurückzuführen. Eine erste Abschätzung der Gefahr für die öffentliche Gesundheit im Zusammenhang mit dem Auftreten von *Alternaria*-Mykotoxinen durch die EFSA ergab, dass zusätzliche Daten zur Toxizität sowie zum Vorkommen von *Alternaria*-Sekundärmetaboliten erhoben werden müssen. Zudem sind viele Komponenten der von *Alternaria*-Schimmelpilzen produzierten Gesamtheit an Metaboliten, dem sogenannten Mykobolom, nach wie vor nicht bekannt und daher auch nicht eingehend untersucht.

Der erste Teil dieser Arbeit umfasst die Entwicklung einer sensitiven LC-MS/MS-Methode zur exakten Quantifizierung der *Alternaria*-Mykotoxine Alternariol, Alternariol-Monomethylether, Tentoxin, Altertoxin I, Alterperylenol und Tenuazonsäure. Die Mykotoxine wurden zum einen mittels Stabilisotopenverdünnungsanalyse, zum anderen mittels Matrixkalibrierung quantifiziert. Anschließend wurde die neu entwickelte Methode für Stärke sowie für frisch pürierte Tomaten validiert. Für alle Analyten konnten in beiden Matrices niedrige

Nachweis- und Bestimmungsgrenzen erreicht werden, was die hohe Sensitivität der neu entwickelten Methode belegt. Zudem konnten konstant hohe Wiederfindungen für jeweils drei unterschiedliche Dotierniveaus bestimmt werden. Ferner zeigte die Methodenvalidierung eine hohe Geräte-, Wiederhol- und Serie-zu-Serie-Präzision, sodass neben der hohen Empfindlichkeit auch die Zuverlässigkeit der Methode bestätigt werden konnte. Anschließend wurde diverse getreide- und tomatenbasierte Babynahrung auf ihre Kontamination mit *Alternaria*-Mykotoxinen hin untersucht. Vereinzelt wurden hohe Gehalte an *Alternaria*-Mykotoxinen nachgewiesen, die möglicherweise eine Gefahr für die Gesundheit von Kleinkindern darstellen könnten. Trotz alledem war der resultierende Mittelwert und Median der Mykotoxinbelastung gering, sodass von keiner erhöhten Gesundheitsgefahr für Kleinkinder auszugehen ist.

Neben der Analyse von *Alternaria*-Mykotoxinen in Nahrungsmitteln wurden im zweiten Teil dieser Arbeit *non-targeted* Studien am Metabolom von *Alternaria*-Pilzen durchgeführt. Mittels Fourier-Transformations-Ionenzyklotronresonanz-Massenspektrometrie (FT-ICR-MS) sollten tiefere Einblicke in das Metabolom erhalten werden. Hierfür wurden *Alternaria alternata* und *Alternaria solani* Isolate in einem speziellen Flüssigmedium kultiviert und anschließend mittels FT-ICR-MS vermessen. Die ultra-hohe Auflösung des FT-ICR-MS ermöglichte eine eindeutige Zuordnung von Summenformeln zu den gemessenen *m*/*z* Signalen. Diese Summenformeln wurden anschließend gegen die Datenbanken *Antibase* und *Kyoto Encyclopedia of Genes and Genomes* abgeglichen, um potenzielle Molekülstrukturen zu erhalten. Darüber hinaus offenbarte eine statistische Auswertung der Daten nicht nur Unterschiede im Metabolismus von *Alternaria alternata* und *Alternaria solani*, sondern auch innerhalb der *Alternaria alternata* Spezies. Parallele LC-MS/MS-Messungen zeigten analoge Unterschiede in der Expression der Mykotoxine Alternariol, Alternariol-Monomethylether, Tentoxin, Altertoxin I, Altertoxin II, Alterperylenol, Stemphyltoxin III und Tenuazonsäure zwischen *Alternaria alternata* und *Alternaria solani*.

I Table of contents

II List of abbreviations

Mycotoxins

ALTP	alterperylenol
AME	alternariol monomethyl ether
AME-3-Glc	alternariol monomethyl ether-3-glucoside
AME-3-S	alternariol monomethyl ether-3-sulfate
AME-9-S	alternariol monomethyl ether-9-sulfate
AOH	alternariol
AOH-3-Glc	alternariol-3-glucoside
AOH-3-S	alternariol-3-sulfate
AOH-9-S	alternariol-9-sulfate
APML	alterperylenepoxide A 9-mercaptolactate
ATX I	altertoxin I
ATX II	altertoxin II
STTX III	stemphyltoxin III
TA	tenuazonic acid
TEN	tentoxin

Abbreviations

A	analyte
A	area
A.	*Alternaria*
a_w	activity of water
b.w.	body weight
c	concentration [g/L]
CE	collision energy
CONTAM	contamination in the food chain
COSY	correlation spectroscopy
d	doublet
Da	Dalton
dd	doublet of doublets
DNA	deoxyribonucleic acid
EFSA	European Food Safety Authority
ESI	electrospray ionization
FT-ICR-MS	Fourier transform ion cyclotron resonance mass spectrometry
Glc	glucoside
HMBC	heteronuclear multiple-bond correlation spectroscopy
HPLC	high performance liquid chromatography
HPLC-UV	high performance liquid chromatography coupled to UV detection
HSQC	heteronuclear single quantum coherence or heteronuclear single quantum correlation
I	intensity
IS	internal standard
IUPAC	International Union of Pure and Applied Chemistry
J	coupling constant
KEGG	*Kyoto Encyclopedia of Genes and Genomes*
LC	liquid chromatography

LC-MS	liquid chromatography coupled to mass spectrometry
LC-MS/MS	liquid chromatography coupled to tandem mass spectrometry
LD_{50}	median lethal dose
LOD	limit of detection
LOQ	limit of quantification
m	mass [g] (or multiplet in NMR spectra)
M	molar mass [g/mol]
m/*z*	mass to charge ratio
MHz	megahertz
MRM	multiple reaction monitoring
MS	mass spectrometry
MS/MS	tandem mass spectrometry
n	amount of substance [mol]
n.d.	not detected
NMR	nuclear magnetic resonance
PCA	principal component analysis
rpm	rotations per minute
RSD	relative standard deviation
S	sulfate
s	singlet
SAM	*S*-adenosyl-methionine
SIDA	stable isotope dilution assay
SNA	synthetic nutrient-poor agar
SPE	solid phase extraction
t	triplet
TDI	tolerable daily intake
TTC	threshold of toxicological concern
UHPLC	ultra-high performance liquid chromatography
UV	ultraviolet

UV-Vis	ultraviolet-visible
V	volume [L]
δ	chemical shift [ppm]
ε	molar extinction coefficient [L mol^{-1} cm^{-1}]

1 Mycological background

Fungi are a heterogeneous group of organisms belonging to the eukaryotes (Moss, 1987). Some fungi exhibit saprophytic or parasitical capabilities on dead organic matter or on living organisms and are classified as molds. In general, molds are not grouped according to systematic characteristics but share physiological and ecological properties (Kück et al., 2009). Common representatives of saprophytic or pathogenic fungi are the genera *Aspergillus*, *Penicillium*, *Fusarium*, *Alternaria*, and *Claviceps* (Steyn, 1995). *Fusarium* species deteriorate crops in the field, while *Penicillium* fungi are responsible for post-harvest decay. On the contrary, *Alternaria* species cause damages both in the field as well as during storage (Ozcelik et al., 1990; Asam et al., 2009). The losses of fruits and vegetables due to fungal post-harvest damages range from 35 to 55% of the total production (Sanzani et al., 2016). Fungal deterioration is accompanied by biochemical changes in agricultural products. Regarding grains, these biochemical alterations include the release of free fatty acids, the decrease of non-reducing sugars, and the degradation of lipids and proteins. Additionally, fungal spoilage is accompanied by the occurrence of musty odors and the formation of off-flavors as well as allergic compounds (Golumbic and Kulik, 1969; Magan and Aldred, 2007).

Apart from the deterioration of the agricultural products, the latter are frequently contaminated with various fungal secondary metabolites. Around 300 fungal secondary metabolites are currently known and some of them are characterized as mycotoxins (Steyn, 1995; Bennett and Klich, 2003). Mycotoxins are natural products of low molecular weight (Bennett and Klich, 2003) leading to adverse effects in animals and humans after oral consumption or dermal contact. These adverse effects are observed even after exposure to low amounts of the mycotoxins (Gallo et al., 2015). Contrarily to primary compounds,

secondary metabolites are not essential for fungal growth and the biological importance of the mycotoxins is not fully understood (Fox and Howlett, 2008). While researchers discuss protective properties of mycotoxins against other organisms (Fox and Howlett, 2008), these secondary metabolites might also contribute to the aggressiveness of the fungi during the colonization on plants (Sanzani et al., 2016). Regarding the above concerns, only around 20 mycotoxins are regularly evaluated in agricultural products for human consumption, namely aflatoxins, ochratoxins, trichothecenes, fumonisins, and patulin (Steyn, 1995). Frequently affected agricultural products comprise cereals, grains, and grain-based products, tomato and tomato-products, sunflower seed and sunflower oil, fruits and fruit products, beer, and wine (Steyn, 1995; EFSA on Contaminants in the Food Chain, 2011; Marin et al., 2013). These fungal metabolites enter the food chain not only via agricultural commodities but also through animal products like meat, milk, and eggs. As animals are frequently exposed to highly contaminated feed, mycotoxins accumulate in animal tissues and organs leading to contaminated animal products (Marin et al., 2013).

The consumption of certain mycotoxins can cause diseases, such as ergotism, which is one of the most well-documented human mycotoxicosis in Europe (Steyn, 1995). The disease is provoked by the oral intake of cereals containing ergot alkaloids, which are produced by *Claviceps purpurea* (Kück et al., 2009). Another well-known mycotoxicosis occurred in the 1930s in Russia and is referred to as alimentary toxic aleukia (Krogh, 1987; Steyn, 1995; Bottalico and Logrieco, 1998). This disease was caused after the consumption of grains that overwintered on the fields and were heavily contaminated with *Fusarium sporotrichioides* (Lutsky et al., 1978; Shephard, 2008).

Various toxicological effects such as carcinogenicity, mutagenicity, teratogenicity, and estrogenicity are described for a multitude of mycotoxins in the literature. The severity of these adverse effects not only depends on the toxicity of the secondary metabolites but also on the incorporated amount (Steyn, 1995). Additionally, possible synergistic effects of various mycotoxins are currently mostly unknown and could increase the risk of mycotoxin contaminated food (Krska et al., 2017). Due to the adverse effects against animal and human health, comprehensive risk assessments are required for the mycotoxins of various fungal genera. In principle, risk assessments constitute hazard identification, hazard characterization, exposure assessments, and risk characterization. The hazard identification comprises epidemiological and toxicological studies including short-term, sub-chronic, chronic, and carcinogenic studies on various animals under controlled conditions. Furthermore, the absorption, the distribution, the metabolism, and the excretion of the mycotoxins as well as the underlying mechanism of health effects are investigated. The hazard characterization distinguishes between non-carcinogenic and carcinogenic mycotoxins and involves the

evaluation of dose response and extrapolation to humans. During exposure assessments, data on the occurrence of mycotoxins as well as on the intake of various contaminated foods are collected and evaluated. Finally, the risk characterization relies on the previously performed hazard identification, hazard characterization, and exposure assessment and includes qualitative and quantitative estimations of the occurrence and severity of adverse effects against humans (Kuiper-Goldman, 2004).

1.1 The genus *Alternaria*

1.1.1 *Alternaria* fungi - morphology and physiology

Alternaria fungi form green to grey, dark brown, or black branched hyphae and felted mycelia (Bottalico and Logrieco, 1998; Kück et al., 2009). The colored mycelium is a result of the production of melanins biosynthesized to protect the fungus from UV irradiation (Kück et al., 2009). Moreover, *Alternaria* species exhibit large pale brown to brown conidia. The conidia are multi-celled with transverse and longitudinal septa (Rotem, 1994; Lawrence et al., 2013) and occur either solitary or in chains (Bottalico and Logrieco, 1998). For reproduction purposes, *Alternaria* fungi produce asexual spores. The conidio spores fall apart during ripening and are spread by the wind even to remote places such as arctic regions or deserts (Rotem, 1994; Kück et al., 2009). Some of the conidio spores can survive on dried wheat grain for almost ten years and around 40% of all spores are able to germinate after one year of storage under dry conditions (Bottalico and Logrieco, 1998). **Figure 1.1** displays microscopic pictures of *A. alternata* and *A. solani* spores from respective suspensions.

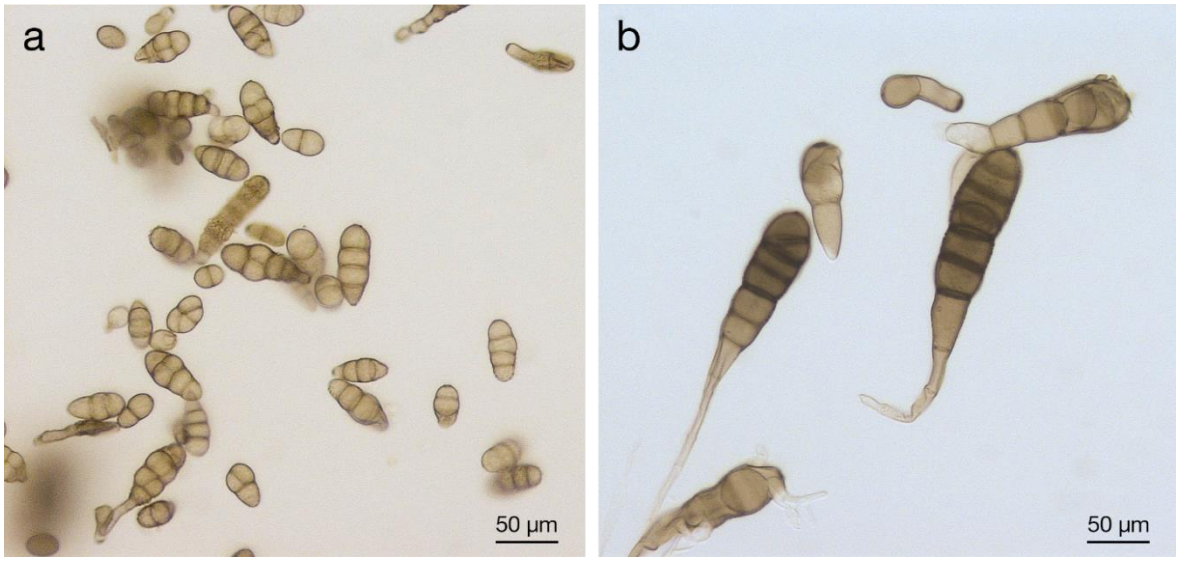


Figure 1.1: Light microscopic pictures of spore suspensions of the *Alternaria* species *A. alternata* (a) and *A. solani* (b).

Apart from asexual reproduction, many fungi produce sexual spores. As the morphology of the organs for the sexual reproduction differs from the ones used for asexual reproduction, great confusion about the morphological classification of the fungi occurred (Kück et al., 2009). In this regard, some species of the genus *Alternaria* were assessed to be the asexual anamorph of the ascomycete *Pleospora* (Thomma, 2003). In general, the genus *Alternaria* is classified into the phylum ascomycetes, class dothideomycetes, and order pleosporales (Lorenz et al., 2012). If no sexual state is determined for an *Alternaria* species, the latter is classified into the phylum *Fungi imperfecti* (Thomma, 2003).

Alternaria growth depends on various environmental conditions such as availability of water, pH, source of nutrition, temperature, and light (da Cruz Cabral et al., 2013). For vegetative growth, an activity of water (a_w) of at least 0.84 is indispensable within a pH range of 2.7–8.0. For sporulation, a minimum a_w of at least 0.90 is required (Bottalico and Logrieco, 1998). Beside sources of carbon such as glucose or sucrose, elements like potassium, phosphor, magnesium, nitrogen, sulfate, calcium, iron, copper, manganese, zinc, and molybdenum are essential for mold growth. *Alternaria* species grow between 2.5–32 °C and exhibit optimum development between 25–28 °C (Kück et al., 2009). Sporulation occurs between 15–33 °C, with optimum sporulation between 25–27 °C. The sporulation of *Alternaria* species is probably triggered by sunlight or UV light during alternating periods of illumination and darkness. The latter favors the release of the spores (Bottalico and Logrieco, 1998). For the infection of plants, high temperatures and free moisture are required (Rotem, 1994). The conidia germinate quickly in the presence of water and the fungi start to colonize the host leaf after entering stomata by producing germ tubes. However, some *Alternaria*

species only colonize on damaged leaves. Seven to ten days after the infection, *Alternaria* species start to sporulate supporting the fast spreading of the fungi (Strandberg, 1992).

1.1.2 *Alternaria* fungi - taxonomy and segregation of species

In 1816, *Alternaria tenuis* was the first species classified into the genus *Alternaria* (Nees von Esenbeck, 1816). Since then, 400-450 *Alternaria* species have been described in the literature (Simmons, 1992), but only 276 species are officially recognized (Simmons, 2007; Lorenz et al., 2012). Over the years, great confusion has arisen regarding the classification of *Alternaria* species. In 1912, Keissler synonymized *Alternaria tenuis* and *Torula alternata* with *Alternaria alternata* (Lawrence et al., 2013). 80 years later, Simmons et al. (1992) summarized the current status and future challenges in the taxonomic classification of *Alternaria*. Conidium characteristics such as size, septation, and pattern of catenation were proposed to form the basis for the identification and classification of *Alternaria* species. Additionally, the genus *Alternaria* was organized into various species-groups (Simmons, 1992). However, as the size of the conidia and the septation vary depending on nutrition, humidity, light, and temperature, the segregation by morphology was considered as unreliable (Logrieco et al., 2009) and some authors claimed a systematic revision within the genus *Alternaria* and the related genera *Stemphylium*, *Embellisia*, *Mimbya*, and *Ulocladium* (Lawrence et al., 2013; Kahl et al., 2015). Especially the species group *A. alternata* might comprise more than one species and, therefore, a taxonomic revision within the genus *Alternaria* is indispensable (Ostry, 2008).

The literature presents various approaches in order to overcome the difficulties in the segregation within the genus *Alternaria*. Simmons and Roberts (1994) introduced the three-dimensional sporulation pattern for the classification of *Alternaria* species. A small window was removed from potato carrot agar and single hyphae with chains of conidia grew into this window during fungal growth. Subsequently, the *Alternaria* species were classified based on the length of conidiophores, conidial shape, and branching type present in the window (Simmons and Roberts, 1994; Kahl et al., 2015). Another approach to segregate *Alternaria* species comprised the analysis of sequences from functional genes such as the translation elongation factor 1-α (TEF1-α). The sequence analysis allowed the differentiation of *A. alternata*, *A. tenuissima*, and *A. arborescence* from *A. infectoria* (Kahl et al., 2015). Besides, the phylogenetic analysis of DNA sequences from *Alternaria* species enabled the segregation of small-spored species from the large-spored *Alternaria* fungi. Nevertheless, the differentiation within the small-spored *Alternaria* species was not possible due to missing variation in the nuclear ribosomal internal transcribed spacer and beta-tubulin sequences (Peever et al., 2017). *Penicillium* and *Aspergillus* species were successfully segregated based on secondary metabolites (Frisvad et al., 2008). Therefore, mycotoxin profiles of *Alternaria*

species were determined (Kahl et al., 2015; Zwickel et al., 2018) and *A. infectoria* was successfully differentiated from other small-spored *Alternaria* species (Andersen et al., 2002; Zwickel et al., 2018). Furthermore, the analysis of secondary metabolites of *A. dauci*, *A. porri*, *A. solani*, and *A. tomatophila* revealed the segregation of *A. dauci*, *A. solani*, and *A. tomatophila* based on species-specific metabolite profiles (Andersen et al., 2008). Nowadays, the coupling of liquid chromatography to high resolution mass spectrometry is gaining more attention to obtain species-specific metabolic profiles. However, only a small percentage of the *Alternaria* secondary metabolites are commercially available and can be utilized for identification purposes (Andersen et al., 2015). Thus, the combination of morphological characteristics, genetics, as well as secondary metabolite profiling might be a promising procedure for the differentiation of *Alternaria* species (Kahl et al., 2015).

1.1.3 *Alternaria* growth - pathologic and economic effects

Alternaria species are distributed widely and their occurrence was confirmed in Iceland, in South Africa as well as in the southern parts of Argentina (Rotem, 1994). Exhibiting mainly saprophytic capabilities, *Alternaria* species are commonly found in the soil, on decaying organic materials (EFSA on Contaminants in the Food Chain, 2011), on decaying wood, wood pulp, and compost (Ostry, 2008). Besides, the molds colonize the surface of walls, wallpapers, textiles, and leather (Kück et al., 2009). In addition to the saprophytic capabilities, *Alternaria* fungi can act as plant pathogens and cause a range of diseases such as stem cancer, leaf blight, or leaf spot on living plants (Visconti and Sibilia, 1994; EFSA on Contaminants in the Food Chain, 2011). Usually, *Alternaria* species are only slightly invasive, but if a host plant is weakened, some *Alternaria* species shift from saprophytic to parasitic growth (Rotem, 1994). The infection of living plants with *Alternaria* fungi results in a slow destruction of parts of the plant as the photosynthetic potential is reduced (Thomma, 2003). Necrotic lesions become visible and are surrounded by a chlorotic halo caused by fungal secondary metabolites (Agarwal et al., 1997; Thomma, 2003). Furthermore, stomatal opening, CO_2 fixation, and the regulation of the chlorophyll content was reported to be negatively affected by an *Alternaria* infection in potato plants (Rotem, 1994). Commonly infected parts of annual plants are leaves, stems, flowers, ears, and fruits (Bottalico and Logrieco, 1998).

Figure 1.2: Early blight in potato caused by *A. solani.*

Infecting plants in the field, large-spored *A. solani* causes early blight on potato (**Figure 1.2**) (Sorauer, 1896) and tomato (El Gobashy et al., 2018), whereas the small-spored *A. alternata* f. sp. *lycopersici* causes stem cancer on tomato (Grogan, 1975). In addition, this species induces leaf spot, fruit rot, and fruit deformation (Malathrakis, 1983). Black mold on tomato and brown spot on potato are caused by *A. alternata* (Logrieco et al., 2009; Leiminger et al., 2015; da Cruz Cabral et al., 2016) and over 100 host plants are reported to be vulnerable to this fungal species (Rotem, 1994). Further diseases are brown spot of tobacco, caused by *A. longipes*, *Alternaria* blotch of apple *(A. mali)*, black spot of Japanese pear *(A. gaisen)* (Scheffer, 1992; Woudenberg et al., 2015), and stem cancer on tomato *(A. arborescence)* (da Cruz Cabral et al., 2016). **Table 1.1** displays the most important *Alternaria* species, the related plant diseases, and the mycotoxins biosynthesized by the respective species. Apart from the mentioned diseases, frequently infected agricultural plants are barley, oats, sorghum, corn, and peanuts (Pero et al., 1973), as well as olives, citrus fruits, and carrots (Logrieco et al., 2009). Fruit imperfection, over ripeness, cold stress, and surface damage support infections by *Alternaria* fungi (Visconti and Sibilia, 1994).

Synthetic antifungals are frequently applied to prevent the plants from infection with plant pathogenic molds. Leiminger and Hausladen (2011) utilized fungicides belonging to strobilurines to control early blight of potato plants. The application of these fungicides resulted in prolonged vitality of the plants and an increase in potato yield (Leiminger and Hausladen, 2011). Unfortunately, some fungi developed resistance to the utilized fungicides and the concentrations of the fungicides need to be increased. Therefore, an increase in the content of residues of the fungicides in food products is observed. To circumvent high concentrations of remaining synthetic residues in foods, plant-derived extracts

exhibiting antifungal activity are increasingly applied. These extracts contain either alkaloids, flavonoids, isoflavonoids, tannins, cumarins, glycosides, or organic acids with antimicrobial activity (da Cruz Cabral et al., 2013).

Apart from the immediate harvest losses in the field, *Alternaria* fungi grow at low temperatures and, thus, are responsible for post-harvest decay during refrigerated transport and storage (Visconti and Sibilia, 1994; Bottalico and Logrieco, 1998).

The fungi not only spoil plants in the field and deteriorate agricultural products during storage but are also considered as emerging human pathogens. Especially immunocompromised patients suffer from diseases caused by *Alternaria* species (Rossmann et al., 1996; Lawrence et al., 2013) including hypersensitivity pneumonitis, bronchial asthma, and skin infections (Pastor and Guarro, 2008). Besides, the spores of *Alternaria* fungi are very potent allergens (Peat et al., 1993; Thomma, 2003; Lawrence et al., 2013) and might cause asthma (Cantani and Ciaschi, 2004).

Table 1.1: Overview of the most important *Alternaria* species, the main diseases caused by the respective species, as well as the mycotoxins biosynthesized by the species groups.

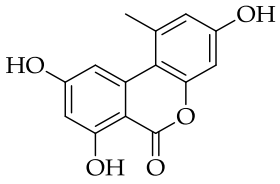
***Alternaria* species**	**Diseases**	**Mycotoxin production**	**References**
A. alternata	black mold on tomato	AOH, AME, TEN, ATX I-III, STTX III, ALT, altenuisol, altenusin, TA	(Montemurro and Visconti, 1992; Bottalico and Logrieco, 1998; Thomma, 2003; Logrieco et al., 2009; da Cruz Cabral et al., 2016)
A. alternata f. sp. lycopersici	stem cancer on tomato	AAL-toxins	(Grogan, 1975; Montemurro and Visconti, 1992; Thomma, 2003)
A. brassicae	infests many vegetables and roses	AOH, AME, TA	(Montemurro and Visconti, 1992; Bottalico and Logrieco, 1998; Thomma, 2003; Logrieco et al., 2009)
A. brassicicola	grows on whole crops	AOH, AME, TA	(Bottalico and Logrieco, 1998; Logrieco et al., 2009)
A. dauci	grows on carrots	AOH, AME	(Montemurro and Visconti, 1992; Bottalico and Logrieco, 1998; Logrieco et al., 2009)
A. infectoria	infests wheat	AOH, AME, TA	(Bottalico and Logrieco, 1998; Logrieco et al., 2009)
A. solani	early blight on potato and tomato	AOH, AME, ALT	(Sorauer, 1896; Montemurro and Visconti, 1992; Bottalico and Logrieco, 1998; El Gobashy et al., 2018)
A. tenuissima	infects tomatoes	AOH, AME, ATX I, ATX III, ALT, TA	(Montemurro and Visconti, 1992, 1992; Bottalico and Logrieco, 1998; Ostry, 2008; Logrieco et al., 2009)

1.2 *Alternaria* mycotoxins

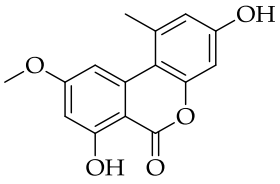
1.2.1 *Alternaria* mycotoxins – classification

During growth on leaves, stems, flowers, and fruits, *Alternaria* species biosynthesize a variety of secondary metabolites that are not essential for fungal survival (Kück et al., 2009). Over the years, up to 70 secondary metabolites produced by various *Alternaria* species have been described in the literature and some of these were further characterized to act as phytotoxins or mycotoxins (EFSA on Contaminants in the Food Chain, 2011; Arcella et al., 2016). The phytotoxins biosynthesized by *Alternaria* fungi are categorized into host-specific and non-host-specific toxins depending on the adverse effects against plants (Thomma, 2003). Host-specific phytotoxins include AAL-toxins, which are detected only on certain plant species and contribute to plant pathogenicity (Thomma, 2003; Yamagishi et al., 2006; EFSA on Contaminants in the Food Chain, 2011). Contrarily, other *Alternaria* toxins such as dibenzo-α-pyrones, tenuazonic acid (TA), as well as perylene quinones are non-host-specific toxins detectable on a wide range of plants and plant materials. Apart from affecting specific metabolism commonly present in plants (Ballio, 1991), mycotoxins cause adverse effects in humans and animals (EFSA on Contaminants in the Food Chain, 2011). Secondary metabolites biosynthesized by *Alternaria* fungi are generally categorized into five structural classes. Group 1, the dibenzo-α-pyrones include alternariol (AOH), alternariol monomethyl ether (AME), and altenuene (ALT), whereas TA belongs to group 2, the tetramic acid derivatives. Group 3, the perylene quinones comprise altertoxin I (ATX I), altertoxin II (ATX II), altertoxin III (ATX III), and stemphyltoxin III (STTX III). Tentoxin (TEN) belongs to the miscellaneous structures (group 4) and toxins solely produced by *Alternaria alternata* f. sp. *lycopersici* are allocated to group 5, the amino pentol esters (EFSA on Contaminants in the Food Chain, 2011; Lorenz et al., 2012). Representatives of the five structural classes are depicted in **Figure 1.3**. Moreover, the chemical names, the molecular formulae, and the molecular weights of the commonly analyzed *Alternaria* mycotoxins are listed in **Table 1.2**. Further chemical and biological data were compiled and depicted by Muntemurro and Visconti (1992).

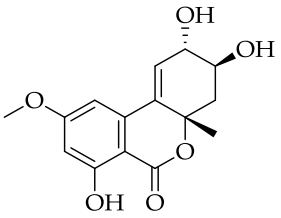
Dibenzo-a-pyrones



alternariol
(AOH)

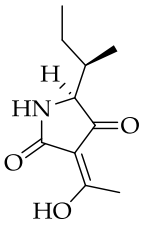


alternariol
monomethylether
(AME)



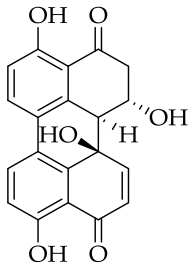
altenuene
(ALT)

Tetramic acid

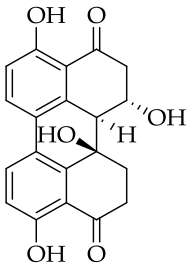


tenuazonic
acid (TA)

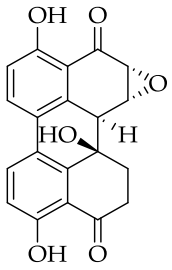
Perylene quinones



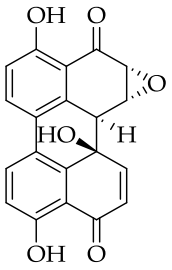
alterperylenol
(ALTP)



altertoxin I
(ATX I)

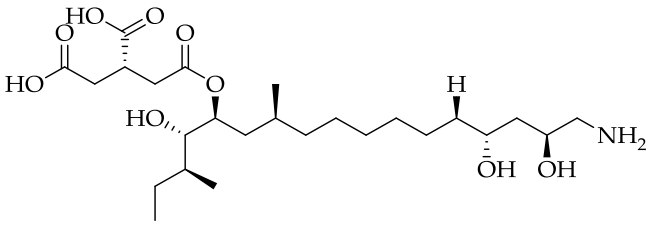


altertoxin II
(ATX II)



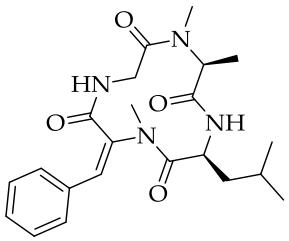
stemphyltoxin III
(STTX III)

AAL toxins



A. alternata f. sp. *lycopersici* toxin TB_1
(AAL TB_1)

Miscellaneous structures



tentoxin
(TEN)

Figure 1.3: Overview of the five structural classes of *Alternaria* mycotoxins. For each structural class, the chemical structure of at least one representative is shown.

Table 1.2: Structural class, abbreviation, chemical name, molecular formula, and molecular weight of the *Alternaria* mycotoxins commonly analyzed.

Chemical class	Toxin	Systematic chemical name	Molecular formula	Molecular weight [g/mol]	References
Benzopyrones	AOH	3,7,9-Trihydroxy-1-methyl-6*H*-dibenzo[*b*,*d*]pyran-6-one	$C_{14}H_{10}O_5$	258.053	(EFSA on Contaminants in the Food Chain, 2011; Escrivá et al., 2017)
	AME	3,7-Dihydroxy-9-methoxy-1-methyl-6H-dibenzo[b,d]pyran-6-one	$C_{15}H_{12}O_5$	272.068	(An et al., 1989; Escrivá et al., 2017)
	ALT	(2*R*,3*R*,4a*R)*-rel-2,3,4,4a-Tetrahydro-2,3,7-trihydroxy-9-methoxy-4a-methyl-6*H*-dibenzo[*b*,*d*]pyran-6-one	$C_{15}H_{16}O_6$	292.095	(EFSA on Contaminants in the Food Chain, 2011)
Perylene quinones	ALTP	(1*S*,12*aR*,12*bS*)-1,4,9,12*a*-tetrahydroxy-2,12*b*-dihydro-1*H*-perylene-3,10-dione	$C_{20}H_{14}O_6$	350.079	(Escrivá et al., 2017)
	ATX I	(1*S*,12a*R*,12b*S*)-1,2,11,12,12a,12b-Hexahydro-1,4,9,12a-tetrahydroxy-3,10-perylenedione	$C_{20}H_{16}O_6$	352.095	(EFSA on Contaminants in the Food Chain, 2011; Escrivá et al., 2017)
	ATX II	(7a*R*,8a*R*,8b*S*,8c*R*)-7a,8a,8b,8c,9,10-Hexahydro-1,6,8c-trihydroxy-perylo[1,2-b]oxirene-7,11-dione	$C_{20}H_{14}O_6$	350.079	(EFSA on Contaminants in the Food Chain, 2011; Escrivá et al., 2017)
	STTX III	(7a*R*,8a*R*,8b*S*,8c*R*)-7a,8a,8b,8c-Tetrahydro-1,6,8c-trihydroxy-peryleno[1,2-*b*]oxirene-7,11-dione	$C_{20}H_{12}O_6$	346.048	(Stack and Mazzola, 1989; Escrivá et al., 2017)
Cyclic tetrapeptides	TEN	Cyclo[*N*-methyl-L-alanyl-L-leucyl-(α*Z*)-α,β-didehydro-*N*-methylphenylalanylglycyl]	$C_{22}H_{30}N_4O_4$	414.227	(Meyer et al., 1971; Escrivá et al., 2017)
Tetramic acids	TA	(5*S*)-3-Acetyl-1,5-dihydro-4-hydroxy-5-[(1*S*)-1-methylpropyl]-2*H*-pyrrol-2-one	$C_{10}H_{15}NO_3$	197.105	(Rosett et al., 1957; Gatenbeck and Sierankiewicz, 1973a)

1.2.2 *Alternaria* mycotoxins – modified forms

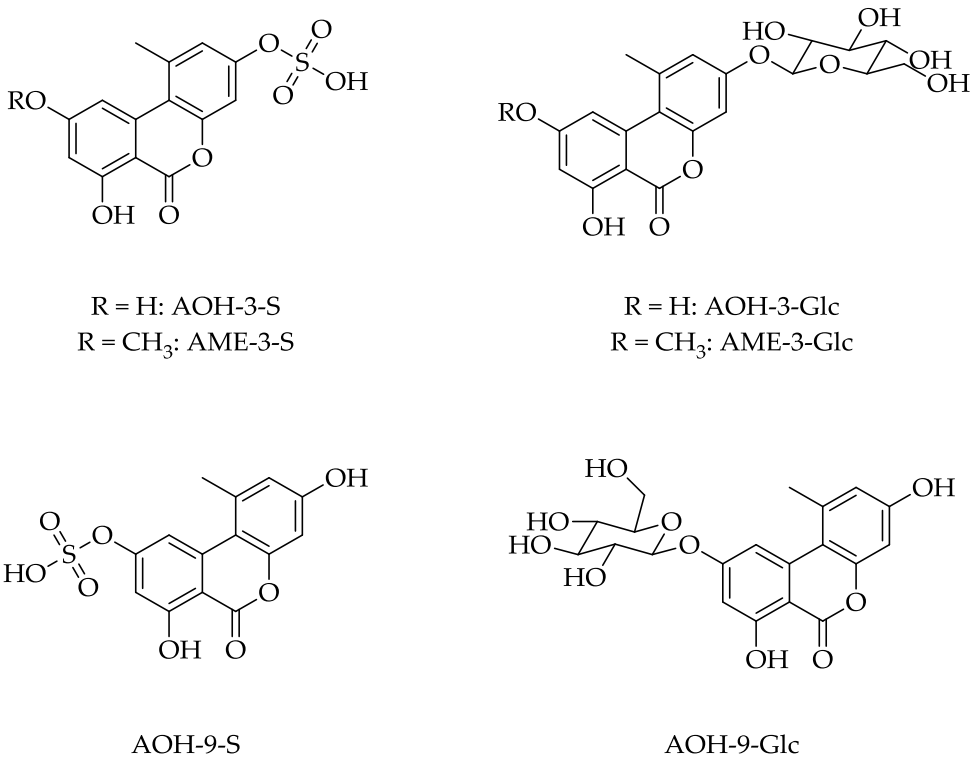
The term "modified mycotoxin" refers roughly to mycotoxins connected to more polar compounds such as glucose, sulfate groups, or glutathione (Rychlik et al., 2014; Righetti et al., 2016). Originally, these conjugated mycotoxins were named "masked mycotoxins" as they escaped routine analysis due to different polarity compared to the free forms. Besides, no reference compounds were available for these modified analytes (Gareis et al., 1990). The modified mycotoxins attracted first attention in the mid-1980s when clinical studies were conducted on animals. Although the contamination level with the free forms of mycotoxins in the feed was low, adverse effects in the animals were observed after feeding. "Masked mycotoxins" were blamed to be responsible for these negative effects as the conjugation might be hydrolyzed in the stomach due to present acid, in the gut by enzymes, or in the intestine by bacteria (Berthiller et al., 2005; Berthiller et al., 2007). The cleavage of masked mycotoxins and the release of the aglycon during digestion was confirmed for zearalenone-4-β-glucopyranoside in pigs (Gareis et al., 1990). Due to the inconsistent usage of the term "masked mycotoxin" in the literature, Rychlik et al. (2014) proposed a comprehensive definition of modified mycotoxins by differentiating between free mycotoxins, matrix-associated mycotoxins, and structurally modified ones. The latter should be divided into "biologically modified" and "chemically modified" with the chemical modifications also differentiating between "thermally formed" and "non-thermally formed" mycotoxins. Moreover, Rychlik et al. (2014) proposed to use the term "masked mycotoxins", if ever, only for conjugates formed by plants (Berthiller et al., 2013; Rychlik et al., 2014).

As mycotoxins constitute xenobiotics to plants, humans, and animals, these compounds are usually detoxified following three different phases. In phase I also termed transformation phase (Berthiller et al., 2007), lipophilic xenobiotics are chemically modified by esterases, amidases, as well as cytochrome P450 dependent monooxygenases. After hydrolysis, oxidation, or other chemical modifications, the activated compounds frequently exhibit the same toxicity as the parent substances. Hydrophilic compounds are usually not affected by phase I metabolism (Coleman et al., 1997; Berthiller et al., 2013). Subsequently, the activated xenobiotics are detoxified in phase II metabolism also termed conjugation phase (Berthiller et al., 2007). The covalent linkage of xenobiotics to hydrophilic compounds such as glucose, malonic acid, and glutathione leads to modified forms exhibiting less toxicity than the parent mycotoxin mainly due to the loss of electrophilic capabilities (Wolf et al., 1996; Coleman et al., 1997; Berthiller et al., 2007; Berthiller et al., 2013). Hydrophilic mycotoxins might directly be conjugated during phase II metabolism. Finally, the modified xenobiotics are exported from the cytosol during phase III metabolism also termed compartmentation phase.

The detoxified mycotoxins are either attached to biopolymers or stored in the vacuole or apoplast of plants, permanently, or excreted by humans or animals (Wolf et al., 1996; Coleman et al., 1997; Berthiller et al., 2013; Rychlik et al., 2014).

Tobacco BY-2 cells are widely accepted as an *in vitro* model for the metabolization of xenobiotics during the plant cell metabolism. Applying AOH and AME to suspension cultures of tobacco BY-2 cells, β-D-glucopyranosides (attached to the 3- or 9- position) as well as their 6'-malonyl derivatives were detected for AOH. Besides, the D-glucopyranoside of AME and its 6'- and 4'-malonyl derivatives were determined (Hildebrand et al., 2015). The subsequent malonylation of glycosylated mycotoxins is considered to be a signal for the transport of detoxified xenobiotics into vacuoles, whereas further glycosylation is supposed to enable incorporation into the cell wall (Sandermann, 1994).

Modifications of dibenzo-α-pyrones with glucose (Glc) and sulfate (S) were described in the literature. *Alternaria* fungi were reported to covalently link sulfate to AOH and AME leading to the formation of alternariol-3-sulfate (AOH-3-S), alternariol-9-sulfate (AOH-9-S), and alternariol monomethyl ether-3-sulfate (AME-3-S). Contrarily, the glucosides of AOH and AME, namely alternariol-3-glucoside (AOH-3-Glc), alternariol-9-glucoside (AOH-9-Glc), and alternariol monomethyl ether-3-glucoside (AME-3-Glc), were formed by plants (Soukup et al., 2016). **Figure 1.4** displays the chemical structure of the modified forms of AOH and AME. Only recently, the modified forms of these dibenzo-α-pyrones were included in multi-mycotoxin methods (Walravens et al., 2014; Walravens et al., 2016; Puntscher et al., 2018). However, as reference standards for the modified mycotoxins are currently not available, the standards are either chemically synthesized (Berthiller et al., 2005; Mikula et al., 2013; Walravens et al., 2014) or isolated from contaminated media and, subsequently, characterized by nuclear magnetic resonance (NMR) spectroscopy (Berthiller et al., 2005).



R = H: AOH-3-S
R = CH_3: AME-3-S

R = H: AOH-3-Glc
R = CH_3: AME-3-Glc

AOH-9-S

AOH-9-Glc

Figure 1.4: Chemical structures of the modified forms of AOH and AME.

1.2.3 *Alternaria* mycotoxins – biosynthetic pathways

Despite the great variety in the chemical structure of fungal secondary metabolites, only a small number of molecules serve as precursors for secondary metabolite production. Depending on the precursor from the primary metabolism and the enzymes involved in the biosynthesis, the secondary metabolites are divided into structural classes such as polyketides, fatty acid derivatives, non-ribosomal peptides, isoprenoids, and alkaloids (Keller et al., 2005; Kück et al., 2009). The dibenzo-α-pyrones belong to the structural class of polyketides and are biosynthesized via the polyketide pathway. Acetyl coenzyme A as well as malonyl coenzyme A originate from the primary metabolism and represent precursors of all polyketides. Acetyl-CoA derives from pyruvate formed during glycolysis, whereas malonyl-CoA is biosynthesized in the cytosol from acetyl-CoA by carboxylation (Gatenbeck et al., 1965; Stinson, 1985). For the formation of fungal polyketides, type I polyketide synthases similar to fatty acid synthases are required (Keller et al., 2005). The enzyme-bound polyketide chain is established by adding six malonyl-CoA to acetyl-CoA via Claisen type condensations and concurrent loss of carbonate (Saha et al., 2012). Subsequently, the poly-β-keto intermediate is further transformed to AOH by aldol type cyclization, aromatization, and lactonization (Saha et al., 2012). Afterwards, alternariol-*O*-methyltransferase catalyzes

the biosynthesis of AME from AOH by methylation utilizing *S*-adenosylmethionine (SAM) as methyl donor (Hiltunen and Söderhäll, 1992). Other dibenzo-α-pyrones are considered to originate from AME after further biotransformation (Stinson, 1985). Altenusin might emerge from AME after a reductive cleavage of the molecule. The subsequent oxidative cleavage of the catechol moiety of altenusin might lead to the formation of altenuic acid IV possibly acting as a precursor of altenuic acids I-III. However, the biosynthetic route of the altenuic acids I-III has never been studied in detail (Kohler and Podlech, 2019). Apart from the formation of altenuic acids, dehydroaltenusin originates from altenusin via oxidation and further reduction of dehydroaltenusin leads to altenuene (Thomas, 1961; Stinson, 1985). The hypothetical biosynthetic route of dibenzo-α-pyrones is displayed in **Figure 1.5**.

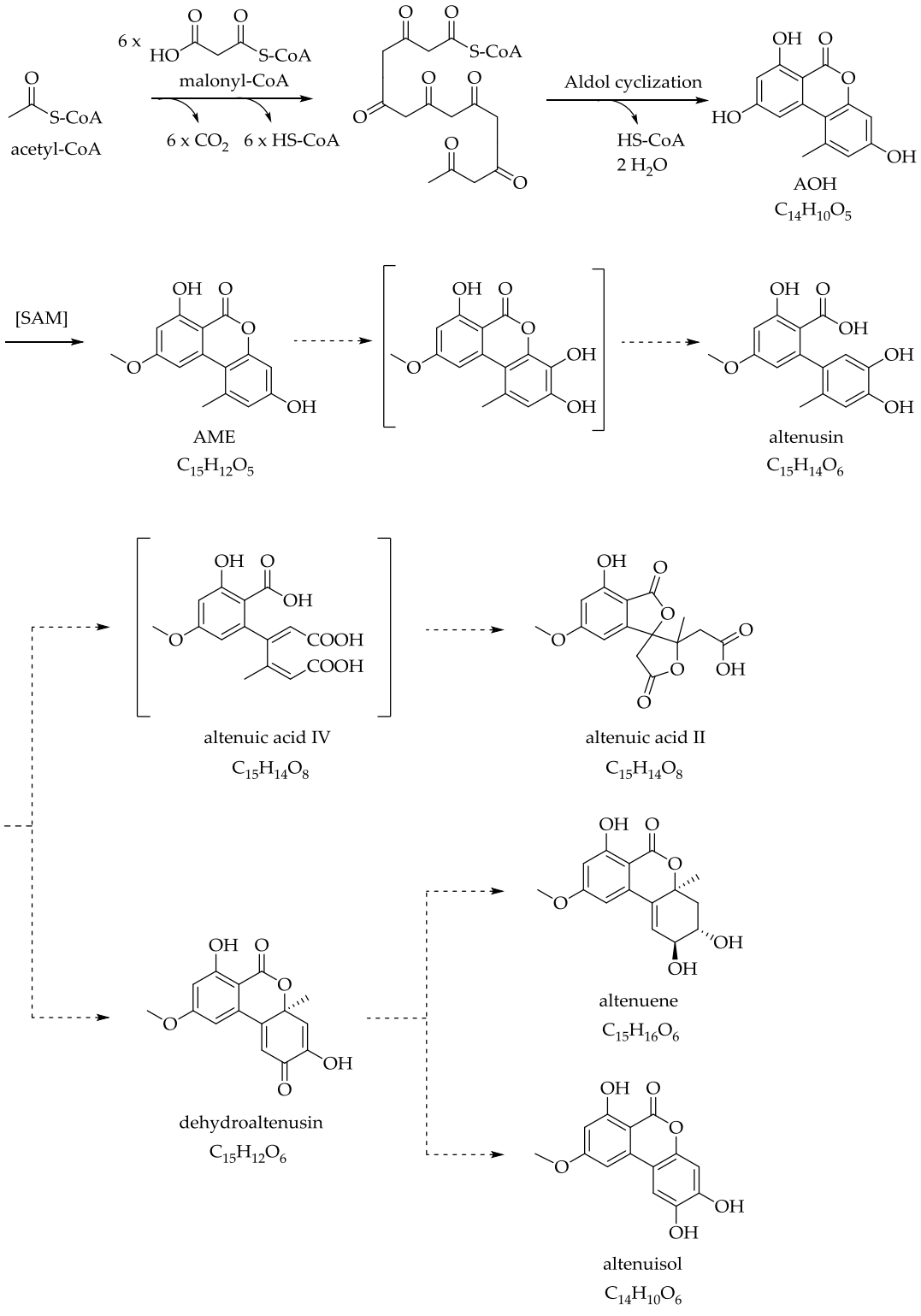


Figure 1.5: Biosynthetic route of dibenzo-α-pyrones. The biosynthetic formation of AME via AOH has been established, but the following biosynthetic route is hypothetical (characterized by dashed arrows) (Harris and Hay, 1977; Stinson, 1985; Saha et al., 2012; Kohler and Podlech, 2019).

TEN belongs to the group of cyclopeptides and four different amino acids are incorporated into the molecule. The biosynthesis of TEN starts with the activation of the amino acids by ATP and their subsequent binding to an enzyme belonging to the synthetases probably also exhibiting an integrated activity of methyltransferase. The amino acids are either methylated when bound to the enzyme or during elongation of the growing peptide chain. Originating from glycine, alanine/methylalanine, phenylalanine/methylphenylalanine, and leucine are consecutively added. Another biosynthetic pathway suggests the linkage of two dipeptides, namely glycine-alanine/methylalanine and phenylalanine/methylphenylalanine-leucine. The enzymatic coupling of the four amino acids leads to the formation of dihydrotentoxin, probably released from the enzyme by cyclization. Finally, TEN is biosynthesized from dihydrotentoxin by dehydrogenation (Ramm et al., 1994).

TA is biosynthesized from one molecule of L-isoleucine and two molecules of acetic acid. The incorporation of two molecules of acetic acid into TA was confirmed by performing ^{14}C labeling experiments (Stickings and Townsend, 1961; Holzapfel, 1980; Stinson, 1985). In the initial step of the biosynthesis, L-isoleucine is transformed via N-acetoacetylation to N-acetoacetyl-L-isoleucine. Subsequently, the five-membered ring of TA is generated (Gatenbeck and Sierankiewicz, 1973b). The enzyme involved in the biosynthesis of TA was identified as TA synthetase 1 (TAS1) (Yun et al., 2015) and probably no further oxidation or reduction steps take place during the formation of TA (Stickings and Townsend, 1961). Gatenbeck et al. (1973a) proved the incorporation of L-valine and L-leucine into the tetramic acid molecule resulting in derivatives of TA. The ^{14}C labeled amino acids L-valine and L-leucine were added to a culture medium of *Alternaria tenuis* and the radioactive labeled tetramic acid derivatives were isolated. Contrarily, L-phenylalanine was not utilized for the biosynthesis of a tetramic acid derivative (Gatenbeck and Sierankiewicz, 1973a).

Only little is known about the biosynthesis of the perylene quinones. Early investigations already excluded the formation of perylene quinones from one long "polyketomethylene" (Chen et al., 1966) and proposed a biosynthetic route via an oxidative coupling of two identical octalones (Chen et al., 1966; Okuno et al., 1983). Similar to the dibenzo-α-pyrones, the perylene quinones originate from acetate and malonate (Kimura and Tsuge, 1993; Bode and Zeeck, 2000) and the incorporation of $^{13}C_2$ labeled acetate into ALTP was confirmed by Okuno et al. (1983). The fungal polyketide synthase catalyzes the biosynthesis of a poly-β-keto intermediate, which is transformed into 1,3,6,8-tetrahydroxynaphthalene. The latter is further modified by reduction reactions and elimination of water resulting in the formation of scytalone, 1,3,8-trihydroxynaphthalene, vermelone, and finally 1,8-dihydroxynaphthalene (Kimura and Tsuge, 1993; Bode and Zeeck, 2000). After the oxidative coupling of two identical molecules of 1,8-dihydroxynaphthalene (Scott, 1965; Okubo et al., 1975; Okuno et al., 1983), the dimerization product is oxidized to perylene quinone (Allport and Bu'Lock,

1960; Chagas et al., 2016). The proposed biosynthetic route of perylene quinone is presented in **Figure 1.6**. Originating from perylene quinone, various perylene quinone derivatives are biosynthesized (Chagas et al., 2016). **Figure 1.7** shows an overview of the structural diversity of the perylene quinone derivatives.

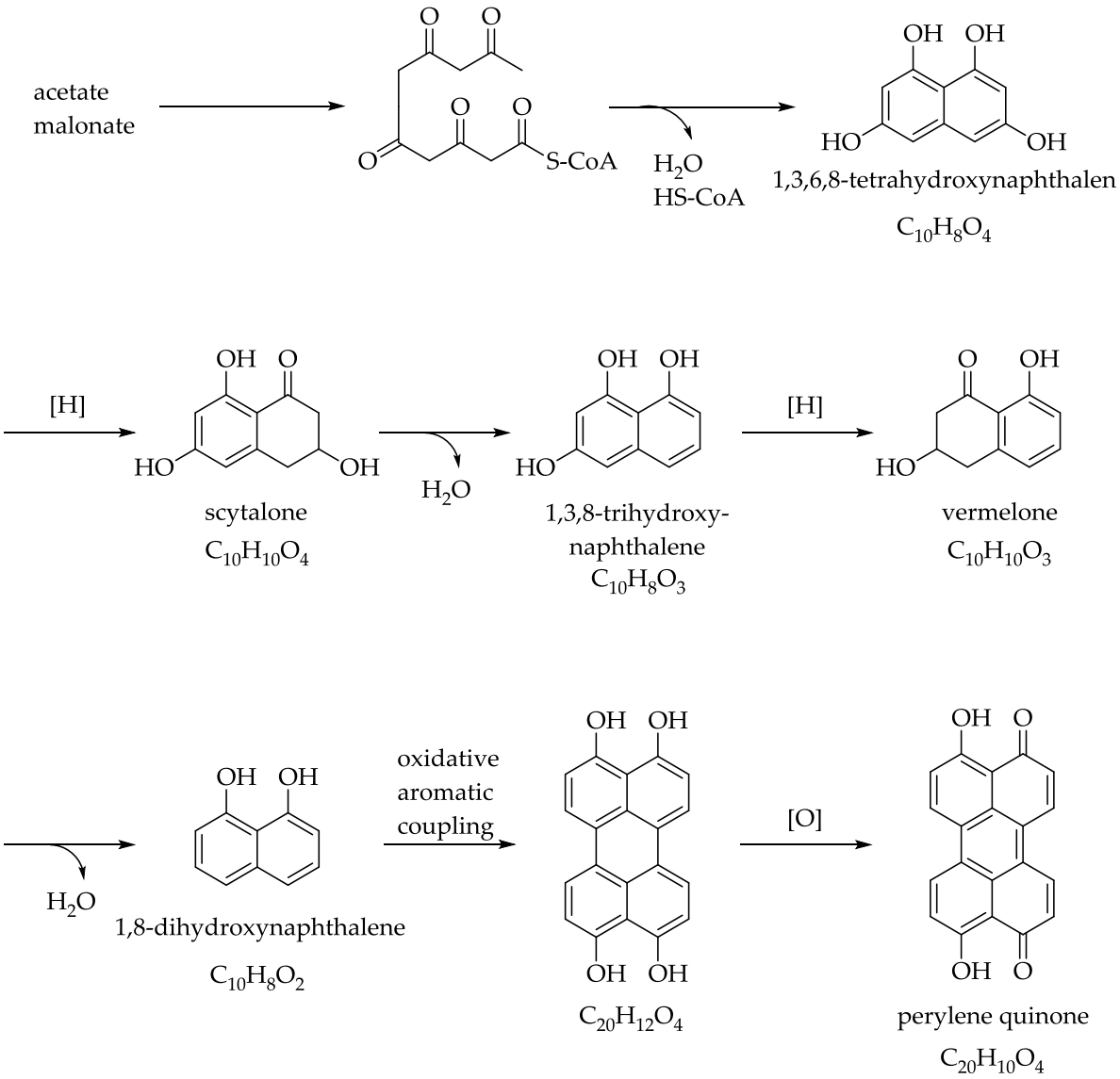
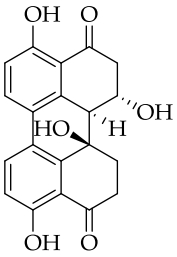
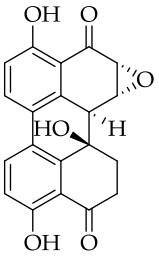


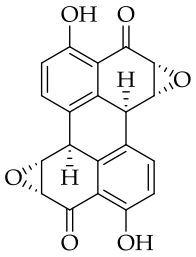
Figure 1.6: Proposed biosynthetic pathway of perylene quinone originating from acetate (Allport and Bu'Lock, 1960; Scott, 1965; Okubo et al., 1975; Kimura and Tsuge, 1993; Bode and Zeeck, 2000; Chagas et al., 2016). [H] represents reduction reactions and [O] equals oxidation reactions.



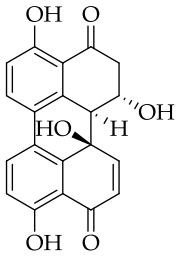
altertoxin I
(Chu 1981;
Podlech et al., 2014)
$C_{20}H_{16}O_6$



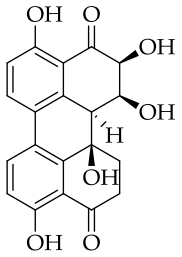
altertoxin II
(Chu 1981;
Podlech et al., 2014)
$C_{20}H_{14}O_6$



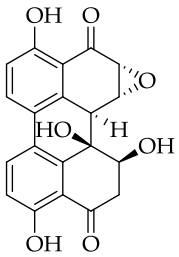
altertoxin III
(Podlech et al., 2014)
$C_{20}H_{12}O_6$



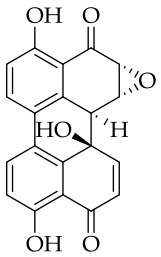
alterperylenol
(Okuno et al., 1983;
Podlech et al., 2014)
$C_{20}H_{14}O_6$



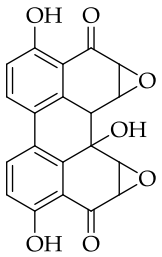
7-*epi*-8-hydroxy-
altertoxin I
(Podlech et al.,
2014)
$C_{20}H_{16}O_7$



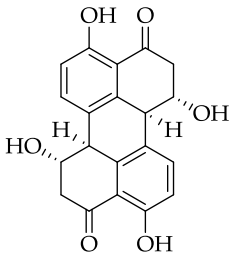
stemphyltoxin I
(Podlech et al., 2014)
$C_{20}H_{14}O_7$



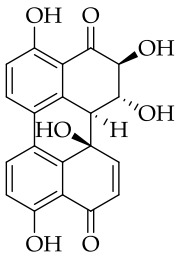
stemphyltoxin III
(Podlech et al., 2014)
$C_{20}H_{12}O_6$



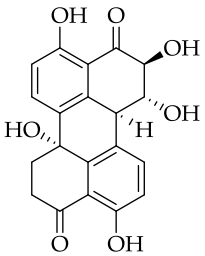
stemphyltoxin IV
(Podlech et al., 2014)
$C_{20}H_{12}O_7$



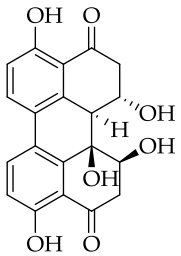
stemphyperylenol
(Podlech et al., 2014)
$C_{20}H_{16}O_6$



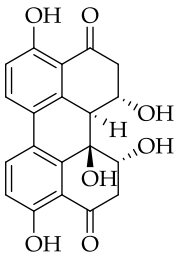
alterlosin I
(Stierle et al., 1989;
Podlech et al., 2014)
$C_{20}H_{14}O_7$



alterlosin II
(Stierle et al., 1989;
Podlech et al., 2014)
$C_{20}H_{16}O_7$



stemphytriol
(Podlech et al., 2014)
$C_{20}H_{16}O_7$



6-*epi*-stemphytriol
(Podlech et al.,
2014)
$C_{20}H_{16}O_7$

Figure 1.7: Structural diversity of perylene quinone derivatives (Chu, 1981; Okuno et al., 1983; Stierle et al., 1989; Podlech et al., 2014). The stereochemistry of stemphyltoxin IV is unknown (Achenbach et al., 1987).

1.2.4 *Alternaria* mycotoxins – toxic effects

Alternaria secondary metabolites not only act as phytotoxins against a variety of plants but also exhibit adverse effects against animals and humans (EFSA on Contaminants in the Food Chain, 2011). However, the toxic effects of *Alternaria* mycotoxins are less frequently investigated than those of other secondary metabolites biosynthesized by the fungal genera *Aspergillus*, *Fusarium*, or *Penicillium* (Logrieco et al., 2009). One toxicological consequence associated with *Alternaria* mycotoxins was the higher incidence of oesophageal cancer observed in the Chinese Linxian province after the consumption of agricultural commodities contaminated with *Alternaria* mycotoxins (Liu et al., 1992; Solhaug et al., 2016). Moreover, the toxicity of extracts of *Alternaria* fungi was investigated and genotoxic as well as mutagenic effects were described *in vitro*. Furthermore, carcinogenic effects were observed after the application of *Alternaria* extracts to experimental animals (Lehmann et al., 2006). Adverse impacts also appeared in poultry, horses, brine shrimps, chick embryos, mice, and rats (Bottalico and Logrieco, 1998).

Although the acute toxicity of AOH, AME, and ALT for mice is low (Pero et al., 1973; Pollock et al., 1982b), various adverse effects are described in the literature for these mycotoxins. A synergistic effect in fetotoxicity in mice was discovered for AOH and AME when administering 25 mg of AOH and AME per kg b.w. to the mice, respectively (Pero et al., 1973; Woody and Chu, 1992; Bottalico and Logrieco, 1998). Further studies on the toxicity of the dibenzo-α-pyrones revealed significantly increasing DNA strand breaking effects of AOH and AME in human carcinoma cells (Fehr et al., 2009). Nevertheless, glucuronidation of AOH and AME eliminated these DNA strand breaking effects (Pfeiffer et al., 2007a). AOH acted as topoisomerase poison against the DNA topoisomerases I and II, with a special selectivity against the IIα isoform by creating DNA topoisomerase intermediates. Although the adverse effect of AME against the catalytic activity of topoisomerase I was much lower compared to AOH, AME revealed equal inhibition to topoisomerase IIα as AOH (Ostry, 2008; Fehr et al., 2009). Additionally, AOH exhibited mutagenic effects in the Ames test (Solhaug et al., 2016) and Brugger et al. (2006) confirmed the mutagenicity of AOH in cultured mammalian cells. In a survey on the estrogenicity of AOH, Lehmann et al. (2006) first demonstrated the estrogenic potential of AOH *in vitro*, but with AOH exhibiting an estrogenicity of only 0.01% of the potential of 17β-estradiol (Lehmann et al., 2006). Investigations on the xenoestrogenic potential of AOH and AME as well as on their modified forms revealed that methylation might increase estrogenicity of hydroxylated benzopyrones *in vitro*. Contrarily, hydroxylation of AOH and AME decreased the estrogenic potential *in vitro* (Dellafiora et al., 2018).

Apart from the toxic effects determined *in vitro*, AOH is also biosynthesized by *Alternaria* fungi to successfully colonize on tomatoes. *Alternaria* species incapable of biosynthesizing AOH were not able to colonize on tomatoes (Graf et al., 2012). Besides, the ability of *Alternaria* fungi to colonize on tomato was eliminated if the biosynthesis of AOH was inhibited by chlorogenic acid produced by tomatoes (Wojciechowska et al., 2014).

Another frequently occurring *Alternaria* mycotoxin is the tetramic acid derivative TA exhibiting the highest acute toxicity among the *Alternaria* mycotoxins (Pero et al., 1973; Bottalico and Logrieco, 1998). The oral LD_{50} to mice was 81 mg/kg for females and 186 mg/kg for males (Pero et al., 1973). Lethal dosing of TA resulted in hemorrhages in one or more organs (Smith et al., 1968) or caused diarrhea and moribund state with subsequent death after two days after administration (Woody and Chu, 1992). Human consumption of TA was related to onyalai, a hematological disorder mainly occurring amongst the Black African population (Steyn and Rabie, 1976). Apart from antiviral effects against enteroviruses, respiratory viruses, and herpes simplex HF (Miller et al., 1963), antitumor, antibacterial, and cytotoxic effects were reported for TA (Gitterman, 1965). By suppressing the release of new proteins from ribosomes, TA also inhibits protein biosynthesis (Shigeura and Gordon, 1963).

TA is also considered to act as a phytotoxin (Bottalico and Logrieco, 1998) and function as a virulence factor for the colonization of *Alternaria* species on various plants (Kang et al., 2017). Beside inhibiting photosynthesis by blocking the photosystem II electron transport (Chen et al., 2007), TA reduced the protein and chlorophyll content in leaves of *Daturia innoxia* and caused chlorosis and necrosis on the latter (Janardhanan and Husain, 1984). In soybeans, the biosynthesis of proteins and nucleic acids was inhibited (Umetsu et al., 1974; Bottalico and Logrieco, 1998).

Regarding the perylene quinones, mainly mutagenic capabilities of ATX I, ATX II, and ATX III have been described in the literature. Mutagenic effects of the perylene quinones were confirmed in the *Salmonella* Ames Test (Scott and Stoltz, 1980; Stack et al., 1986), which showed that the mutagenicity against *Salmonella typhimurium* increased from ATX I to ATX II, and subsequently ATX III (Stack and Prival, 1986; Bottalico and Logrieco, 1998). The higher mutagenicity of ATX III compared to ATX I was explained by an increase in the number of epoxide groups (Fleck et al., 2012). STTX III also comprises an epoxide group (Fleck et al., 2012) and exhibited mutagenic effects against *Salmonella typhimurium* TA98 and TA1537 (Davis and Stack, 1991). The high mutagenicity of ATX II against mammalian cells *in vitro* was first demonstrated by Fleck et al. (2012) and exceeded the mutagenicity of AOH and AME by a factor of 50 (Fleck et al., 2012). As the chemical structure exhibits an epoxide group, ATX II might directly form covalent adducts with DNA without metabolic

activation (Fleck et al., 2012). The altertoxins and especially ATX II inhibited the topoisomerase I and II resulting in DNA strand breaks (Lorenz et al., 2012). In addition to mutagenicity, strong genotoxic effects in mammalian and human cells were reported for ATX II (Fleck et al., 2012; Schwarz et al., 2012), and high cytotoxicity against HeLa cells was observed for ATX I and ATX II (Bottalico and Logrieco, 1998). The lethal dose of ATX I and ATX II to mice was 200 mg/kg b.w. with toxic effects reported to be inactivity, hemorrhages, and blood in the cerebral ventricles (Pero et al., 1973).

TEN is a phytotoxin inhibiting the cyclic photophosphorylation in chloroplasts (Arntzen, 1972). Due to specific binding to the F_1 part of the chloroplast ATP-synthase (Delaforge et al., 1997), the synthesis of ATP from ADP is blocked by the phytotoxin (Steele et al., 1978; Thomma, 2003). Studies on the recognition and metabolism of TEN by rat- and yeast-expressed P-450 3A enzymes *in vitro* resulted in the N-demethylation of TEN accompanied by the interconversion of TEN to isoTEN (Delaforge et al., 1997). So far, no data on the toxicity of TEN against mammals has been available (Liu and Rychlik, 2013; Asam and Rychlik, 2015).

1.2.5 *Alternaria* mycotoxins – absorption, distribution, and metabolism

Apart from *in vitro* investigations, some studies focused on the *in vivo* metabolism of *Alternaria* mycotoxins. Early surveys on the metabolism of *Alternaria* mycotoxins used ^{14}C-labeling to determine absorption and excretion rates. As most of the ^{14}C-labeling was detected in the feces after oral application of ^{14}C-labeled AME to rats, only a small percentage of AME appeared to be absorbed via the gastrointestinal tract. The absorbed AME was extensively metabolized and either excreted as very polar compounds via urine or exhaled as CO_2 (Pollock et al., 1982a; Woody and Chu, 1992). High excretion rates of AOH via feces were also determined after an oral application of ^{14}C-labeled AOH to mice. 5-11% of the applied AOH was excreted via urine. Apart from AOH, very low amounts of 2-hydroxy-AOH, 4-hydroxy-AOH, 8-hydroxy-AOH, and 10-hydroxy-AOH were determined in the urine of mice. The blood also contained very low amounts of AOH, 2-hydroxy-AOH, 4-hydroxy-AOH, and 10-hydroxy-AOH (Schuchardt et al., 2014). In a recently published survey, Puntscher et al. (2019) investigated the *in vivo* metabolism of a mixture of *Alternaria* mycotoxins. An *Alternaria* extract comprising a multitude of mycotoxins was applied to rats, orally. Subsequently, the feces as well as the urine were analyzed for *Alternaria* mycotoxins. 9% of the AOH dosage was excreted via feces and 6–10% was excreted via urine. Contrarily, AME was found in high concentrations in the feces with an excretion rate of 87%. AME was only partially absorbed, and 23% of the absorbed AME was determined as AME-3-S in urine. Contrarily, only low amounts of ATX I and ALTP were determined in

the urine (0.5 and 0.2%) and in the feces (9 and 3%). ATX II and STTX III were neither detected in the urine nor in the feces. TA was highly excreted via urine with percentages of up to 55% (Puntscher et al., 2019). A first study focusing on the excretion rates of TA in humans was conducted by Asam et al. (2013a). Tomato juice and sorghum-based infant cereals naturally contaminated with TA were ingested by two volunteers and the excretion rates of TA were determined. Six hours after the oral consumption, 54–81% of TA were determined in urine and after 24 h, the excretion rate ranged from 87–93%. However, it remained unclear whether the remaining 10% of TA were metabolized, bound, or excreted after the 24 h covered by the study (Asam et al., 2013a).

To simulate *in vivo* intestinal absorption and metabolism of AOH and AME in human intestinal epithelial cells, differentiated Caco-2 cells were incubated with the two dibenzo-α-pyrones. After incubation, glucuronides of AOH and AME were detected both in the apical and basal compartments of the cells, whereas the formed sulfates were only located in the apical compartments. As the intestinal absorption of AME was poor, it was concluded that AME might not reach the portal blood. Contrarily, AOH was absorbed rapidly and might enter the portal blood *in vivo* (Burkhardt et al., 2009). Apart from the absorption and metabolism of AOH and AME, cultured Caco-2 cells were utilized to investigate the permeation and metabolism of ATX I, ATX II, ALTP, and STTX III. After the incubation of the Caco-2 cells with ATX II and STTX III on the apical side, these mycotoxins were partly metabolized to ATX I and ALTP. However, ATX I and ALTP were not transformed at all. The permeability of the perylene quinones from the apical to the basal side decreased from ATX I to ALTP, ATX II, and subsequently STTX III. Since STTX III, ATX II, and ALTP exhibit α,β-unsaturated carbonyl groups and/or epoxide groups, Fleck et al. (2014) concluded that these perylene quinones might covalently bind to cell components. Overall, it was assumed that ATX I and ALTP were well absorbed from the intestinal tract into the blood *in vivo*. Contrarily, the permeation of ATX II and STTX III into the blood was low, but the mycotoxins might be absorbed after their transformation into ATX I and ALTP. Besides, ATX II and STTX III potentially unfold their mutagenic effects primarily in the intestinal tract (Fleck et al., 2014).

Another study investigated the formation of hydroxylated dibenzo-α-pyrones. During the incubation of microsomes from rat, human, and porcine liver with AOH and AME, the mycotoxins were hydroxylated and the 2-hydroxy, 4-hydroxy, 8-hydroxy, and 10-hydroxy metabolites of AOH and AME were generated. As the hydroxylated metabolites potentially form reactive semi-quinones and quinones or undergo redox cycling (Pfeiffer et al., 2007b), DNA adducts or oxygen species might be produced (EFSA on Contaminants in the Food Chain, 2011). To inactivate the hydroxylated dibenzo-α-pyrones, conjugations with glucuronides and sulfates are frequently observed (Pfeiffer et al., 2007b; Burkhardt et al., 2011).

It was concluded that conjugation reactions with glucuronic acid are a major pathway for detoxification and metabolic disposal of AOH and AME (Pfeiffer et al., 2009).

2 Analytical background

The present thesis focused on targeted LC-MS/MS analyses of *Alternaria* mycotoxins. Additionally, non-targeted metabolomics investigations using Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS) were conducted to comprehensively determine primary and secondary metabolites biosynthesized by *Alternaria* fungi. The term 'targeted' refers to analytical procedures focusing on the detection of a few metabolites only (Forcisi et al., 2013). Contrarily, applications aiming at a profound detection of all low molecular weight compounds of a biological system belong to non-targeted metabolomics investigations (Fiehn, 2002; Patti et al., 2012; Bueschl et al., 2017). Due to the differences in the concepts of targeted and non-targeted analytics, the following chapter is divided into two parts. The first part deals with all aspects of targeted analyses of *Alternaria* mycotoxins including the extraction and purification of the analytes as well as their qualitative and quantitative measurements. Additionally, the prevalence of *Alternaria* mycotoxins in various foods is reviewed and, subsequently, the current status of risk evaluations of these mycotoxins is described. The section on non-targeted metabolomics studies on *Alternaria* metabolites comprises the techniques applied in metabolomics investigations, current applications, the principle of FT-ICR-MS, as well as data handling and visualization.

2.1 Targeted analysis of *Alternaria* mycotoxins

Agricultural products, foods, and feeds are frequently contaminated with *Alternaria* mycotoxins inducing adverse effects on human and animal health (EFSA on Contaminants in the Food Chain, 2011). Currently, occurrence data on *Alternaria* mycotoxins is too limited to

allow the European Food Safety Authority to set tolerable daily intakes (TDI). Therefore, there is an urgent need to develop sensitive analytical methods for the accurate quantification of these mycotoxins in various food matrices (EFSA on Contaminants in the Food Chain, 2011).

2.1.1 Analytical methods – an overview

Over the past decades, various analytical methods for the detection of *Alternaria* mycotoxins have been developed. Rapid, sensitive, and cheap approaches for the semi-quantitative and quantitative analysis of mycotoxins represent immunoaffinity methods such as enzyme-linked immuno sorbent assays (ELISAs). ELISA relies on specific antigen-antibody coupling and the target molecule is determined either directly or indirectly (Escrivá et al., 2017). One of the first ELISAs regarding *Alternaria* mycotoxin analytics was developed for AAL-toxin-TA (Szurdoki et al., 1996). Since then, also AOH and TA have successfully been determined by applying ELISA. AOH was analyzed in maize and animal feed and the limit of quantification (LOQ) of AOH was 20 μg/kg (Burkin and Kononenko, 2011). Additionally, AOH was determined in diluted wine, apple juice, and tomato juice without any further sample preparation or clean-up (Ackermann et al., 2011). Although AOH and AME share similar chemical structures, an ELISA has not been developed for the detection of AME (Man et al., 2017). Furthermore, a competitive direct enzyme immunoassay was established for TA. The analyte was extracted from apple juice and tomato matrix using ethyl acetate. However, TA needed to be acetylated prior to analysis (Gross et al., 2011).

Thin-layer chromatography (TLC) was one of the first analytical procedures allowing a chromatographic separation of the analytes. Although TLC is characterized by advantages such as rapidity, simplicity in sample preparation and analyte detection, as well as cost-effectiveness (Man et al., 2017), only very high limits of detection (LODs) for AOH, AME, and TA of 100, 100, and 700 μg/kg in tomato were determined (Hasan, 1995). Solvent mixtures such as chloroform-acetone (88:12) and toluene-ethyl acetate-formic acid (6:3:1) served as mobile phases during the chromatographic separation of the *Alternaria* mycotoxins on TLC plates. AOH and AME were visualized by irradiation with UV light, whereas TA was detected due to its quenching capabilities of fluorescence under short-wavelength UV (Visconti et al., 1986; Visconti and Sibilia, 1994). Coupling high-performance TLC to densitometry allowed an improved chromatographic separation of *Alternaria* mycotoxins. Additionally, the sensitivity of the detection was enhanced and resulted in LODs for AOH and AME of 60 μg/kg, respectively (Matysik and Giryn, 1996).

Apart from TLC, gas chromatographic (GC) separation coupled to various detectors has been applied for the analysis of *Alternaria* mycotoxins. Due to their polar and non-volatile

properties, derivatization of the *Alternaria* mycotoxins prior to the GC separation is indispensable (Man et al., 2017). Scott et al. (1997) utilized heptafluorobutyrylation or trimethylsilylation to derivatize AOH, AME, ALT, ATX I, and TA. In general, GC measurements of derivatized analytes are accompanied by many disadvantages such as matrix interferences and poor repeatability. Moreover, the sample preparation is both laborious and time-consuming and induces high costs owing to the usage of expensive derivatization reagents. Therefore, GC is not applied widely in the determination of *Alternaria* mycotoxins (Hickert et al., 2016b; Man et al., 2017).

Nowadays, especially high performance liquid chromatography (HPLC) and subsequent fluorescence detection, ultraviolet (UV) detection, diode array detection (DAD), or mass spectrometric detection (MS) is widely utilized in the analysis of *Alternaria* mycotoxins (Stack et al., 1985; Visconti and Sibilia, 1994; Andersen and Frisvad, 2004; EFSA on Contaminants in the Food Chain, 2011; Man et al., 2017). Many applications use reversed-phase C18 columns to separate the analytes chromatographically (Visconti and Sibilia, 1994; Battilani et al., 2009). TA is most challenging toward chromatographic separation. The tetramic acid derivative is a strong acid and a metal chelating compound (Scott, 2001). Hence, zinc (II) sulfate is frequently added to the mobile phase when analyzing TA by HPLC-UV (Scott and Stoltz, 1980; Man et al., 2017). Coupling HPLC to UV detection, AOH, AME, and TA were analyzed in wheat grains. The limit of detection for AOH, AME, and TA was 50, 50, and 80 µg/kg, respectively (Azcarate et al., 2008).

Novel approaches in the analysis of *Alternaria* mycotoxins are exclusively based on HPLC coupled to tandem mass spectrometry (LC-MS/MS). Mass spectrometers not only allow a selective and specific detection of chemically diverse compounds but also exhibit improved sensitivity compared to UV and fluorescence detection (EFSA on Contaminants in the Food Chain, 2011; Lee et al., 2015; López et al., 2016). Electrospray ionization (ESI) rather than atmospheric pressure chemical ionization (APCI) is favored due to the higher sensitivity of ESI (Man et al., 2017). For the mass spectrometric analysis of TA, a derivatization method using 2,4-dinitrophenylhydrazine was developed by Siegel et al. (2009), as zinc (II) sulfate is incompatible with mass spectrometric detection. The derivatization resulted in a reliable chromatographic and mass spectrometric behavior of the obtained TA hydrazone (Siegel et al., 2009; Asam et al., 2011a). Besides, lower LODs and LOQs of the TA hydrazone were obtained compared to TA without derivatization (Siegel et al., 2009). Nowadays, TA is increasingly analyzed without derivatization, as it is included in multi-mycotoxin methods (Noser et al., 2011; Walravens et al., 2016; Zwickel et al., 2016; Puntscher et al., 2018).

Multi-mycotoxin methods are gaining more and more attention, as these approaches aim at a simultaneous analysis of a broad range of different fungal metabolites (Capriotti et al.,

2012). A recently published multi-mycotoxin method comprised the *Alternaria* mycotoxins TA, AOH, AME, TEN, ALT, ATX I, ATX II, altenuisol, iso-altenuene, altenuic acid III, and the AAL toxins TB1 and TB2 (Zwickel et al., 2016). Beside the free forms, Walravens et al. (2014) and Puntscher et al. (2018) also included the modified forms of AOH and AME, namely AOH-3-Glc, AOH-9-Glc, AME-3-Glc, AOH-3-S, and AME-3-S, in their multi-mycotoxin methods. Due to the extensive optimization of the multi-step gradient, the eluents, and the temperature, Puntscher et al. (2018) succeeded in chromatographically separating the isomers ALT and iso-ALT as well as the glucosides of AOH.

In addition to analytical methods focusing on the determination of *Alternaria* mycotoxins only, ALT, altenusin, AOH, AME, altersolanol, ATX I, TEN, and TA were included in a multi-mycotoxin method designed for the analysis of 295 bacterial and fungal metabolites in total (Malachová et al., 2014). Although multi-methods allow the simultaneous analysis of a variety of mycotoxins belonging to different chemical classes, the applications display diverse disadvantages. The extraction of the analytes is chosen as non-selective as possible and the clean-up of the analytes is reduced to a minimum or omitted. Thus, multi-mycotoxin approaches suffer from incomplete analyte extraction resulting in low recoveries of some mycotoxins during method validations. Besides, co-extracted matrix components cause signal suppression or enhancement during mass spectrometric detection of the analytes leading to reduced accuracy and precision of the method (Vishwanath et al., 2009; Capriotti et al., 2012; Malachová et al., 2014).

2.1.2 Mass spectrometry – mycotoxin identification

The focus in the analysis of mycotoxins is on the correct identification of the analytes. Therefore, a guidance document on the identification of mycotoxins in food and feed was published in 2016. In this guidance document, chromatographic separation and subsequent mass spectrometric detection were considered to be the method of choice for the accurate identification of the analytes (SANTE/12089/2016, 2016). During the chromatographic separation, the retention time of the analytes should exhibit at least twice the retention time of the void volume of the column (EC 2002/657/EG, 2002; SANTE/12089/2016, 2016). Applying external calibration, a maximum time shift of ± 0.2 min between the target analyte in sample matrix and the calibration standard measured in the same sequence is tolerated. If ^{13}C-labeled internal standards are used for the quantification of mycotoxins, the maximum time shift between the analyte and its ^{13}C-labeled analogue should not exceed ± 0.05 min (SANTE/12089/2016, 2016).

Applying mass spectrometry, the mycotoxins are identified based on their retention time and two mass transitions. The mass transitions need to be specific and selective (EC

2002/657/EG, 2002; SANTE/12089/2016, 2016) with specificity ensuring the correct determination of one analyte without any disturbance due to other components. Contrarily, selectivity aims at accurate identifications of multiple analytes present in one sample without disturbances (Kromidas, 2011). The fragmentations are recorded as peaks in the ion chromatograms of the analytes. In general, one mass transition is utilized for the quantification of an analyte and is termed 'quantifier'. The second mass transition is used for confirmation purposes and is referred to as 'qualifier'. The peaks of the two fragmentations should overlap and have a similar shape. Besides, the ion ratio of the quantifier and qualifier mass transition should lie within ± 30% (relative) compared to that obtained from the calibration standards from the same sequence (SANTE/12089/2016, 2016).

2.1.3 Mass spectrometry – mycotoxin quantification

Although mass spectrometric detection provides many advantages, LC-MS/MS frequently suffers from ion-suppression. In order to compensate for this effect and to achieve reliable results, quantitative determinations have to be corrected by either using matrix-matched calibration, standard addition, or stable isotope dilution assays (SIDAs) (Jessome and Volmer, 2006; Rychlik and Asam, 2008; Asam et al., 2009). Matrix-matched calibrations are generated by spiking various concentrations of the analyte under investigation to a 'blank matrix', i.e. a matrix free from the target compound. Only if the blank matrix and the sample matrix exhibit identical compositions, equal ion suppression during measurements can be assumed and the matrix effect can be fully compensated for. Several calibration points must be prepared for each analyte and –after sample preparation and LC-MS/MS measurements– a calibration curve is calculated from the data using a suitable regression model, preferably linear regression. As matrix-matched calibration requires respective calibrations for each analyte and matrix, this procedure is very laborious and time-consuming (Jessome and Volmer, 2006).

Beside matrix-matched calibrations, standard addition might also be applied for the accurate quantification of mycotoxins. After the sample preparation, varying amounts of the analyte are spiked to the sample extract and the latter is measured again. Although reliable quantitative results are obtained, standard-addition is also a time-consuming procedure (Jessome and Volmer, 2006).

In contrast to matrix-matched calibration and standard addition, SIDAs allow reliable and yet fast quantifications of the analytes. Internal standards labeled with stable isotopes differ from the target analytes only in their composition of isotopes. The isotopes ^{2}H, ^{13}C, and ^{15}N are mostly used. Apart from their molecular weight, they exhibit nearly identical physical

and chemical properties as the respective unlabeled compounds. In general, the stable isotopically labeled standards are added to the sample prior to mycotoxin extraction. After the addition of the internal standards, the ratio of labeled and unlabeled compounds remains identical throughout the entire analytical procedure. Therefore, losses during the sample preparation, variations in injection and chromatography as well as matrix effects during the mass spectrometric detection can be compensated for. As the native and labeled analytes only differ in their molecular weight, mass spectrometry allows the differentiation between the target analyte and the internal standard (Jessome and Volmer, 2006; Rychlik and Asam, 2008). SIDAs are the most accurate method for the determination of mycotoxins as even trace amounts of analytes can be quantified accurately. Besides, low LODs and LOQs, good recoveries, and outstanding precisions are achieved during method validations. However, only a few stable isotopically labeled internal standards are commercially available (Asam and Rychlik, 2015).

Many SIDAs have been developed to determine *Alternaria* mycotoxins in various food matrices such as fruit juices (Asam et al., 2009), cereal, fruit, and vegetable products (Asam et al., 2011b). For AOH and AME, the [$^{2}H_{4}$]-labeled internal standards were synthesized by a palladium-catalyzed protium-deuterium exchange from the unlabeled toxins. Utilizing these internal standards for the accurate quantification of AOH and AME in beverages resulted in very low LODs of 0.03 and 0.01 µg/kg and LOQs of 0.09 and 0.03 µg/kg, respectively (Asam et al., 2009). The ^{13}C-labeled isotopologs of the dibenzo-α-pyrones were biosynthesized by cultivating *A. alternata* in a modified Czapek-Dox medium containing [$^{13}C_{6}$]-glucose and [$^{13}C_{2}$]-acetate. [$^{13}C_{14}$]-AOH and [$^{13}C_{15}$]-AME were obtained and a SIDA for the accurate quantification of AOH and AME in cereals was established by Liu and Rychlik (2015).

For TA, two different stable isotopically labeled internal standards were synthesized in the literature. [$^{13}C_{6}$,^{15}N]-TA was synthesized from [$^{13}C_{6}$,^{15}N]-L-isoleucine following conversion to its methyl ester and subsequent acetoacetylation. After an intramolecular Claisen condensation, the stable isotopically labeled compound [$^{13}C_{6}$,^{15}N]-TA was obtained (Asam et al., 2011a). This internal standard allowed the accurate quantification of TA in tomato products (Asam et al., 2011a), fruit juices, cereals, spices (Asam et al., 2012), cereal products (Walravens et al., 2014), and infant foods (Asam and Rychlik, 2013). Another internal standard for TA was synthesized by Lohrey et al. (2013). The [$^{13}C_{2}$]-labeled TA was generated via a three-step synthesis starting from a condensation reaction of *N*-Boc-L-isoleucine and Meldrum's acid with subsequent thermal cyclization and decarboxylation. The labeling was introduced via 3-*C*-acylation using ^{13}C-labeled acetyl chloride. Finally, [$^{13}C_{2}$]-TA allowed the accurate determination of TA in tomato and pepper products (Lohrey et al., 2013).

Liu et al. (2015) developed a SIDA for various perylene quinones in grain and spice matrix. The [$^{13}C_{20}$]-labeled isotopologs of ATX I, ATX II, ALTP, and STTX III were biosynthesized during cultivating *A. alternata* in a modified Czapek-Dox medium. After the biosynthesis, the internal standards were extracted from the medium and purified by preparative HPLC (Liu and Rychlik, 2015).

TEN was quantified using [$^{2}H_{3}$]-TEN after a chemical total synthesis of the latter. The deuterium label was introduced into the molecule in the last synthetical step before cyclization. Besides, stable isotopically labeled internal standards of dihydrotentoxin and isotentoxin were synthesized. The deuterated internal standards were applied to the accurate quantification of TEN, dihydrotentoxin, and isotentoxin in various foods such as cereals, juices, oils, sauces, and spices (Liu and Rychlik, 2013).

2.1.4 Sampling, analyte extraction, and analyte purification

Prior to a qualitative and quantitative mass spectrometric analysis, several consecutive steps comprising sampling, mycotoxin extraction, and clean-up of the analytes must be performed.

In general, mycotoxins are distributed heterogeneously in agricultural products. Thus, proper sampling is indispensable to obtain samples representing the true mycotoxin content of the entire sample lot. Multiple samples from the same batch should be combined and homogenized thoroughly. Subsequently, this homogenized sample is divided into laboratory samples. After exhaustive homogenization, several samples are taken for the analysis of the mycotoxins. In general, the sampling of liquid matrices is easier than of solid materials (Blanc, 2006; Köppen et al., 2010). Further information and guidelines on the correct and representative sampling of various food matrices contaminated with mycotoxins are provided in the Commission Regulation (EC) 401/2006 (EC 401/2006, 2006).

Over the years, various procedures for the extraction and purification of *Alternaria* mycotoxins from solid matrices have been established. The extraction was conducted using either pure organic solvents such as methanol (Scott et al., 2012), acetonitrile, acetone or ethyl acetate (Schade and King, 1984; Andersen and Frisvad, 2004) or solvent mixtures such as methanol/acetonitrile/water (10/45/45; v/v/v) acidified utilizing o-phosphoric acid (Noser et al., 2011; Siciliano et al., 2015), acetonitrile/water (84/16; v/v) (Asam et al., 2011b; Liu and Rychlik, 2015), acetonitrile/water/acetic acid (79/19.5/1.5; v/v/v) (Walravens et al., 2014), or acetonitrile/water/formic acid (49/50/1; v/v/v) (Hickert et al., 2016b). TA requires an extraction with acidified solvent mixtures (Schade and King, 1984). Depending on the composition of the matrix, a defatting step using pentane or hexane might be implemented (Scott,

2001; Walravens et al., 2014; Liu and Rychlik, 2015; Puntscher et al., 2018). In addition to the mycotoxins, disturbing compounds might also be extracted applying the procedures mentioned above (Köppen et al., 2010). Therefore, the *Alternaria* mycotoxins are purified using solvent partitioning, solid phase extraction, or solid phase micro extraction (Ostry, 2008; Battilani et al., 2009). Commonly applied solid phases are C18 (Asam et al., 2011b) and combined amino propyl/C18 materials (Scott et al., 2012). The consecutive implementation of C18 and aminopropyl cartridges was published by Delgado et al. (1996). Additionally, solid phases containing diatomaceous earth (Zwickel et al., 2016) and hydrophilic-lipophilic balanced cartridges (Siciliano et al., 2015) were utilized in the literature.

The QuEChERS (quick, easy, cheap, effective, rugged, and safe) approach was originally developed to extract pesticides from fruits and vegetables. After homogenizing a sample with water, acetonitrile is added to extract the analytes. The subsequent addition of high amounts of $MgSO_4$ and NaCl induces a phase separation between the organic and the aqueous phase. Very polar compounds remain in the latter phase, whereas the analytes are transferred to the organic phase (Anastassiades et al., 2003). In 2010, Rasmussen et al. (2010) transferred the methodology to various mycotoxins produced by *Fusarium*, *Aspergillus,* as well as *Alternaria* species in silage. For AOH, AME, and TA only low average recoveries of 78, 79, and 37% were obtained during method validation (Rasmussen et al., 2010). A few years later, Dzuman et al. (2014) achieved good results applying QuEChERS to various *Fusarium*, *Alternaria*, *Penicillium*, and *Aspergillus* toxins. After the extraction of AOH, AME, TEN, and altenuene using acetonitrile, the analytes were subjected to further purification utilizing C18 silica sorbents. The recoveries of the *Alternaria* mycotoxins varied between 74 and 108% (Dzuman et al., 2014). Moreover, the QuEChERS approach was applied to determine *Alternaria* mycotoxins in lyophilized tomato products. The tomato products were mixed with water and the mycotoxins were extracted using acetonitrile. After the addition of $MgSO_4$ and Na_2SO_4 and a subsequent phase separation between acetonitrile and water, an aliquot of the organic phase was evaporated, the residue was reconstituted in 100 µL of mobile phase, and the analytes were analyzed by LC-MS/MS without any further purification (Walravens et al., 2016).

Beside QuEChERS, so-called dilute-and-shoot approaches and subsequent LC-MS/MS analyses became increasingly popular to simultaneously determine a vast range of mycotoxins (López et al., 2016). As purification procedures discriminate against groups of mycotoxins depending on their chemical structure, extractions and purifications are chosen as nonselective as possible (Capriotti et al., 2012; Krska et al., 2017). However, the direct analysis of the extracts without any sample clean-up might induce signal suppression or enhancement during the ionization in the mass spectrometer due to remaining matrix components (Sulyok et al., 2010). Sulyok et al. (2006) developed a multi-mycotoxin LC/ESI-

MS/MS method to analyze 39 mycotoxins in wheat and maize grains, simultaneously. The analytes were extracted using a mixture of acetonitrile/water/formic acid (79/20/1; v/v/v). The extract was diluted with mobile phase and introduced to the analytical instrument without further purification (Sulyok et al., 2006). Malachová et al. (2014) established and validated an LC-MS/MS method for the simultaneous detection of 295 fungal and bacterial metabolites. Again, the analytes were extracted for 90 minutes using a mixture of acetonitrile/water/acetic acid (79/20/1; v/v/v). The supernatant was diluted and directly injected into the analytical instrument without any further purification (Malachová et al., 2014).

2.1.5 Method validation

Once an analytical method is established, the suitability of the latter for the accurate identification and quantification of target compounds needs to be proven via method validation. Method validation usually comprise selectivity and specificity, LOD, LOQ, and recovery of the analytes as well as precision and robustness of the method. Furthermore, certified reference materials allow the determination of the accuracy of the method. However, the results are only valid for the matrix under investigation (EC 2002/657/EG, 2002; Thompson et al., 2002; Malachová et al., 2014).

The LOD and LOQ constitute important parameters during method validation. The analytes are spiked to a 'blank' matrix free of the compounds under investigation. The LOD represents the lowest concentration level that can be differentiated from the blank matrix, significantly. Contrarily, the LOQ specifies the concentration level above which mycotoxin contents can be determined with defined recovery and precision (Vogelgesang, 1987; Vogelgesang and Hädrich, 1998). The LODs and LOQs are frequently determined based on signal/noise ratios. Accordingly, a signal/noise ratio of 3 represents the LOD, whereas a signal/noise ratio of 9 or 10 constitutes the LOQ (Kromidas, 2011). Beside signal/noise ratios, the LODs and LOQs of various mycotoxins can be defined more accurately following a procedure of Vogelgesang and Hädrich (1998). Starting from an estimated LOD, the analyte is spiked to a blank matrix in four increasing concentrations with the highest one equaling around ten times the lowest one. Each level is prepared in multiplicate. After the sample preparation and measurements, the signal intensity of the analyte is plotted against the content and a calibration curve is obtained. Applying prediction intervals with $\alpha = 0.05$ below and above the calibration curve, 95% of all possible future signal intensity of an analyte at a given concentration are covered. The maximum peak intensity detected during the analysis of a blank matrix is represented by the edge of the upper prediction interval at content zero of the calibration curve. By transforming this maximum signal intensity into a content via the calibration function the LOD is received. According to Vogelgesang and

Hädrich (1998), three different LOQ values can be calculated for one analyte. The first LOQ only relies on a sufficient distance between LOD and LOQ, whereas the other two LOQs also consider complete recovery and limited variability during the analyte determination. After calculating the three LOQs, the highest value of these should serve as the LOQ for further quantitative analyses (Vogelgesang and Hädrich, 1998; Hädrich and Vogelgesang, 1999).

Another parameter commonly determined during method validation is the recovery of analytes. Various amounts of the analyte are spiked to the blank matrix and the recovery is calculated by dividing the amount found in the sample by the amount originally added to the sample prior to the sample preparation. As the recovery varies depending on the content of the analyte, the recovery should be evaluated over a wide range of the analyte content (Thompson et al., 1999).

Beside LODs, LOQs, and recoveries, various precisions are commonly determined during method validations by calculating the relative standard deviations of multiple quantitative measurements. The precision of the instrument is specified after the repeated analysis of one sample. The intra-day precision represents the variation between several identical samples prepared within one day. Finally, the inter-day precision requires multiple preparations of one sample on subsequent days or weeks (Kromidas, 2011).

2.1.6 *Alternaria* mycotoxins in food and feed

Alternaria mycotoxins are not included in routine analyses and are not legislatively regulated on a European level either. Therefore, these mycotoxins are considered as "emerging" (Vaclavikova et al., 2013; Puntscher et al., 2018). Commonly analyzed *Alternaria* mycotoxins constitute AOH, AME, TEN, and TA and these contaminants are frequently detected in tomato and cereal-based food products (Patriarca et al., 2007; Battilani et al., 2009; Asam et al., 2011b). The content of TA usually exceeds the contents of other *Alternaria* mycotoxins present in a sample. Noser et al. (2011) analyzed 85 tomatoes and tomato-based products from the Swiss market for their AOH, AME, TEN, ATX I, ALT, and TA contamination. Very high contents of TA were determined in tomato purée and tomato concentrates with a maximum content of 790 µg TA/kg in one sample of tomato purée. The mean and median in the group of tomato purée and tomato concentrates were 165 µg/kg and 46 µg/kg, respectively. In addition to the tetramic acid derivative, AOH and AME were frequently identified in the tomato and tomato-based samples with maximum contents of 30 µg/kg of AOH and 9 µg/kg of AME (Noser et al., 2011). Lopez et al. (2016) analyzed various food commodities such as fresh apples, olives, dried figs, sunflower seeds, fresh tomatoes, tomato sauces, and cereals for their contamination with ALT, AME, AOH, TEN, and TA. ALT was not detected

in any of the samples. High concentrations of TA were detected in dried figs (up to 2345 μg/kg) and all sunflower seeds contained TA with contents of up to 449 μg/kg. AOH, AME, and TA were detected in tomato sauces and processed tomato juices with contents of 25, 7.8, and 462 μg/kg, respectively. Cereals frequently contained TEN with a maximum content of 14 μg/kg (López et al., 2016). A study dealing with the *Alternaria* mycotoxin contamination in cereal-based foods was conducted by Scott et al. (2012). The analysis of various flours and brans, breakfast cereals, bread, and infant foods from Canada revealed frequent occurrence of AOH and AME in these products. The AOH and AME contamination in flour and bran ranged from "not detected" to 63 μg/kg and from "not detected" to 8.9 μg/kg, respectively. Up to 35 μg/kg of AOH and 12 μg/kg of AME were determined in breakfast cereals. Besides, Scott et al. (2012) analyzed infant foods, which contained up to 4.4 μg/kg of AOH and up to 9.0 μg/kg of AME (Scott et al., 2012). As infants are especially endangered by *Alternaria* mycotoxins (EFSA on Contaminants in the Food Chain, 2011), Asam et al. (2013) analyzed various infant food samples for their TA contamination. TA was detected in all teas for infant consumption and 17.5 μg/L of TA were determined in a fennel tea infusion. All purée infant foods investigated were contaminated with TA. Relatively low amounts of TA were determined in infant cereals based on wheat and oats, whereas products based on sorghum or millet were highly contaminated with contents of up to 1200 μg/kg TA (Asam and Rychlik, 2013).

One of the few studies focusing on the analysis of perylene quinones in foods was conducted by Liu et al. (2015). The perylene quinones were quantified using the [$^{13}C_{20}$]-labeled standards. ATX I and ALTP were discovered in organic whole spelt flour and organic whole rye flour, and paprika powder. ATX II, ATX III, and STTX III were not detected in any of the food products, but ATX II was determined in sorghum feed (Liu and Rychlik, 2015).

Over the last years, the analyses of modified *Alternaria* mycotoxins have been increasingly reported. Walravens et al. (2014) developed a UHPLC-ESI-MS/MS method for the simultaneous detection of free and conjugated *Alternaria* mycotoxins in cereals and cereal-based foodstuffs. TA was the most frequently detected toxin in rice and oat flake samples with contents ranging from 1.90 to 113 μg/kg and from 2.13 to 39 μg/kg, respectively. Rice additionally contained TEN and AOH in contents of 3.6 to 15.6 μg/kg and 1.83 to 2.97 μg/kg. AME as well as the modified forms of AOH and AME, namely AOH-3-Glc, AOH-3-S, AME-3-Glc, and AME-3-S, were not detected in any of the rice and oat flake samples (Walravens et al., 2014). Two years later, the same group determined AOH-3-S and AME-3-S in contents of up to 8.7 and 9.9 μg/kg in tomato concentrates. AOH-3-Glc and AME-3-Glc were not detected in tomato juices, tomato sauces, and tomato concentrates (Walravens et al., 2016).

Shortly thereafter, Puntscher et al. (2018) detected AOH-9-Glc, AOH-3-S, and AME-3-S in naturally contaminated tomato sauce above the respective LOQs. AOH-3-Glc was not detected in any of the food samples although lower LODs were determined for AOH-3-Glc compared to AOH-9-Glc (Puntscher et al., 2018).

Beside TA, *Alternaria* species also biosynthesize its diastereomer *allo*-tenuazonic acid (*(5**R**,8S)*-3-acetyl-5-*sec*-butyltetramic acid). Most analytical methods are incapable of distinguishing between TA and *allo*-TA, especially after derivatization with DNPH. Hickert et al. (2015) isolated and purified TA and its isomer *allo*-TA and developed a stable isotope dilution HPLC-MS/MS method for the determination of both isomers. The newly established procedure was utilized to investigate tomato products. The amounts of *allo*-TA in these samples ranged from 1.5 to 270 µg/kg while TA was detected in amounts ranging from 5.3 to 550 µg/kg. As *allo*-TA did not exhibit cytotoxic properties during toxicological investigations, the risk originating from TA (mainly detected as a sum of TA and *allo*-TA) in food products might be overestimated (Hickert et al., 2015). *Allo*-TA was not only biosynthesized by *Alternaria* fungi but was also formed in aqueous solutions and occurred during the chemical synthesis of the stable isotopically labeled internal standard due to the epimerization of TA (Siegel et al., 2010; Asam et al., 2011a; Asam and Rychlik, 2015).

Apart from the occurrence in food products, *Alternaria* mycotoxins frequently contaminate feeds. Therefore, Streit et al. (2013) developed a multi-mycotoxin method to analyze AOH, AME, TEN, TA, and ATX I in various feeds and feed ingredients. AOH, AME, and TEN were determined in around 80% of the samples, respectively, whereas TA and ATX I were detected in 65 and 42% of the feeds and feed ingredients, respectively. Although some samples exhibited high contaminations of 733 µg/kg of AME, 221 µg/kg of AOH, 76 µg/kg of TEN, 1983 µg/kg of TA, and 65 µg/kg of ATX I, the median ranged from 1.1 to 3.9 µg/kg for AOH, AME, TEN, and ATX I. For TA, the median was 68 µg/kg (Streit et al., 2013).

2.1.7 Risk evaluations on *Alternaria* mycotoxins

Until today, no maximum limits for *Alternaria* mycotoxins in food and feed have been established, although a first risk assessment was conducted for the EU in 2011 (EFSA on Contaminants in the Food Chain, 2011; Arcella et al., 2016). As only deficient toxicological information was available, neither TDIs nor provisional maximum tolerable daily intakes (PMTDI) were established for any of the *Alternaria* mycotoxins (Ostry, 2008; EFSA on Contaminants in the Food Chain, 2011). Apart from the lack of sufficient data on toxicology 'no observed adverse effect levels' (NOAEL) have not been reported in the literature (EFSA on Contaminants in the Food Chain, 2011). Solely for TA, the lowest observed adverse effect level (LOAEL) of 1.25 mg TA/kg b.w. per day was derived from a study conducted in 1978.

Giambrone et al. (1978) orally administered TA to chickens over three weeks and the administration resulted in weight reduction and intestinal hemorrhages (Giambrone et al., 1978). In 2016, Rychlik et al. (2016) claimed a factor of at least 1000 between the LOAEL of TA determined in animal studies and the daily dietary short-term exposure of the tetramic acid derivative dose in humans to exclude any risk. For long-term exposure, a factor of 5000 was required (Rychlik et al., 2016).

The scientific opinion elaborated by the EFSA in 2011 focused on AOH, AME, TEN, and TA, only (EFSA on Contaminants in the Food Chain, 2011). Even though only limited data on toxicology are available for these toxins, the chemical structures of AOH, AME, TEN, and TA were known and, therefore, the EFSA applied the threshold of toxicological concern (TTC) approach to evaluate the relative level of concern to human health originating from these toxins (Kroes et al., 2004; EFSA on Contaminants in the Food Chain, 2011). As AOH and AME show genotoxic potential, their TTC value was set to 2.5 ng/kg b.w. per day. The EFSA evaluation concluded that the estimated mean chronic dietary exposures of AOH and AME exceeded the TTC value of 2.5 ng/kg b.w. per day and, hence, the authority required additional compound-specific toxicity data for AOH and AME. TEN and TA were not considered to be genotoxic. Thus, their TTC value was set to 1500 ng/kg b.w. per day and the mean chronic dietary exposure estimates did not exceed the TTCs. Due to the missing occurrence and toxicological data, no TTC could be established for other *Alternaria* mycotoxins (EFSA on Contaminants in the Food Chain, 2011). A subsequent scientific report was published in 2016. The statement contained data on the contamination situation of AOH, AME, TEN, and TA with over 24,000 analytical results in total. High contaminations of AOH were determined in buckwheat and oat samples with means ranging from 27.9 to 33.1 µg/kg and from 35.3 to 39.7 µg/kg. AME was frequently detected in grains and grain-based products, whereas TEN was mainly determined in fruits and fruit products as well as fruit and vegetable juices. The content of TA exceeded those of the other *Alternaria* mycotoxins and the tetramic acid derivative was frequently detected in tomato-based products. The dietary exposures were estimated to be lower for AOH compared to the survey in 2011, whereas for AME, TEN, and TA the dietary exposures were higher compared to 2011 (Arcella et al., 2016). As a lot of the data collected were left-censored (results below the LOD/LOQ), Arcella et al. (2016) required more analytical data to be obtained for *Alternaria* mycotoxins in fruits and fruit products, tomatoes and tomato-based foods, and cereal-based food for infants (Arcella et al., 2016). Infants are especially endangered by the intake of foods contaminated with mycotoxins. Thus, Asam et al. (2013) determined the contents of TA in various infant food products. Surprisingly, high levels of TA were determined in sorghum/millet-based foods (Asam and Rychlik, 2013). Therefore, the Bavarian Health and Food Safety Authority limited the TA content in sorghum/millet-based infant foods to

500 µg/kg (Rychlik et al., 2016). To introduce maximum levels for *Alternaria* mycotoxins in food and feed on a European level, sensitive and precise multi-methods for the quantification of the food contaminants need to be established. Thus, not only commercially available analytical standards for the *Alternaria* mycotoxins are required, but the EFSA also claimed the standardization of analytical methods as well as the introduction of reference materials to perform inter-laboratory validation studies. These demands aim at obtaining valid quantitative results for the *Alternaria* mycotoxins and at reducing uncertainty originating from the left-censored data (EFSA on Contaminants in the Food Chain, 2011; Lorenz et al., 2012; Asam and Rychlik, 2015; Arcella et al., 2016). Besides, feeds should be included in quantitative analyses and the carry-over from feed to food products such as meat, milk, and eggs should be investigated (Lorenz et al., 2012). Moreover, the completion of additional toxicological studies should also implement *in vivo* surveys to estimate the adverse effects after oral administration of *Alternaria* mycotoxins to humans (Lorenz et al., 2012). Furthermore, the potential synergistic effects of simultaneously occurring mycotoxins should be investigated (Krska et al., 2017).

2.2 Non-targeted metabolomics studies on *Alternaria* metabolites

Metabolomics belongs to the so-called -omics techniques and have emerged lately compared to genomics, transcriptomics, and proteomics (Klein and Heinzle, 2012; Bueschl et al., 2013). Non-targeted metabolomics studies aim at a comprehensive qualitative and quantitative analysis of all low molecular weight compounds of an organism or biological system (Fiehn, 2002; Patti et al., 2012; Bueschl et al., 2017). Due to the comprehensive analysis of small molecules, metabolomics approaches support the detection of metabolic changes caused by diseases and improve the determination of biomarkers. Besides, microorganisms are frequently investigated and variations in the metabolome due to different phenotypes and cell cycle stages or environmental factors can be identified (Marshall and Hendrickson, 2008; Lucio et al., 2011). In addition to these metabolic differences the compounds themselves exhibit great structural diversity and occur over a vast dynamic range. Therefore, the utilization of only one analytical procedure is currently insufficient to cover the entire metabolome at once and multiple analytical approaches need to be combined to determine the metabolites comprehensively (Birkemeyer et al., 2005; Klein and Heinzle, 2012).

2.2.1 Metabolomics – the techniques

Metabolomics techniques mainly rely on the nuclear magnetic resonance spectroscopic or the mass spectrometric detection of small molecules (< 1500 Da) (Moritz et al., 2013; Markley et al., 2017). NMR measurements are highly reproducible and non-destructive enabling inter alia *in vivo* analyses. Although NMR exhibits lower sensitivity compared to mass spectrometry, metabolites can be analyzed quantitatively over a wide dynamic range. Furthermore, NMR-based metabolomics techniques allow the analysis of compounds that are difficult to ionize (Markley et al., 2017).

Metabolomics investigations based on mass spectrometric detection are either conducted by a direct injection of samples into the ionization source of the mass spectrometer or after a chromatographic separation and subsequent MS analysis. While gas chromatography coupled to MS allows the quantitative analysis of a few hundred metabolites only (Roessner et al., 2001), the combination of liquid chromatography and MS supports a more comprehensive detection of chemically diverse metabolites including non-volatile and thermally labile compounds (Forcisi et al., 2013). Common stationary phases for the chromatographic separation of metabolites are reversed-phase materials such as C8 and C18 (Forcisi et al., 2013; Bueschl et al., 2017). Various mass analyzers are applied in non-targeted metabolomics investigations, including time-of-flight (TOF), Orbitrap™, and Fourier transform ion cyclotron resonance (FT-ICR) mass analyzers (Forcisi et al., 2013). For the ionization of the analytes, electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) are the techniques of choice (Ermer and Vogel, 2000). While TOF and Orbitrap™ coupled to LC enable the chromatographic separation of isobars and isomers, the latter cannot be differentiated by applying direct infusion FT-ICR-MS. Besides, FT-ICR-MS suffers from molecules competing for ionization, a problem that can be partly circumvented by chromatographically separating the compounds prior to mass spectrometric detection (Forcisi et al., 2013; Bueschl et al., 2017). Despite the aforementioned disadvantages, FT-ICR-MS combines ultra-high resolution ($R = 10^5$-10^6) (He et al., 2001; Bossio and Marshall, 2002; Brown et al., 2005) and superior mass accuracy allowing the differentiation of mass differences of less than an electron (He et al., 2001; Forcisi et al., 2013). Therefore, measured mass signals can be assigned unambiguously to elemental compositions. This is not possible by applying TOF and Orbitrap™ (Marshall et al., 1998; Brown et al., 2005; Forcisi et al., 2013). FT-ICR-MS also covers a wide dynamic range of metabolites and exhibits great sensitivity (Brown et al., 2005; Müller et al., 2013). However, the structural identification of metabolites with known elemental compositions remains the bottleneck in metabolomics research (Dunn et al., 2013; Roullier-Gall et al., 2014b).

Nowadays, metabolomics represents an emerging discipline in the analysis of mycotoxins (Krska et al., 2017). Recent applications of TOF and Orbitrap™ covered the mycotoxin production of fungi on foods (Hickert et al., 2016a) as well as plant-fungi interactions (Bueschl et al., 2012; Rychlik et al., 2017). Moreover, a UHPLC-Orbitrap™ analysis was applied for the detection of 32 mycotoxins of various fungal genera in a beer matrix. The mycotoxins were quantified using matrix-matched calibration and the method was validated. Positively and negatively charged ions of the analytes were detected in one single run (Zachariasova et al., 2010). In general, high-resolution full scan spectra allow retrospective data analysis and comprehensive sample profiling (Zachariasova et al., 2010; Krska et al., 2017), as well as the detection of unexpected compounds (Capriotti et al., 2012; Zwickel et al., 2018).

2.2.2 Metabolomics - stable isotope labeling

An approach commonly applied in LC-HRMS-based metabolomics studies is stable isotope labeling (SIL) (Bueschl et al., 2017). This technique aims at improving the detection of biologically-derived metabolites by artificially labeling these compounds with stable isotopes. Native and stable isotope-enriched biological samples are mixed and analyzed by mass spectrometry. The native and the stable isotope labeled metabolites exhibit equal retention times during chromatographic separation, but can be differentiated by a mass spectrometric analysis (Winder et al., 2011; Klein and Heinzle, 2012).

Early applications of isotope labeling utilized either radioactive tracers or deuterium. As deuterium incorporated into metabolites might interchange with hydrogen from water (Klein and Heinzle, 2012), ^{13}C-labeling has become the method of choice over the last years due to the ubiquitous presence of carbon atoms in biological metabolites (Klein and Heinzle, 2012; Bueschl et al., 2017). Occasionally, also ^{15}N and ^{34}S stable isotopes are utilized (Klein and Heinzle, 2012). The stable isotopes are incorporated into metabolites and enriched in the latter *in vivo*. Microorganisms need to be cultivated in parallel on chemically defined media comprising only native or ^{13}C-enriched carbon sources (Birkemeyer et al., 2005; Bueschl et al., 2017). During parallel cultivation under identical conditions, equal metabolization of native and ^{13}C-enriched carbon sources by the microorganism is assumed (Klein and Heinzle, 2012; Bueschl et al., 2017). The native and ^{13}C-labeled samples are mixed and, subsequently, analyzed by LC-HRMS. Corresponding native and ^{13}C-labeled metabolites are grouped based on identical retention times during chromatographic separation. The mass spectra comprise mass signals of metabolites exhibiting native carbon isotopic distributions as well as ^{13}C-enriched isotopic distributions (Bueschl et al., 2017). If the retention time of native and ^{13}C-labeled metabolites are identical and, additionally, mass signals

of the two isotopic distributions of native and corresponding ^{13}C-labeled metabolites are present in the mass spectra, these metabolites are considered true biological origin. The number of carbons of the respective compounds can be calculated from the mass shifts between the *m*/*z* signals of monoisotopic native and corresponding monoisotopic fully ^{13}C-labeled metabolites (Birkemeyer et al., 2005; Bueschl et al., 2013). Therefore, SIL not only enhances the detection of biologically-derived metabolites but also improves molecular formula annotation (Kluger et al., 2013; Bueschl et al., 2017). However, SIL approaches are expensive due to the high costs of uniformly (U)-^{13}C-labeled carbon sources. Additionally, SIL procedures are extremely laborious as the microorganisms need to be cultivated in parallel (Bueschl et al., 2013).

2.2.3 FT-ICR-MS – the principle

The present thesis mainly focusses on the FT-ICR mass spectrometric analysis of primary and secondary metabolites of *Alternaria* fungi. Thus, the principle of FT-ICR-MS is described in the following.

After the ionization of the fungal metabolites applying ESI, the ions are introduced into a homogenous magnetic field of a superconducting magnet. Charged ions moving within a homogenous magnetic field experience the Lorentz force, a force in opposite direction to the centrifugal force. Due to the Lorentz force, the charged particles follow a curved trajectory (i.e. the cyclotron movement) and are trapped within the magnetic field. To support the trapping of the ions, a weak electric field orthogonal to the magnetic field is applied, additionally. During the movement on circular trajectories, the cyclotron frequencies of the ions are constant and are only directly related to mass, charge, and strength of the magnetic field. Therefore, each *m*/*z* exhibits an individual cyclotron frequency at a given strength of the magnetic field. The radius of the circular movement of the ions is enlarged by applying an oscillating dipolar electric field orthogonal to the magnetic field. If the frequency of the electric field matches the cyclotron frequency, the ions absorb energy and the radii of the circular trajectories increase linearly (Marshall et al., 1998; Gross, 2013; Moritz et al., 2013). To excite all ions within the ICR cell simultaneously, broadband excitation with a frequency-sweep excitation or Stored-Waveform-Inverse-Fourier-Transform (SWIFT) excitation is applied (Marshall et al., 1998; Gross, 2013). Due to the excitation and the increase of the radii, the ions pass detector electrodes located at the edge of the ICR cell. While passing the electrodes, the ions induce a detectable image current. The image currents are digitized, transformed into frequency domains using Fourier transform, and, finally, converted to mass spectra (Marshall et al., 1998; Gross, 2013; Moritz et al., 2013).

The implementation of FT-ICR-MS allowed the determination of alterations between *Chlamydia pneumoniae*-infected and non-infected Hep-2 cells (Müller et al., 2013) and the identification of variations in the growth stages of bacteria (Lucio et al., 2011). Additionally, FT-ICR-MS was utilized in the analysis of microorganisms to determine growth-specific metabolites of *E. coli* (Takahashi et al., 2008), to compare extracellular profiles of different yeast-bacteria interactions (Liu et al., 2016), and to differentiate fungi of the genus *Suillus* (Heinke et al., 2014). Furthermore, surveys were conducted on the ripening stages of strawberries (Brown et al., 2005), the characterization of barrel-aged whiskeys (Roullier-Gall et al., 2018), and the determination of the origin and the vintage of wines (Roullier-Gall et al., 2014a). The application of advanced -omics techniques on foods was defined as "foodomics". This approach aims inter alia at determining the nutritional value, sensation, functionality, origin, and authenticity of food products. Besides, foodomics approaches might contribute to the safety of agricultural products as new food contaminants might be determined and data for proper risk assessments might be generated (Rychlik et al., 2017).

2.2.4 FT-ICR-MS – measurements and data handling

Non-targeted metabolomics investigations aim at a comprehensive detection of the vast chemical diversity of metabolites. However, the comprehensive detection of the metabolites is limited as sample collection and preparation discriminate against various compounds and, therefore, should be chosen as nonselective as possible (Courant et al., 2014). Depending on the samples analyzed, sampling, quenching, and storage need to be optimized to cover the metabolites comprehensively. Once the samples are collected, the metabolites need to be extracted from the matrix. Polar metabolites are usually extracted from solid matrices using pure solvents such as methanol or solvent mixtures such as methanol and water (Müller et al., 2013; Smirnov et al., 2016). Liquid samples usually only require dilutions prior to analysis (Roullier-Gall et al., 2014b; Roullier-Gall et al., 2015). Clean-up steps such as solid phase extraction prior to mass spectrometric analysis might also be indispensable in some applications (Nielsen et al., 2003; Schmitt-Kopplin et al., 2010; Courant et al., 2014). Nevertheless, only a minimum number of sample preparation steps should be conducted to prevent the introduction of contaminations (Forcisi et al., 2013).

During non-targeted mass spectrometric analysis, several m/z signals are detected for one biologically-derived metabolite due to the formation of various adduct ions and in-source fragments as well as the presence of isotopologs (Brown et al., 2009; Bueschl et al., 2013; Dunn et al., 2013; Bueschl et al., 2014). Apart from these m/z signals, chemical noise and background signals constitute a high proportion of the signals of one mass spectrum (Keller et al., 2008). After exporting the mass signals, the latter are stored in a data matrix. Because

of the background signals and noise, the data matrix needs to be cleaned by applying a special program of de-noising (Kanawati et al., 2017). Subsequently, the data matrix is subjected to unsupervised or supervised multivariate statistics (Moritz et al., 2013). Principal component analysis (PCA) is an unsupervised statistical method depicting the variation within a data matrix on newly defined axes of principal components. The first component explains most variation within the data matrix, whereas the subsequent components illustrate descending variation in the data. Applying PCA, an overview of the relations between samples is generated and outliers can be detected (Miller and Miller, 2011; Bro and Smilde, 2014). For classification purposes of samples in non-targeted metabolomics investigations, partial least squares-discriminant analysis (PLS-DA), orthogonal partial least squares (OPLS), and support vector machine (SVM) are utilized (Moritz et al., 2013).

During FT-ICR-MS measurements, metabolites are detected at superior resolution and mass accuracy. Therefore, unique molecular formulae can be assigned unequivocally to measured *m*/*z* signals. Biological samples contain metabolites interconnected via enzymatic and chemical reactions (Breitling et al., 2006a; Breitling et al., 2006b). These connections form the basis for a mass difference network to assign molecular formulae to measured *m*/*z* signals (Tziotis et al., 2011). The mass difference network comprises nodes and edges with the nodes representing metabolite candidates (*m*/*z* signals) and edges constituting biochemical reactions expressed as mass differences between substrates and products. The mass differences are predefined in a mass difference list, covering common biochemical reactions such as (de-)hydroxylation, oxidation, and reduction. Reference masses serve as starting points of the network. Molecular formulae are assigned if a mass difference between a reference mass and an *m*/*z* signal from the data matrix matches the mass differences from the mass difference list. Mass signals, which cannot be connected to other metabolite candidates via biochemical reactions are excluded from the network. Molecular formulae usually comprise C, H, N, O, S, and P (Tziotis et al., 2011; Forcisi et al., 2013).

As the assigned molecular formulae alone do not give hints on the chemical structure of a metabolite, *van Krevelen* diagrams categorize metabolites into compound classes. After the annotation of elemental compositions to the mass signals, the hydrogen to carbon ratios (H/C) and the oxygen to carbon ratios (O/C) are calculated. The H/C and O/C ratios of each molecular formula are plotted on a two-dimensional *van Krevelen* diagram. Different compound classes have their characteristic H/C and O/C ratios in the *van Krevelen* diagram and, hence, the molecular formulae can be categorized into these compound classes based on their H/C and O/C ratios (van Krevelen, 1950; Wu et al., 2004; Roullier-Gall et al., 2014b).

Matching the elemental compositions to entries of databases allows the identification of possible structural candidates of a molecular formula. Commonly used databases are the

Human Metabolome Database (HMDB) (Wishart et al., 2009), *METLIN* (Smith et al., 2005), and the *Kyoto Encyclopedia of Genes and Genomes (KEGG)* (Kanehisa et al., 2012). *KEGG* combines data from metabolites, reactions, enzymes, and genes to understand the interactions of molecules in biological systems better (Go, 2010). Besides, the metabolites are displayed on metabolic pathway maps (Takahashi et al., 2008). Natural compounds derived from microorganisms and higher fungi are stored in the database *Antibase* (Lang et al., 2008; Nielsen et al., 2011). 95–98% of the fungal metabolites described in the literature are included in this database (Nielsen et al., 2011). However, matching molecular formulae to entries of databases only gives clues on the chemical structure of a metabolite (Patti et al., 2012). For one elemental composition, several isomers are present hampering the identification of a compound (Chen et al., 2008). The clear identification of metabolites is only possible using authentic reference compounds analyzed under identical conditions (Sumner et al., 2007; Dunn et al., 2013). Although more and more metabolites are included in various databases, the latter are far from covering all existing compounds. It was estimated that databases cover 2% of the total existing metabolites, only (Green et al., 2008). To identify metabolites not included in databases, their chemical structure needs to be elucidated *de novo* (Patti et al., 2012; Dunn et al., 2013).

3 Motivation and objectives of the thesis

Alternaria mycotoxins exhibit a pronounced toxicological potential against animals and humans exposed to contaminated feeds and foods resulting in a multitude of adverse effects. However, the data on occurrence and toxicity are currently insufficient to set tolerable daily intakes or maximum levels in foods and feeds for any of the *Alternaria* mycotoxins. As the *Alternaria* mycotoxins are not yet routinely analyzed, they are considered as "emerging mycotoxins". Only a few mycotoxins biosynthesized by *Alternaria* fungi are investigated regularly, but most of the metabolites of the *Alternaria* mycobolome have not been characterized yet and their toxicological potential remains unknown.

The first aim of the thesis was to develop a sensitive LC-MS/MS method to accurately quantify *Alternaria* mycotoxins in food products. For this purpose, a sample preparation protocol as well as a sensitive LC-MS/MS method should be developed to quantitatively determine the dibenzo-α-pyrones AOH and AME, the perylene quinones, TEN, and TA in cereal- and tomato-based foods using either SIDAs or matrix-matched calibrations. To prove the accuracy and the reliability of the newly developed method, the sample preparation protocol, and the LC-MS/MS method should be subjected to method validations. A selection of food products should be analyzed for *Alternaria* mycotoxins to obtain an actual overview on the current contamination situation of foods with the mycotoxins.

Another aim of the present thesis was the comprehensive analysis of the *Alternaria* mycobolome applying a non-targeted approach by FT-ICR-MS. Various *Alternaria* species belonging to *Alternaria alternata* and *Alternaria solani* should be analyzed by FT-ICR-MS to

determine similarities as well as differences in the metabolite production between the two species. Discriminating metabolites, which were only produced by one of the two *Alternaria* species, should be isolated and the chemical structure should be identified by MS and ^{1}H-, ^{13}C-, and 2D-NMR. Besides, inter-species variations of *Alternaria alternata* should be determined in the non-targeted approach. Additionally, variations in the mycotoxin profiles of *Alternaria alternata* and *Alternaria solani* should be determined by targeted LC-MS/MS.

4 Materials and Methods

4.1 Chemicals, reagents, and preparation of stock solutions

Analytical standards of AOH, AME, TEN, and TA copper (II) salt were purchased from Sigma-Aldrich (Steinheim, Germany). For the release of TA from its copper(II) salt, the protocol in the literature was followed (Shephard et al., 1991; Siegel et al., 2009). ATX I, ATX II, ALTP, and STTX III were extracted from rice inoculated with *A. alternata* (Liu and Rychlik, 2013). The perylene quinones were purified by preparative HPLC and characterized by MS and NMR analyses (Liu and Rychlik, 2013). The stable isotopically labeled internal standards, namely [$^{2}H_{4}$]-AOH and [$^{2}H_{4}$]-AME, were synthesized as reported earlier (Asam et al., 2009) and [$^{13}C_{6}$,^{15}N]-TA was synthesized following the protocol in the literature (Asam et al., 2011a). Subsequently, the isotopically labeled internal standards were chromatographically purified and characterized by MS and NMR.

The unlabeled and labeled *Alternaria* mycotoxins were dissolved in acetonitrile (for AOH, AME, and TEN) or methanol (for ATX I, ALTP, and TA) to obtain concentrations of 10 to 100 µg/mL, respectively. For the preparation of samples and for the method validation, the stock solutions were further diluted. The respective concentrations of the stock solutions were confirmed by UV spectroscopy (Genesys, 10S, UV-Vis spectrophotometer, Thermo Fisher Scientific, Madison, Wisconsin, USA) using the extinction coefficients listed in the literature (Zwickel et al., 2016). The available amounts of ATX II and STTX III (both solved in methanol) were not detectable by UV-Vis. Thus, these mycotoxins were only qualitatively included in the LC-MS/MS method. All solutions were stored at -20 °C in the dark until usage.

Details on the utilized chemicals and solvents for the sample preparation as well as LC-MS/MS and FT-ICR-MS measurements are listed in the materials and methods section of the respective manuscripts.

4.2 Targeted analyses of *Alternaria* mycotoxins in infant food products

In this chapter, the sample preparation protocol for the quantitative analysis of AOH, AME, TEN, ATX I, ALTP, and TA in cereal- and tomato-based food matrices is described. Additionally, details on the LC-MS/MS measurements, the calibration and quantification, and the method validation according to Vogelgesang and Hädrich are presented (Vogelgesang and Hädrich, 1998). The validated method was applied to accurately quantify the *Alternaria* mycotoxins in infant foods and these results were published in the manuscript "Quantitation of six *Alternaria* toxins in infant foods applying stable isotope labeled standards" (Chapter 5.1).

4.2.1 LC-MS/MS analysis

The chromatographic separation of the analytes was performed using a Shimadzu Nexera X2 UHPLC system (Shimadzu, Kyoto, Japan) comprising a liquid chromatograph (LC-30AD), a communication-bus-module (CBM-20A), an autosampler (SIL-30AC), a degasser (DGU-20A$_{5R}$), a column oven (CTO-20AC), and a diode array detector (SPD-M30A). The parameters for the chromatographic separation of AOH, AME, TEN, ATX I, ATX II, STTX III, and ALTP were based on Liu and Rychlik (Liu and Rychlik, 2013, 2015). The column used was a HyperClone BDS-C18 column (150 x 3.2 mm, 3 µm, 130 Å, Phenomenex, Aschaffenburg, Germany) protected by a C18-guard column (4 x 2.0 mm ID, Phenomenex, Aschaffenburg, Germany) operating at 30 °C. A binary gradient elution with acetonitrile/2-propanol/water (17.5/17.5/65; v/v/v) as solvent A and methanol as solvent B at a constant flow rate of 0.2 mL/min was applied. After an initial time of 3 min at 0 % B, the gradient raised linearly to 100 % B in the following 19 min. Thereafter, the gradient remained at 100 % B for further 1 min. The mobile phase composition returned to 0 % B within the next 2 min and the column was equilibrated for 5 min. The injection volume was 10 µL. An additional diverter valve after the column allowed the introduction of the column effluent into the mass spectrometer solely from 6.9 min to 19.5 min to prevent the instrument from matrix contamination.

TA was analyzed in an additional LC-MS/MS run due to the higher polarity of the molecule compared to the other *Alternaria* mycotoxins. The parameters for the qualification and quantification of TA were based on Asam et al. (2013b). The column used was a Gemini-

NX C18 column (150 x 4.6 mm, 3 µm, 110 Å, Phenomenex, Aschaffenburg, Germany) protected by a Gemini-NX C18-guard column (4 x 3.0 mm ID, Phenomenex, Aschaffenburg, Germany) operating at 40 °C. A binary gradient elution with 5 mM ammonium formate (pH 9) as solvent A and methanol as solvent B at a constant flow rate of 0.5 mL/min was applied. After an initial time of 3 min at 5 % B, the gradient raised linearly from 5 % B to 100 % B in 5 min and remained at 100 % B for 2 min. Thereafter, the gradient returned to 5 % B within 3 min and the column was equilibrated for 10 min. The injection volume was 10 µL. An additional diverter valve after the column allowed the introduction of the column effluent into the mass spectrometer solely from 6.5 min to 9.5 min to prevent the instrument from matrix contamination. As the UHPLC system allowed automated column switching and fourfold solvent selection, both methods could be run in the same sequence.

The LC was connected to a triple quadrupole mass spectrometer (LCMS-8050, Shimadzu Corporation, Kyoto, Japan) that operated in the negative electrospray ionization (ESI) mode for all analytes. The heating gas flow, the nebulizing gas flow, and the drying gas flow were adjusted to 10 L/min, 3 L/min, and 10 L/min, respectively. Further parameters of the interface were set as follows: heat block temperature 400 °C, desolvation line temperature 250 °C, interface temperature 300 °C, interface voltage 4 kV, and collision-induced dissociation gas pressure 270 kPa. For the quantification of the analytes, the scheduled multiple reaction monitoring (MRM) mode for MS/MS measurements was applied. The parameters for the fragmentation of each analyte were optimized via direct infusion of standard solutions of AOH, AME, TEN, ATX I, ATX II, ALTP, STTX III, and TA at concentrations of 1 µg/mL, respectively. The first mass transition listed in **Table 4.1** served as quantifier, whereas the second one was utilized as qualifier, respectively. The optimized voltages and collision energies of each analyte are listed in **Table 4.1**. The data acquisition and the data analysis were carried out using the *LabSolutions* software (Shimadzu, Kyoto, Japan).

Table 4.1: Optimized precursor and fragment ions as well as fragmentation conditions of the *Alternaria* mycotoxins. The first mass transition listed in the table served as quantifier, whereas the second one was utilized as qualifier, respectively.

Analyte	Precursor ion *m/z*	Product ion *m/z*	Q1 Pre-bias [V]	CE [V]	Q3 Pre-bias [V]
ATX I	351.20	297.20	25	28	19
		314.20	27	32	21
ALTP	349.30	261.25	18	28	15
		303.00	18	19	18
AOH	257.30	213.00	15	23	12
		215.05	14	24	13
[$^{2}H_4$]-AOH	261.30	217.00	15	23	12
		218.05	14	24	13
TEN	413.30	141.05	15	23	23
		271.30	15	17	17
ATX II	349.40	313.25	16	25	12
		331.30	20	15	20
STTX III	347.20	329.20	17	22	21
		301.10	17	35	18
AME	271.30	256.20	20	23	25
		228.20	19	30	13
[$^{2}H_4$]-AME	275.30	260.20	20	23	25
		232.20	19	30	13
TA	196.30	112.05	22	26	20
		139.00	14	22	11
[$^{13}C_6$,^{15}N]-TA	203.25	113.05	22	26	20
		142.00	14	22	11

4.2.2 Calibration and quantitation

The *Alternaria* mycotoxins AOH, AME, and TA were quantified in cereal- and tomato-based infant food products using stable isotopically labeled internal standards. The [$^{2}H_4$]-labeled isotopologs were used as internal standards for AOH and AME, whereas the [$^{13}C_6$,^{15}N]-labeled isotopolog was utilized for TA. The quantification of AOH, AME, and TA was based on calibration functions received after thorough mixing varying amounts of analyte (A) with constant amounts of internal standard (IS) and subsequent LC-MS/MS measurements. The calibration functions were calculated by plotting molar ratios [n(A)/n(IS)] against peak area ratios [A(A)/A(IS)]. Due to the high natural variation in the content of the *Alternaria* mycotoxins in the cereal- and tomato-based food products, the calibration functions for these food matrices covered molar ratios [n(A)/n(IS)] of 0.01–100 (1:100, 1:50, 1:10, 1:5, 1:2, 1:1, 2:1, 5:1, 10:1, and 100:1). The linearity of the respective calibration function was verified using Mandel's fitting test (Mandel, 1984). The linearity was confirmed for AOH, AME, and TA between the molar ratios of 0.01–100, respectively. **Table 4.2** displays the range of molar ratios as well as the calibration functions of AOH, AME, and TA.

Table 4.2: Calibration functions for the quantification of AOH, AME, and TA. The mass transitions for the quantifier utilized for the quantification of AOH, AME, and TA are listed in **Table 4.1**.

Analyte	Internal standard	Food matrix	Molar ratio [n(A)/n(IS)]	Calibration function
AOH	[$^{2}H_4$]-AOH	cereal, tomato	0.01–100	y = 0.471x + 0.001
AME	[$^{2}H_4$]-AME	cereal, tomato	0.01–100	y = 0.7833x + 0.002
TA	[$^{13}C_6$,^{15}N]-TA	cereal, tomato	0.01–100	y = 1.898x + 0.0062

No stable isotopically labeled standards were available for TEN, ATX I, and ALTP. To compensate for signal suppression or enhancement in the mass spectrometer due to co-eluting matrix components, these mycotoxins were quantified using matrix-matched calibration. TEN, ATX I, and ALTP were quantified in cereal- and tomato-based infant foods. As all analyzed flours from various supermarkets contained *Alternaria* mycotoxins, potato starch served as a "blank matrix" for cereal-based infant foods. Freshly prepared tomato purée from whole tomatoes was free of *Alternaria* mycotoxins and, therefore, served as a "blank matrix" for tomato-based infant food products.

To obtain matrix-matched calibration functions, increasing amounts of TEN, ATX I, and ALTP were spiked to the blank matrices (**Table 4.3**). The extraction of the mycotoxins as well as the reduction of the matrix components were performed following the protocol for cereal- and tomato-based products. The samples were analyzed by LC-MS/MS and the calibration curves were constructed by plotting peak areas [A(A)] against the respective contents of the analyte [c(A)]. The calibration function was received via linear regression and the linearity was confirmed using Mandel's fitting test (Mandel, 1984).

Table 4.3: Matrix-matched calibration functions for TEN, ATX I, and ALTP in various matrices. The mass transitions for the quantifier utilized for the quantification of TEN, ATX I, and ALTP are listed in **Table 4.1**.

Analyte	Matrix	Linear range [µg/kg]	Calibration function
TEN	cereal	0.1–100	y = 62470x + 3530.3
TEN	tomato	0.1–100	y = 140854x – 30794
ATX I	cereal	0.4–20	y = 90828x – 28363
ATX I	tomato	0.4–20	y = 16240x + 5409
ALTP	cereal	0.6–20	y = 400847x + 145323
ALTP	tomato	0.4–20	y = 326116x – 6746.3

The mycotoxin contamination in cereal- and tomato-based infant food samples was either calculated by using the respective calibration function (AOH, AME, and TA) or by using the matrix-matched calibration function (TEN, ATX I, and ALTP).

4.2.3 Method validation

The sample preparation protocol for cereal- and tomato-based infant foods and the LC-MS/MS method were validated according to Vogelgesang and Hädrich (Vogelgesang and Hädrich, 1998). For the determination of the LODs and LOQs of the *Alternaria* mycotoxins in various matrices, potato starch and the blank tomato matrix were utilized. AOH, AME, TEN, ATX I, ALTP, and TA were spiked to the blank matrices at four different levels, respectively. Potato starch was spiked with AOH (1.0, 3.0, 7.0, and 10 µg/kg), AME (0.1, 0.3, 0.7, and 1.0 µg/kg), TEN (0.1, 0.4, 0.7, and 1.0 µg/kg), ATX I (0.8, 2.0, 5.0, and 8.0 µg/kg), ALTP (0.4, 1.6, 2.8, and 4.0 µg/kg), and TA (2.5, 8.0, 16, and 25 µg/kg), whereas the blank tomatoes were spiked with AOH (0.15, 0.6, 1.0, and 1.5 µg/kg), AME (0.015, 0.06, 0.1, and 0.15 µg/kg), TEN (0.15, 0.6, 1.0, and 1.5 µg/kg), ATX I (0.5, 2.0, 3.5, and 5.0 µg/kg), ALTP

(0.4, 1.6, 2.8, and 4.0 µg/kg), and TA (1.5, 6.0, 10, and 15 µg/kg). Each level was prepared in triplicate. For the extraction of the mycotoxins and the reduction of the matrix components, the sample preparation protocol of the respective matrices was followed and the samples were analyzed by LC-MS/MS. The mycotoxin content was either calculated by using the respective calibration function (AOH, AME, and TA) or by applying the matrix-matched calibration function (TEN, ATX I, and ALTP).

The recoveries for each mycotoxin were determined in starch and tomato matrix at three different levels, respectively. To determine the recovery rates of each analyte, potato starch was spiked with AOH (3.0, 7.0, and 10 µg/kg), AME (0.3, 0.7, and 1.0 µg/kg), TEN (0.4, 0.7, and 1.0 µg/kg), ATX I (2.0, 5.0, and 8.0 µg/kg), ALTP (1.6, 2.8, and 4.0 µg/kg), and TA (15, 100, and 200 µg/kg) and the blank tomato purée was spiked with AOH (0.6, 1.0, and 1.5 µg/kg), AME (0.06, 0.1, and 0.15 µg/kg), TEN (0.6, 1.0, and 1.5 µg/kg), ATX I (2.0, 3.5, and 5.0 µg/kg), ALTP (1.6, 2.8, and 4.0 µg/kg), and TA (50, 100, and 200 µg/kg). Each level was prepared in triplicate. For the extraction of the mycotoxins and the reduction of the matrix components the sample preparation protocol of the respective matrix was followed and the samples were analyzed by LC-MS/MS. The mycotoxin content was either calculated by using the respective calibration function (AOH, AME, and TA) or by applying the matrix-matched calibration function (TEN, ATX I, and ALTP). The recovery was calculated for each toxin as follows:

R[%] = found amount [µg/kg] / spiked amount [µg/kg] x 100.

$$R\ [\%] = \frac{found\ amount\ \left[\frac{\mu g}{kg}\right]}{spiked\ amount\ \left[\frac{\mu g}{kg}\right]}\ x\ 100 \tag{4.1}$$

To determine inter-injection, intra-day, and inter-day precisions for the *Alternaria* mycotoxins in starch and tomato matrix, naturally contaminated infant food samples were analyzed. A seven-grain infant food product contained TEN (23 µg/kg), ALTP (6 µg/kg), and TA (114 µg/kg) and was spiked with AOH (10 µg/kg), AME (1.5 µg/kg), and ATX I (6 µg/kg). An organic tomato sauce contained AOH (75 µg/kg), AME (9 µg/kg), and TA (490 µg/kg) and was spiked with TEN (8 µg/kg), ATX I (5 µg/kg), and ALTP (6 µg/kg).

The inter-injection precisions were determined after multiple injections of one sample (n = 5) into the LC-MS/MS instrument and subsequently calculating the relative standard deviation (RSD [%]) of the multiple injections. The intra-day precisions were determined after triple injection of one sample (prepared in triplicate) into the LC-MS/MS. For the inter-

day precisions, one sample was prepared in triplicate over three weeks and injected in triplicate into the LC-MS/MS. The mycotoxin content was either calculated by using the respective calibration function (AOH, AME, and TA) or by applying the matrix-matched calibration function (TEN, ATX I, and ALTP).

4.2.4 Analysis of cereal- and tomato-based infant food products

For the analysis of infant foods, 1 g of thoroughly homogenized sample was weighed into a 50 mL centrifuge tube. The isotopically labeled internal standards (100 µL of [$^{2}H_{4}$]-AOH (0.1 µg/mL), 50 µL of [$^{2}H_{4}$]-AME (0.1 µg/mL), and 100 µL of [$^{13}C_{6}$,^{15}N]-TA (1.0 µg/mL)), 15 mL of a mixture of acetonitrile/water (84/16; v/v), and 0.2 mL of formic acid were added. After the extraction of the mycotoxins by applying horizontal shaking for 30 min (175 rpm), the sample was centrifuged (room temperature, 1690 g, 5 min). The residue was extracted again following the protocol above and, subsequently, a third extraction step was conducted as follows: 15 mL of a mixture of acetonitrile/methanol/water (50/25/25; v/v/v) and 0.3 mL of formic acid were added to the residue and the latter was extracted by horizontal shaking (30 min, 175 rpm). After filtering, the extracts were combined and rotary evaporated to dryness at 40 °C. The residue was reconstituted in 12 mL of water (adjusted to pH 5.5 using formic acid) and the mycotoxins were purified by solid phase extraction (Discovery® DSC-C18, 500 mg, 6 mL, Sigma-Aldrich, Bellefonte, PA, USA). Initially, the C18 material was washed with 6 mL of methanol and conditioned with 6 mL of water (adjusted to pH 5.5 using formic acid). The sample was loaded onto the column completely, and interfering matrix was removed by washing the column with 12 mL of water. The column was dried under vacuum and the analytes were eluted with 6 mL of methanol and 9 mL of methanol + 2% ammonium hydroxide. After rotary evaporation to dryness at 40 °C, the residue was reconstituted in 1 mL of methanol/water (1/1; v/v). Remaining matrix precipitated during the night at -20 °C. The sample was membrane filtered (0.22 µm) and analyzed by LC-MS/MS.

Various infant foods (n = 25) were collected from several supermarkets and drugstores in Germany. Cereal-based infant food products contained mainly spelt, oat, millet, rice, and wheat, whereas infant food in jars comprised either vegetables or fruits. Besides, tomato products for infant consumption were included in the survey.

4.3 Non-targeted analysis of the *Alternaria* mycobolome

4.3.1 Preparation of synthetic nutrient-poor agar (SN agar)

For the preparation of SN agar, 0.2 g of glucose, 0.5 g of magnesium sulfate heptahydrate, 0.5 g of potassium chloride, 1 g of potassium dihydrogen phosphate, 1 g of potassium nitrate, and 0.2 g of sucrose were dissolved in 100 mL of water. The pH was adjusted to 5.5 using 600 µL of sodium hydroxide (1 mol/L). 22 g of agar were dissolved in 900 mL of water. Both solutions were combined and the medium was autoclaved at 121 °C for 20 min (Leslie and Summerell, 2006; Metz et al., 2019).

4.3.2 Isolation of *Alternaria* fungi from plant material

A. alternata isolates derived from naturally infected plant material such as potato leaves (Uelzen, Germany, isolate 1), tomato leaves (Aitrang, Germany, isolate 2), and moldy tomatoes (Aitrang, Germany, isolate 3). The *A. solani* isolate originated from potato leaves (Kirchheim, Germany). All fungi were isolated in 2015.

Parts of the dried tomato and potato leaves, as well as pieces of moldy tomatoes were placed on SN agar (Leslie and Summerell, 2006; Metz et al., 2019). After two weeks of cultivation (22 °C, 65% relative humidity, alternation of black light exposure and darkness, 12 hours each), single spores were isolated from the overgrown agar plates (Metz et al., 2019). The single spores were transferred to SN agar and cultivated at 22 °C and 65% relative humidity for two weeks (alternation of black light exposure and darkness, 12 hours each). The overgrown agar plates with pure isolates were used for further experiments.

4.3.3 Cultivation of *Alternaria* isolates for FT-ICR-MS analysis

The *A. alternata* isolates 1–3 and the *A. solani* isolate were cultivated in a synthetic liquid medium to obtain samples for FT-ICR-MS measurements. The liquid medium consisted of 0.2 g/L of ammonium sulfate, 0.3 g/L of calcium nitrate tetrahydrate, 4.0 g/L of glucose, 0.02 g/L of iron sulfate heptahydrate, 0.25 g/L of magnesium sulfate heptahydrate, 0.25 g/L of potassium chloride, 0.5 g/L of potassium dihydrogen phosphate, 0.66 g/L of sodium acetate trihydrate, and 2.0 g/L of sodium nitrate. The pH of the liquid medium was adjusted to pH 5.5 using formic acid. A volume of 35 mL of the liquid medium were transferred to polycarbonate Erlenmeyer flasks and autoclaved at 121 °C for 20 min.

After cooling of the sterile liquid medium, the latter was inoculated with defined spore suspensions of *A. alternata* and *A. solani*. To obtain spore suspensions of the different isolates, 3 mL of detergent solution (0.5% tween® 20) were pipetted on the respective overgrown agar plates and, subsequently, the mycelium and the spores were scratched. The spore suspensions were adjusted to 8.75×10^5 spores/mL for *A. alternata* and to 2×10^5 spores/mL for *A. solani* using a Thoma chamber (Leslie and Summerell, 2006). The liquid medium was inoculated with 25 µL of the spore suspensions of the *A. alternata* isolates and with 100 µL of the spore suspension of the *A. solani* isolate to receive equal amounts of total spores for all fungal isolates. By adding 25 µL of pure detergent solution to the liquid medium, control samples were obtained. The control samples covered contaminations in the FT-ICR-MS spectra originating from chemicals, solvents, and plastic and glass surfaces. The fungal cultures as well as the control samples were prepared in replicates of five, respectively. To allow sterile sampling during the cultivation process, the polycarbonate Erlenmeyer flasks were sealed with septa. The fungal cultures and the control samples were cultivated for eleven days in the dark (26 °C, 110 rpm) (Liu and Rychlik, 2015) and the isolates were exposed to artificial daylight for half an hour a day.

4.3.4 Metabolite extraction for FT-ICR-MS analysis

A volume of 3 mL of the liquid medium were sterilely taken using cannulas and syringes after four, seven, nine, and eleven days of cultivation, respectively. The samples were centrifuged (15.000 x g, 10 min) to separate the liquid medium from fungal mycelium. Afterwards, the pH of the supernatant was adjusted to pH 2 using formic acid. As the liquid medium still contained a multitude of salts harmful to the ESI source of the FT-ICR-MS instrument, the liquid medium was desalted by solid phase extraction (Discovery® DSC-8, Supelco, Bellefonte, PA, USA). Prior to sample loading (2 mL), the C8 material was washed with 1 mL of methanol and conditioned with 1 mL of water (adjusted to pH 2 using formic acid). The C8 material was washed with 5 mL of water (adjusted to pH 2 using formic acid) and the analytes were eluted with 1 mL of methanol.

Additionally, the mycelium of the fungal cultures was analyzed by LC-MS/MS and FT-ICR-MS. The surface of the mycelium was thoroughly washed with water to remove remaining liquid medium. The disruption of the fungal cells was performed by nucleo spin bead tubes (type A, 0.6–0.8 mm, Machery Nagel, Düren, Germany). The nucleo spin bead tubes were thoroughly washed with 5 mL of water and 5 mL of methanol to avoid contamination of the samples originating from the surface of the bead tubes. 200 mg of the mycelium were used for the disruption of the cells and 1 mL of an ice-cold methanol/water mixture (90/10, v/v) was added to the mycelium. The disruption of the cells was performed at 6800 rpm

(4 x 30 s) utilizing a Precellys homogenizer (bertin instruments, Montigny-le-Bretonneux, France). To improve the disruption of the cells, the homogenizer was cooled to -10 °C using liquid nitrogen. Remaining cell particles were removed by centrifugation (21.000 x g, 10 min).

As the analysis of the cell extracts resulted in salt clusters in the FT-ICR-MS spectra, a desalting step was inevitable. To ensure comparability between the liquid medium and the extract of the mycelium, the latter was desalted following the same protocol as for the desalting of the liquid medium. The extract of the fungal cells was dried under nitrogen and the residue was reconstituted in 2 mL of water (adjusted to pH 2 using formic acid). A DSC-8 cartridge (Discovery® DSC-8, Supelco, Bellefonte, PA, USA) was washed with 1 mL of methanol and conditioned with 1 mL of water (adjusted to pH 2 using formic acid). The waterous extract of the fungal cells was loaded onto the column and the latter was washed with 5 mL of water (adjusted to pH 2 using formic acid). The elution of the analytes was performed with 1 mL of methanol. The samples of the liquid medium and the mycelium were diluted with methanol (1/10; v/v) prior to injection. In total, 120 samples were analyzed.

4.3.5 FT-ICR-MS analysis

Ultrahigh-resolution mass spectra were acquired on a Bruker Solarix Fourier Transform Ion Cyclotron Resonance Mass Spectrometer (FT-ICR-MS) (Bruker Daltonics GmbH, Bremen, Germany) equipped with a 12 Tesla superconducting magnet (Magnex Scientific Inc., Yarnton, GB). Fungal samples were directly injected into an APOLO II electrospray ionization source (Bruker Daltonics GmbH, Bremen, Germany) at a syringe flow rate of 120 µL/h using a Gilson autosampler (Gilson, Inc., Meddleton, WI, USA). The mass spectrometer operated in the negative ionization mode and was externally calibrated using ion clusters of arginine (10 mg/L in methanol) before measurements. Mass spectra were acquired with a time domain transient of four mega words in size and covered a mass range from *m/z* 150 to *m/z* 1000 Da. 300 scans were accumulated for each sample. Mass spectrometric parameters were set as follows: capillary voltage 3600 V, spray shield voltage -500 V, drying gas flow rate 4.0 L/min. The ion accumulation time was 0.3 s and a resolving power of 600.000 at *m/z* 300 was achieved. After the measurements, each mass spectrum was internally calibrated based on a calibration list of *Alternaria* metabolites covering an *m/z* range of 160 to 730 Da. The *Data Analysis* Version 4.2 (Bruker Daltonics GmbH, Bremen, Germany) was used to process raw spectra. Mass lists were generated with a signal to noise ratio of 7 and a relative intensity threshold of 0.01%. The exported mass lists were de-noised from the

well-known Gibbs sidelobes (wiggles) by the use of a special program of denoising (Kanawati et al., 2017).

4.3.6 Molecular formula annotation

The molecular formula annotation was performed only on the *m/z* signals that occurred in at least two out of five biological replicates using an in-house written software tool named *NetCalc*. *NetCalc* links *m/z* values (metabolite candidates) via mass differences deriving from biochemical reactions between substrates and products (Tziotis et al., 2011). For the annotation process, the mass differences were specified in a mass difference list covering 191 reaction-equivalent mass differences such as oxidation, reduction, and hydroxylation (Tziotis et al., 2011; Forcisi et al., 2013). Originating from 41 predefined reference masses (*Alternaria* metabolites) the mass difference network was established. The molecular formula of a measured *m/z* value was assigned if a mass difference of the mass difference list matched the mass difference between one of the reference masses and the *m/z* value. On the basis of this new molecular formula assignment, the network was further established (Tziotis et al., 2011; Forcisi et al., 2013). Any *m/z* signals deriving from isotopes and masses with an unusual mass defect were excluded from the network (Tziotis et al., 2011; Moritz et al., 2017). The elemental composition of the assignments comprised C, H, N, O, P, and S. In total, 4467 monoisotopic elemental compositions (55%) were assigned with 3285 molecular formulae exhibiting an annotation error within ± 0.2 ppm (74%) (Forcisi et al., 2015; Moritz et al., 2017). Due to the formation of adduct ions during the FT-ICR-MS measurements, 402 chloride adducts were removed and, finally, 2883 molecular formulae were obtained for further investigations. The established mass difference network was displayed using *Gephi 0.9.2* software (Bastian et al., 2009).

4.3.7 Database assignments

The assigned molecular formulae were matched to entries of the database *Antibase* (Laatsch, 2017), as this database contains more than 40,000 natural compounds, derived only from microorganisms and higher fungi (Lang et al., 2008; Nielsen et al., 2011). About 95–98% of the metabolites for *Alternaria* and other genus of fungi described in the literature are listed in the database (Nielsen et al., 2011). Besides, the molecular formulae obtained during the annotation process using the mass difference network were checked against the entries of the *Kyoto Encyclopedia of Genes and Genomes (KEGG)* database (http://www.genome.jp/kegg/compound/) (Kanehisa et al., 2012). Contrarily to *Antibase*, *KEGG* collects data about metabolites, reactions, enzymes, and genes for a better understanding of the

molecular interactions in biological systems (Go, 2010).The *KEEG* database includes 13,000 metabolites which are connected to known metabolic pathways (Takahashi et al., 2008).

The determination of the elemental compositions during the assignment process allowed the calculation of hydrogen to carbon ratios (H/C) as well as of oxygen to carbon ratios (O/C) for each molecular formula. The H/C ratios of the molecular formulae were plotted against the respective O/C ratios resulting in specific locations of the compounds in a two-dimensional v*an Krevelen* diagram. As various classes of compounds have their specific locations on the diagram (Roullier-Gall et al., 2014b), *van Krevelen* diagrams were used to display the variations in the metabolic profile of the *Alternaria* fungi.

Only *m*/*z* values detected in at least four out of five biological replicates were subjected to statistical evaluations. To reduce the complexity of the data and to display the variation in the data as a new set of new independent variables, PCA, an unsupervised statistical method, was applied. The implementation of the PCA using *Simca-P 9.0* software (Umetrics, Sweden) provided an overview of the complex data set and allowed the detection of outliers and relation between samples (Bro and Smilde, 2014).

Volcano plots allowed the determination of the most discriminative molecular formulae between the *A. alternata* and the *A. solani* isolates (Li Wentian, 2012). To create volcano plots, the $-\log_{10}$ p-value of the measured intensities was plotted against the $\log_2$ fold change (ratio of averaged intensities of measured *m*/*z* signals).

4.4 Biosynthesis, extraction, purification, and characterization of alterperylenepoxide A 9-mercaptolactate (APML)

In the following, the biosynthesis, extraction, purification, as well as characterization of the fungal metabolite APML is described. This metabolite attracted attention as it was one of the most discriminating metabolites between *A. alternata* and *A. solani* in the non-targeted metabolomics experiments and was only produced by *A. alternata*. Furthermore, due to MS/MS fragmentation experiments utilizing FT-ICR-MS, the structure of the metabolite was assumed to be related to the known perylene quinones.

4.4.1 MS/MS spectra of APML

Due to the ultra-high resolution of FT-ICR-MS, the experimental *m*/*z* signal of 471.07550 was assigned to the molecular formula $C_{23}H_{20}O_9S$ (theoretical $[M\text{-}H]^-$: 471.07553; mass error of annotation: -0.064 ppm). The MS/MS fragmentation of this *m*/*z* signal was conducted on the FT-ICR-MS and the fragments were recorded after applying a collision energy of 20 eV.

The ion accumulation time was adjusted to 1.5 s. The obtained fragments were compared to the LC-MS/MS fragmentation patterns of ATX II and ALTP and showed a great overlap. Therefore, a chemical structure similar to ATX II and ALTP was assumed for the metabolite with the molecular formula $C_{23}H_{20}O_9S$.

4.4.2 Biosynthesis of APML

In order to obtain a high quantity of secondary metabolites of *A. alternata*, including APML, the isolate 1 was cultivated on rice medium. 25 g of parboiled rice were weighed in a polycarbonate Erlenmeyer flask, 15 mL of water were added, and the rice medium was autoclaved (121 °C, 20 min). After the cooling of the sterile rice medium, the latter was inoculated with defined spore suspensions of the *A. alternata* isolate 1. The spore suspensions were obtained by pipetting 3 mL of detergent solution (0.5% tween® 20) on the overgrown agar plate of isolate 1 and the mycelium as well as the spores were scratched. The spore suspension was adjusted to 1.25 x 10^6 spores/mL (Leslie and Summerell, 2006) and 25 µL of the suspension were added to the rice medium. During seven days of cultivation (26 °C, 110 rpm), the fungi were exposed to artificial daylight for half an hour a day. In total, 56 Erlenmeyer flasks (25 g of rice each) were cultivated.

4.4.3 Extraction and purification of APML

After seven days of cultivation, the rice was totally covered with fungal mycelium. After thorough homogenization of the overgrown rice, the fungal metabolites were extracted for 1 h at 250 rpm using 1 L of ethyl acetate/methanol (1/1; v/v) on a horizontal shaker. The extraction was repeated twice. The extracts were decanted, combined, and evaporated to dryness using a rotary evaporator at 30 °C.

To purify the metabolites, the residue was dry-loaded onto a column of silica gel (125 g, Mesh 70-230, pore size 60 Å, particle size 63–200 µm, Sigma-Aldrich, Steinheim, Germany) equilibrated with dichloromethane/methanol (9/1; v/v). The metabolites were gradually eluted with 200 mL of dichloromethane/methanol (9/1; 7/1; 3/1; 1/1; 1/3; 1/7; 0/1; v/v). In total, 30 fractions (50 mL each) were collected and the fractions containing the metabolite $C_{23}H_{20}O_9S$ were identified by LC-MS/MS analysis. The fractions 20-29 were combined, evaporated, and dry loaded on to a second column of silica gel (125 g) pre-conditioned with dichloromethane/methanol (9/1; v/v). The metabolites were gradually eluted with 400 mL of dichloromethane/methanol (9/1; 3/1; 1/1; 0/1; v/v). 30 fractions were collected (50 mL each) and the fractions containing APML were identified by LC-MS/MS. The fractions 18-29 were combined and the volume of the combined fractions was reduced using rotary

evaporation at 30 °C. The residue was further purified using a column of C18 material (50 g, end-capped, Chromabond, Machery-Nagel GmbH & Co. KG, Düren, Germany). After the equilibration with acetonitrile/water (10/90; v/v), the metabolites were gradually eluted using 50 mL of acetonitrile/water (10/90; 15/85; 20/80; 25/75; 30/70; 40/60; 60/40; 80/20; 100/0; v/v) and the eluates were collected in 90 fractions (5 mL each). Fraction 47 was identified to contain APML and the latter was further purified using a reversed-phase, semi-preparative HPLC column.

The preparative separation was performed on a Merck-Hitachi LaChrom HPLC System (Tokyo, Japan) and the column used was a YMC-Pack Pro C18 column (150 x 10 mm, S-5 µm, 12 nm, YMC Europe GmbH, Dinslaken, Germany) protected by a C18-guard column (4 x 2.0 mm ID, Phenomenex, Aschaffenburg, Germany). Binary gradient elution with water + 0.1% formic acid as solvent A and acetonitrile as solvent B at a constant flow rate of 1.25 mL/min was applied. After an initial time of 3 min at 25 % B for the first 3 min, the gradient raised linearly from 25 % B to 65 % B in the following 15.5 min and, finally, raised to 100 % B during 1 min. The gradient remained at 100% B for 3 min and, subsequently, the mobile phase returned to 25 % B within the next 1.5 min. The column was equilibrated for further 3 min. 100 µL were used as injection volume and the UV absorption was measured at 254 nm. APML eluted after 17.2 min, was collected, and, after evaporation of the solvents at 30 °C, a yellow, amorphous solid was obtained.

4.4.4 Qualitative and quantitative NMR analysis

The purified APML was dissolved in 600 µL of acetonitrile-d_3 and transferred into an NMR tube (5 x 178 mm, USC-Tubes, Bruker, Faellanden, Switzerland). ^{1}H (400 MHz), ^{13}C (100 MHz), and 2D NMR (^{1}H–^{1}H COSY, HSQC, HMBC) experiments for details on the chemical structure were performed on a Bruker AV III (298 K, Bruker, Rheinstetten, Germany). The spectra were referenced to the residual signal of the acetonitrile-d_3 solvent.

To calculate the absolute concentration of APML, quantitative ^{1}H nuclear magnetic resonance spectroscopy (qNMR) according to Frank et al. (2014) was performed on the Bruker AV III. Tyrosine (purity ≥ 99%, Sigma-Aldrich, Steinheim, Germany) was used as external standard. Choosing the signals of the protons H-5 (δ_H 6.89 ppm), H-9 (δ_H 4.21 ppm) and H-8a (δ_H 3.89 ppm) of APML, the quantification was performed using the *ortho*-aromatic protons of the tyrosine standard at 7.10 ppm. For the calculation of the concentration of APML, the ERETIC 2 function of *TopSpin 3.6.0* (Bruker BioSpin, Faellanden, Switzerland) was applied. The accuracy of the qNMR measurement was within an error limit of ± 2%.

4.4.5 UV-Spectroscopy

The absorption maxima of APML were determined at a concentration of 15.0 µg/mL via full scan (200–450 nm, steps: 1 nm) using a UV-Vis spectrophotometer (Genesys, 10S, Thermo Fisher Scientific, Madison, Wisconsin, USA). For each absorption maximum (λ = 217.0, 258.0, 291.0, and 381.0 nm), the extinction coefficients were determined by recording the absorptions of APML at various concentrations. APML was diluted to concentrations of 5.0 µg/mL, 10 µg/mL, and 15 µg/mL (each in triplicate) using acetonitrile and the dilutions were measured against acetonitrile. The molar extinction coefficients ε were calculated for each dilution using the equation:

$$\epsilon = \frac{absorbance \cdot 1000}{concentration \left[\frac{mmol}{L}\right] \cdot pathlength\,[cm]} \tag{4.2}$$

5 Results

5.1 Quantitation of Six *Alternaria* Toxins in Infant Foods Applying Stable Isotope Labeled Standards

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The published manuscript was inserted into Appendix A.

5.1.1 Summary

Fungi of the genus *Alternaria* frequently infect crops and vegetables resulting in harvest losses in the field as well as during storage. Beside the immediate harvest losses, *Alternaria* fungi produce a variety of mycotoxins with a pronounced toxicological potential.

In order to determine the current contamination situation of various agricultural foods with *Alternaria* mycotoxins, a sample preparation protocol for AOH, AME, TEN, ATX I, ALTP,

and TA was extensively optimized to extract the latter analytes from cereal- and tomato-matrix. After the mycotoxin extraction and purification using solid phase extraction, the mycotoxins were analyzed by LC-MS/MS and quantified using either a SIDA or a matrix-matched calibration. To prove the reliability and robustness of the newly developed method, the sample preparation, as well as the LC-MS/MS measurements were validated. Utilizing potato starch (as a model for cereals) and fresh tomato purée as blank matrices for the method validation, limits of detection ranging from 0.05 to 1.25 µg/kg for starch and from 0.01 to 1.36 µg/kg for fresh tomatoes were determined. The limits of quantification ranged from 0.16 to 4.13 µg/kg for starch and from 0.02 to 5.56 µg/kg for tomatoes. Additionally, the recoveries of the mycotoxins were specified and varied between 83 and 108% for starch and between 95 and 111% for tomatoes. The outstanding intra-day precisions and inter-day precisions of 1 to 4% and 3 to 8% RSD in both matrices proved the stability and the robustness of the method.

The newly developed sample preparation protocol and the LC-MS/MS method were applied to determine the *Alternaria* mycotoxin contamination in various cereal based infant foods, jars containing vegetables and fruits, as well as tomato products for infants (n = 25). While AOH, ATX I, and ALTP were only detected rarely, the infant food products were frequently contaminated with TA, AME, and TEN. High contents were calculated for TA, whereas only low contents of AME and TEN were determined. Scattered infant food products were highly contaminated with TA and AOH and the consumption of these individual products might pose a risk to the health of infants. However, the mean and median of each individual *Alternaria* mycotoxin did not indicate a toxicological risk to infants.

5.1.2 Author Contributions

Marina Gotthardt designed the experiments for the mycotoxin extraction form cereal matrix and for the optimization of the mycotoxin purification by solid phase extraction. Furthermore, Marina Gotthardt planned the experiments for the method validation in cereal and tomato matrix and evaluated all LC-MS/MS data. Besides, Marina Gotthardt calculated the *Alternaria* mycotoxin contents in the infant food products. Marina Gotthardt wrote the manuscript, designed all figures, and revised the manuscript in accordance to the comments of the reviewers.

Stefan Asam and Michael Rychlik supported the development of the experimental design. Besides, Stefan Asam participated in the writing process of the manuscript. Under the supervision of Marina Gotthardt, the students Klara Gunkel, Roland Kietz, and Elisabeth Baumann as well as the trainee Atefeh Fooladi Moghaddam performed the experiments.

5.2 Comprehensive Analysis of the *Alternaria* Mycobolome Using Mass Spectrometry Based Metabolomics

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Authors: Marina Gotthardt, Basem Kanawati, Frank Schmidt, Stefan Asam, Richard Hammerl, Oliver Frank, Thomas Hofmann, Philippe Schmitt-Kopplin, Michael Rychlik

The published manuscript and the Supporting Information were inserted into Appendix B and C.

5.2.1 Summary

Alternaria fungi biosynthesize a variety of secondary metabolites and some of these fungal compounds have been characterized to act as mycotoxins exhibiting adverse effects against animals and humans. However, many *Alternaria* secondary metabolites have not been identified yet and their toxicological potential remains unknown.

Various fungal isolates of the species *A. alternata* and *A. solani* were cultivated in a chemically defined liquid medium. After the desalting of the fungal extracts, the latter were subjected to non-targeted analysis by FT-ICR-MS. Additionally, the non-targeted measurements were complemented by a targeted analysis of the *Alternaria* mycotoxins by LC-MS/MS. The ultra-high resolution of the FT-ICR-MS instrument allowed the clear assignment of molecular formulae comprising C, H, N, O, P, and S to 35% of the measured *m/z* signals. After the molecular formulae assignments, the elemental compositions were matched to the entries of the database *Antibase*. Nevertheless, only 3% of the molecular formulae were allocated to fungal metabolites. An additional database search against the entries of the *KEGG* database resulted in 18% of assignments of the molecular formulae to general cellular metabolites. The non-targeted measurements allowed the clear distinction between the fungal species *A. alternata* and *A. solani* and some discriminating metabolites between the two species were putatively identified by performing a database search against *Antibase*. Additionally, one of the discriminating metabolites between *A. alternata* and *A. solani* was isolated, characterized by MS and ^{1}H, ^{13}C, and 2D-NMR, and identified as alterperylenepoxide A-9-mercaptolactate. The results of the non-targeted measurements were supported by LC-MS/MS measurements, as the mycotoxin profiles of the *Alternaria* isolates also revealed great differences.

5.2.2 Author contributions

Marina Gotthardt designed the study. Furthermore, Marina Gotthardt performed the cultivation of the fungi as well as the sample preparation. Marina Gotthardt analyzed the fungal samples by FT-ICR-MS and LC-MS/MS. Additionally, Marina Gotthardt designed the extraction and purification procedure of APML. Besides, Marina Gotthardt wrote the manuscript, designed all figures, and revised the manuscript in accordance to the comments of the reviewers.

Michael Rychlik and Philippe Schmitt-Kopplin supported the development of the experimental design. Basem Kanawati contributed to the FT-ICR-MS measurements. Under the supervision of Marina Gotthardt, Frank Schmidt accomplished the extraction and purification of APML. Frank Schmidt, Richard Hammerl, and Oliver Frank performed the NMR analyses.

6 Discussion

In the present thesis, two analytical approaches, namely targeted and non-targeted analysis, were pursued to investigate metabolites biosynthesized by *Alternaria* fungi.

Following the targeted approach, the extraction and purification of six *Alternaria* mycotoxins from two food matrices was optimized and a sensitive LC-MS/MS method for the accurate identification and quantification of these analytes was developed. Low LODs and LOQs were determined during the method validation proving the sensitivity of the method for all analytes under investigation. Besides, the high recoveries and outstanding precisions demonstrated the accuracy and robustness of the newly developed method. Studies on the *Alternaria* mycotoxin contamination in infant foods are currently rare (Scott et al., 2012; Asam and Rychlik, 2013). Thus, the new method was applied to evaluate the contamination situation of infant foods with six *Alternaria* mycotoxins for the first time. Surprisingly, scattered infant food products contained very high amounts of AOH and TA constituting a possible risk to the health of infants. However, the overall mean and median of the mycotoxin contamination was low. The consumption of these infant food products was evaluated and did not constitute a risk to the health of infants on average.

In the non-targeted approach, primary and secondary metabolites biosynthesized by *A. alternata* and *A. solani* isolates were investigated by ultra-high resolution FT-ICR-MS for the first time. The ultra-high resolution of FT-ICR-MS allowed the unambiguous assignment of molecular formulae to the measured mass signals by mass difference network analysis. Subsequently, database searches provided structural hints for some of the molecular formulae. The database *Antibase* was utilized to assign the elemental compositions to

secondary metabolites of *Alternaria* fungi described in the literature. A database search in *KEGG* allowed the allocation of the molecular formulae to metabolites of the primary metabolism. However, only a small percentage of the molecular formulae was covered by the two different databases and the identification of the fungal metabolites was only possible for some of the *Alternaria* mycotoxins due comparison with the targeted LC-MS/MS measurements. Applying targeted LC-MS/MS investigations, variations in the mycotoxin profiles of the *Alternaria* isolates were identified. Additionally, statistical evaluations of the FT-ICR-MS data allowed the determination of similarities and differences in the metabolic capabilities of the *A. alternata* and *A. solani* isolates and these similarities and differences were putatively identified applying a database search in *Antibase*. Finally, one of the metabolites solely biosynthesized by *A. alternata* was laboriously extracted and purified applying several consecutive purification steps. In-depth NMR and UV measurements allowed the identification of the metabolite APML. The very detailed results of the NMR and UV measurements of APML were published for the first time.

In the following sections, the analytical methods applied in this thesis, the achieved results, as well as the conclusions drawn from the results will be discussed.

6.1 Analytical methods

6.1.1 Analyte extraction and purification

The objectives of targeted and non-targeted investigations are contradictory to each other. In targeted analyses, the analyte extraction and the sample preparation are designed and optimized for a small number of analytes only (Forcisi et al., 2013). In contrast, non-targeted applications rely on a minimalistic and non-selective sample preparation to cover as many metabolites as possible (Courant et al., 2014).

In the present targeted investigation of six *Alternaria* mycotoxins, three consecutive extraction steps were inevitable to guarantee a quantitative analyte coverage from two different food matrices. The implementation of only one extraction step during the method development resulted in insufficient recoveries for most of the analytes. While the dibenzo-α-pyrones, TEN, and TA were already extracted, sufficiently, applying two extractions using an acidified acetonitrile/water mixture (84/16; v/v), the comprehensive extraction of the perylene quinones was only possible by implementing a third extraction step using a mixture of acetonitrile/methanol/water. In the literature, *Alternaria* mycotoxins are frequently extracted using acidified solvent mixtures such as acetonitrile/water/acetic acid, acetonitrile/water/formic acid, methanol/water/acetic acid, or acetonitrile/water/methanol (Noser

et al., 2011; Walravens et al., 2014; Siciliano et al., 2015; Hickert et al., 2016b; Puntscher et al., 2018). However, many applications only perform one extraction step (Walravens et al., 2014; Siciliano et al., 2015; Hickert et al., 2016b; Zwickel et al., 2016; Puntscher et al., 2018). Thus, low recoveries of only 50 % were determined for AME, TEN, STTX III, and TA in tomatoes, sun flower seeds, and bakery products (Noser et al., 2011; Hickert et al., 2016b; Puntscher et al., 2018).

After the extraction of the mycotoxins, matrix components potentially contaminating the ESI source of the analytical instrument were removed by SPE in the present thesis. A C18 cartridge served as solid phase and methanol was utilized for the elution of the analytes. However, a second elution step under basic solvent conditions was inevitable to quantitatively elute TA from the C18 material, finally enabling the simultaneous purification of TA together with the other *Alternaria* mycotoxins. In the literature, multi-mycotoxin applications, which also include TA, often waive the analyte purification by SPE (Walravens et al., 2014; Hickert et al., 2016b; Puntscher et al., 2018). Other studies utilized solid phases of diatomaceous earth or Bond Elut Plexa cartridges for the simultaneous purification of TA and other *Alternaria* mycotoxins (Noser et al., 2011; Zwickel et al., 2016). However, Liu et al. (2015) simply omitted TA in their analysis of *Alternaria* mycotoxins.

The extraction and purification procedure of the present method resulted in low LODs and LOQ, good recoveries, and outstanding precisions during the method validation for all analytes. However, the application of three consecutive extraction steps as well as the implementation of SPE is very time-consuming and contradictory to cost effectiveness. To improve the time- and cost effectiveness during the analysis of *Alternaria* mycotoxins, QuEChERS and the dilute-and-shoot approach were applied. Both techniques rely on the implementation of only one extraction step and a subsequent purification of the extracts is omitted (Walravens et al., 2016; Puntscher et al., 2018). While the QuEChERS procedure resulted in a quantitative extraction of the dibenzo-α-pyrones, their modified forms, ATX I, TEN, and TA from fruit and vegetable juices and lyophilized products (Walravens et al., 2016), the dilute-and-shoot approach suffered from an insufficient extraction of the *Alternaria* mycotoxins from various matrices. Thus, low recoveries of 19 and 28% were determined for altenusin and STTX III in wheat flour. Beside the partly low recoveries, also the intra-day precisions of the quantitative measurements were non-satisfactory resulting in intra-day precisions of 45, 53, and 83% RSD for ALTP, AME-3-Glc, and ATX I in wheat flour (Puntscher et al., 2018).

The non-targeted investigations in the present thesis focused on the comprehensive analysis of primary and secondary metabolites biosynthesized by *Alternaria* fungi. In general, the metabolites of a biological sample are highly diverse and exhibit different capabilities

regarding hydrophilicity/hydrophobicity, volatility, chemical reactivity, and stability. Thus, the sampling, sample preparation, and analytical measurements clearly discriminate for some of the metabolites or for classes of compounds. Consequently, the analyte extraction should be chosen as non-selective as possible and the sample preparation should be reduced to a minimum in non-targeted metabolomics investigations (Courant et al., 2014; Nielsen and Larsen, 2015). Liquid samples or biofluids are usually diluted and directly analyzed by FT-ICR-MS allowing a high metabolite coverage (Courant et al., 2014; Roullier-Gall et al., 2014b; Roullier-Gall et al., 2015; Roullier-Gall et al., 2018). Nevertheless, in some cases, clean-up steps such as a desalting of the liquid samples by SPE might be indispensable prior to mass spectrometric analysis (Nielsen et al., 2003; Schmitt-Kopplin et al., 2010; Lucio et al., 2011; Courant et al., 2014) although losses of metabolites might occur (Lucio et al., 2011). In the present thesis, the *A. alternata* and *A. solani* isolates were cultivated in a chemically defined liquid medium comprising salts and carbon sources essential for fungal growth. However, the salts of the liquid medium hampered the direct injection of the samples into the ESI source of the FT-ICR-MS. Thus, a desalting step prior to the mass spectrometric analysis of the fungal metabolites was indispensable despite the potential loss of fungal metabolites. Liquid-liquid partitioning was not promising, as the extraction of the metabolites from the aqueous matrix by dichloromethane led to contaminations of the fungal extracts in the present thesis. Thus, SPE was preferred for the desalting of the liquid medium and C8 material served as stationary phase. The pH of the liquid medium was adjusted to acidic conditions prior to the desalting as more than 50% of all metabolites exhibit acidic moieties (Nielsen and Larsen, 2015).

Beside the analysis of liquid media, the *Alternaria* mycotoxins present inside the fungal cells were also analyzed in the present thesis. The fungal cells were disrupted using Nucleo Spin bead tubes (Machery Nagel, Düren, Germany) and a Precellys® Homogenizer (bertin instruments, Montigny-le-Bretonneux, France) and the analytes were extracted most efficiently using an ice-cold methanol/water-mixture. A subsequent solid phase extraction of the cell extracts prevented the ESI source from contaminations due to remaining salts. In the literature, the metabolites are usually extracted from solid matrices utilizing pure organic solvents such as chloroform (Cano et al., 2013), ethyl acetate (Heinke et al., 2014), and dichloromethane (Nielsen and Larsen, 2015). Besides, solvent-water mixtures such as (ice-cold) methanol/water are frequently applied (Müller et al., 2013; Bazanella et al., 2017; Roullier-Gall et al., 2018). To cover hydrophilic and hydrophobic metabolites simultaneously, chloroform-methanol or chloroform-methanol-water mixtures were utilized (t'Kindt et al., 2009; El Rammouz et al., 2010).

6.1.2 Targeted and non-targeted measurements

Over the years, LC-MS/MS has developed to one of the most frequently applied methods for the qualitative and quantitative analysis of mycotoxins. Nowadays, this technique is considered as the method of choice for the accurate identification of fungal-derived compounds (EFSA on Contaminants in the Food Chain, 2011; SANTE/12089/2016, 2016; Man et al., 2017). In the present thesis, LC-MS/MS was utilized to quantitatively determine dibenzo-α-pyrones, perylene quinones, TEN and TA. The *Alternaria* mycotoxins AOH, AME, TEN, ATX I, and ALTP were chromatographically separated utilizing a HyperClone BDS C18 column as solid phase. A mixture of acetonitrile/2-propanol/water (17.5/17.5/65; v/v/v) served as mobile phase A and pure methanol was utilized as solvent B. As TA is a strong acid and a very polar compound, the tetramic acid derivative exhibited too low retention in the prevailing chromatographic system and an additional measurement was indispensable. The utilization of basic solvent conditions (pH = 9) of mobile phase A allowed a constant chromatographic behavior of TA during the measurements on a Gemini-NX C18 column and the addition of ammonium formate to the waterous solvent increased the sensitivity during the mass spectrometric measurements. In the literature, many investigations focused on the analysis of TA after the derivatization of the molecule using 2,4-dinitrophenylhydrazine, as the derivative exhibited both constant chromatographic and mass spectrometric behavior (Siegel et al., 2009; Asam et al., 2011a; Asam et al., 2012). Nowadays, TA is more and more included in multi-mycotoxin methods without previous derivatization (Noser et al., 2011; Walravens et al., 2016; Zwickel et al., 2016; Puntscher et al., 2018). Multi-mycotoxin methods, which include TA as a target analyte, rely either on acidified solvent conditions (Walravens et al., 2014; Hickert et al., 2016b) or on basic conditions during chromatographic separation (Zwickel et al., 2016; Puntscher et al., 2018), as an impaired peak shape was determined for TA during chromatographic separation with neutral solvents (Walravens et al., 2014). Beside TA, also the modified forms of AOH and AME, namely AOH-3-Glc, AOH-9-Glc, AME-3-Glc, AOH-3-S, and AME-3-S, are more and more included in multi-mycotoxin methods. Due to the more polar capabilities of the modified forms compared to the free forms, the gradient elution usually started with 5 or 10% organic phase to ensure sufficient retention of AOH-3-Glc, AOH-9-Glc, AME-3-Glc, AOH-3-S, and AME-3-S on C18 solid phases (Walravens et al., 2014; Puntscher et al., 2018).

ATX II and STXX III were qualitatively included in the present targeted investigation, but neither a method validation nor quantitative measurements could be performed due to the lack of sufficient amounts of reference compounds. As only AOH, AME, TEN, ALT, TA, and the AAL toxins TB1 and TB2 are currently commercially available, reference substances of the perylene quinones, modified dibenzo-α-pyrones, and of other *Alternaria* mycotoxins

are either chemically synthesized (Altemöller et al., 2006; Nemecek et al., 2012; Nemecek et al., 2013; Walravens et al., 2014) or obtained after a preparative purification of extracts of *Alternaria* fungi (Fleck et al., 2012; Schwarz et al., 2012; Fleck et al., 2014; Liu and Rychlik, 2015).

Targeted analyses usually aim at accurate quantifications of the analytes under investigation. As mass spectrometric measurements frequently suffer from ion suppression, either standard addition, matrix-matched calibration, or SIDA are applied to obtain reliable quantitative results (Jessome and Volmer, 2006; Rychlik and Asam, 2008; Asam et al., 2009). In the present thesis, SIDAs were performed for AOH, AME, and TA utilizing the stable isotopic labeled standards [$^{2}H_{4}$]-AOH, [$^{2}H_{4}$]-AME, and [$^{13}C_{6}$,^{15}N]-TA. Due to lacking deuterated or ^{13}C-labeled internals standards of TEN, ATX I, and ALTP, matrix-matched calibrations were applied for the latter analytes. In general, SIDAs allow most accurate quantifications of the analytes (Rychlik and Asam, 2008), but good results were also obtained for the mycotoxins quantified by matrix-matched calibration in the present investigation. A great obstacle in the development of SIDAs is the poor commercial availability of the stable isotopic labeled internal standards. Hence, the internal standards are usually either chemically synthesized (Asam et al., 2009; Asam et al., 2011a; Liu and Rychlik, 2013; Lohrey et al., 2013) or are obtained after biosynthesis during fungal cultivation on labeled medium (Liu and Rychlik, 2015).

Prior to sample analysis, the suitability and the accuracy of novel methods need to be proven by method validations. In the present investigation, potato starch served as blank matrix for the method validation as no cereal flour from the supermarket was free from *Alternaria* mycotoxins. Besides, fresh tomato purée was chosen as a blank matrix to represent the matrix of fruit and vegetable based infant food in jars and tomato products. The LODs and LOQs of the *Alternaria* mycotoxins were determined according to Vogelgesang and Hädrich (Vogelgesang, 1987; Vogelgesang and Hädrich, 1998). Low LODs of 0.05 to 1.25 µg/kg and 0.01 to 1.36 µg/kg were determined for AOH, AME, TEN, ATX I, ALTP, and TA in starch and fresh tomato purée. The LOQs ranged from 0.16 to 4.13 µg/kg and from 0.02 to 5.56 µg/kg in starch and tomato matrix. The LODs and LOQs of the *Alternaria* mycotoxins in starch matrix were similar to those determined in starch, wheat, rice, oat, and barley matrix in the literature (Walravens et al., 2014; Liu and Rychlik, 2015; Puntscher et al., 2018). Compared to the literature (Zwickel et al., 2016; Puntscher et al., 2018), very low LODs and LOQs were determined for AME and TEN in tomato matrix in the present thesis. Due to the low LODs and LOQs of TA in starch as well as in the tomato matrix in the present investigation, the derivatization of the tetramic acid derivative utilizing 2,4-dinitrophenylhydrazine was not required (Siegel et al., 2009; Asam et al., 2011a). Beside the LODs and LOQs, recoveries were determined for the *Alternaria* mycotoxins at three different spiking

levels, respectively. Although high recoveries were determined for all analytes at different spiking levels in the present study, improved recoveries might be obtained for TEN applying a SIDA. In the present investigation, the recoveries of TEN ranged from 83 to 91% applying matrix-matched calibration, whereas Liu et al. (2013) calculated recoveries of 98 to 112% utilizing [$^{2}H_{3}$]-TEN as internal standard. Various precisions of the newly developed method were specified during the method validation. The inter-injection precisions were below 4% for all analytes in both matrices. The intra-day precisions ranged from 1 to 3% and the inter-day precisions varied from 3 to 8%. The precisions of the present investigation were comparable to those obtained by Zwickel et al. (2016). However, if the *Alternaria* mycotoxins were analyzed following a dilute-and-shoot approach, intra-day precisions of up to 83% were determined (Puntscher et al., 2018). During the method validation, the accuracy of the method is usually specified using certified reference materials (EC 2002/657/EG, 2002; Thompson et al., 2002; Capriotti et al., 2012; Malachová et al., 2014). However, no reference material for *Alternaria* mycotoxins is currently available. Thus, the accuracy of the present method could not be determined.

In contrast to targeted surveys, non-targeted investigations aim at comprehensive determinations of all metabolites of biological samples (Dettmer et al., 2007). Although the sensitivity, dynamic range, scan speed, resolution, as well as the accuracy of various mass analyzers have evolved over the years (Vos et al., 2007; Han et al., 2008; Okazaki and Saito, 2012; Nakabayashi et al., 2013), the comprehensive analysis of the entire chemical diversity of a biological sample is still not feasible in current non-targeted metabolomics investigations (Dettmer et al., 2007). In the present thesis, FT-ICR-MS was utilized to analyze the mycobolome of various *Alternaria* isolates at ultra-high mass resolution ($R = 10^{5}$-10^{6}) (He et al., 2001; Bossio and Marshall, 2002; Brown et al., 2005) and great mass accuracy enabling the distinction of mass differences of less than an electron mass (Marshall et al., 1998; He et al., 2001; Forcisi et al., 2013). Therefore, isobaric compounds could be separated and elemental compositions could be assigned to the measured *m*/*z* signals (Okazaki and Saito, 2012). The sensitivity of the ion detection was improved by accumulating 300 scans for each sample. Thus, ions of low relative abundance were detected in the present investigation. However, FT-ICR-MS exhibits a lower mass limit of *m*/*z* 125 Da, which is rather high compared to other mass analyzers such as modern TOF instruments capable of analyzing metabolites of *m*/*z* 20 Da (Forcisi et al., 2013). Hence, many metabolites biosynthesized by *Alternaria* fungi exhibiting a mass below 125 Da were not determined in the present thesis. Another disadvantage of FT-ICR-MS constitutes the long scanning time required for the ultra-high resolution of a mass spectrum. Therefore, coupling FT-ICR-MS to chromatographic systems is not possible without the loss of ultra-high resolution (Moritz et al., 2013).

As isomers cannot be separated by direct injection mass spectrometry, FT-ICR-MS lacks the ability to identify compounds with identical molecular formula (Forcisi et al., 2013).

Another obstacle in mass spectrometric analyses involves the suppression or enhancement of ions due to matrix components (Sulyok et al., 2006; Scalbert et al., 2009) with ESI being more affected than APCI (Annesley, 2003). However, ESI and APCI allow the ionization of metabolites without the destruction of heat-functional groups or chemically unstable substructures. Besides, compounds with high vapor points and of high molecular weights can be ionized (Okazaki and Saito, 2012).

Although ESI is a soft ionization technique and only little ion fragmentation occurs during the measurements, several ions might be formed for one biological metabolite. Apart from $[M+H]^+$ and $[M-H]^-$ ions, adducts with neutral solvent molecules or metal and alkali ions such as $[M+Na]^+$ and $[M+K]^+$ might be generated during the ionization (Keller et al., 2008; Forcisi et al., 2013; Bueschl et al., 2014). Additionally, the isotope signals of a biological metabolite are present in the mass spectra (Forcisi et al., 2013; Bueschl et al., 2014). Beside the multitude of mass signals originating from only one biological metabolite, many background signals might be detected in a mass spectrum originating from contaminations and impurities as well as FT artifacts during measurements hampering the correct identification of biologically-derived mass signals (Keller et al., 2008; Trötzmüller et al., 2011; Kanawati et al., 2017).

6.2 *Alternaria* mycotoxin contamination in infant foods

Infants represent the most endangered group of consumers regarding an *Alternaria* mycotoxin contamination in food products (EFSA on Contaminants in the Food Chain, 2011). Thus, the newly developed LC-MS/MS method was utilized to accurately quantify six *Alternaria* mycotoxins in cereal-based infant foods in the present thesis. As the extraction and purification of the mycotoxins were totally applicable to tomato matrix, jars and tomato-based products were also investigated for *Alternaria* mycotoxins. Strikingly, almost all the cereal-based infant foods as well as the jars and tomato products contained the fungal secondary metabolites. TA was determined most frequently in both sample types and was quantified in 17 out of 19 cereal-based samples as well as in all tomato-based products above the respective LOQs. The content of TA ranged from 5.66 to 149 µg/kg in the cereal-based products, whereas the tomato sauce and tomato soup tested contained 505 and 8.73 µg/kg of TA, respectively. AME and TEN were determined frequently in the cereal-based products with contents varying from 0.23 to 0.58 µg/kg and 0.30 to 7.53 µg/kg, respectively. The content of AOH ranged from 4.73 to 7.17 µg/kg. ATX I and ALTP were detected above the LOQs in only one cereal-based infant food product, respectively. The

Alternaria mycotoxin contamination in the jars and the tomato soup was mainly low. However, the tomato sauce contained 54.2 µg/kg of AOH, 7.56 µg/kg of AME, 0.44 µg/kg of TEN, and 505 µg/kg of TA, respectively, and we called the manufacturer's attention to this specific product. Despite the partly highly contaminated infant foods, the mean and median of the *Alternaria* mycotoxin contamination in the cereal-based products, jars, and tomato products were low.

So far, only scattered investigations have been conducted on the *Alternaria* mycotoxin contamination in infant food products. One of these surveys focused on the analysis of AOH and AME in cereal-based infant foods from the Canadian market. AOH and AME were determined in all samples analyzed and the contents of AOH were similar to those determined in the present investigation. However, the contents of AME determined by Scott et al. (2012) exceeded those of the present thesis by a factor of up to 30 (Scott et al., 2012). A study, conducted by Asam and Rychlik (2013), focused on the analysis of TA present in various cereal-based infant food products, jars, and teas for infant consumption. Low contents of TA were determined in the tea products and the jars. While the infant foods based on wheat, oat, barley, and spelt contained low contents of TA, the products based on millet were highly contaminated with TA with contents ranging from 130 to 1200 µg/kg (Asam and Rychlik, 2013). Due to the high contamination rates in millet-based infant foods, Rychlik et al. (2016) claimed a maximum contamination level of millet-based infant food products of 500 µg/kg of TA. However, the TA contents in the millet-based products of the present study were far below this maximum level. Thus, we concluded that the manufacturers already optimized their quality control regarding the TA contamination in millet-based products. Comparing the contents of the *Alternaria* mycotoxins of the present tomato sauce to tomato products analyzed in the literature, similar contents were determined for AOH, AME, and TA in tomato sauces (Walravens et al., 2016; Puntscher et al., 2018) and a tomato purée (Noser et al., 2011). Nevertheless, the tomato products from the literature were not considered for infant consumption (Noser et al., 2011; Walravens et al., 2016; Puntscher et al., 2018).

As some of the *Alternaria* mycotoxins were omnipresent in the infant foods of the present investigation as well as in the literature, the manufacturers should include these mycotoxins into their routine analyses of agricultural raw materials and manufactured food products. The present investigation covered 25 cereal-based infant foods and jars of different manufacturers and from different supermarkets and drug stores. However, more products should be analyzed to obtain a comprehensive overview on the actual contamination situation of the infant foods with *Alternaria* mycotoxins. In addition to the mycotoxins AOH, AME, TEN, ATX I, ALTP, and TA, the modified forms of AOH and AME, namely AOH-3-

Glc, AOH-9-Glc, AME-3-Glc, AOH-3-S, and AME-3-S, should also be included in the present method as these modified forms might contribute to the toxicological impacts of the free forms after oral consumption (Berthiller et al., 2005; Berthiller et al., 2007). However, only AOH, AME, TEN, ALT, TA, and the AAL toxins TB1 and TB2 are currently commercially available. Thus, reference compounds of the perylene quinones and the modified forms of AOH and AME need to be synthesized chemically or prepared biosynthetically by cultivating *Alternaria* fungi and subsequent purification of the analytes (Walravens et al., 2014; Liu and Rychlik, 2015).

6.3 Risk evaluations on *Alternaria* mycotoxins

Until today, neither maximum limits in food and feed nor TDIs or PMTDIs have been established for *Alternaria* mycotoxins due to the insufficient availability of data on toxicology and occurrence. In a first risk assessment on AOH, AME, TEN, and TA, conducted by the EFSA in 2011, the TTC approach was applied to assess the relative level of concern to human health originating from these toxins (EFSA on Contaminants in the Food Chain, 2011). The TTC for TEN and TA was set to 1500 ng/kg b.w. per day, as the two mycotoxins are not considered to be genotoxic. Contrarily, AOH and AME were proven to be genotoxic and, therefore, the TTC of AOH and AME was set to 2.5 ng/kg b.w. per day (EFSA on Contaminants in the Food Chain, 2011).

In the present thesis, a risk assessment was conducted on the basis of the mycotoxin contents of AOH, AME, TEN, and TA determined in the cereal- and tomato-based infant foods and in jars. Occasionally, high contents of AOH were detected in one spelt (7.17 µg/kg) and one multi-grain product (4.73 µg/kg), respectively, and in the tomato sauce (54.2 µg/kg). Considering the weight of infants and the size of a portion as suggested by the manufacturers, the TTC of AOH of 2.5 ng/kg b.w. per day would be exceeded many times over when consuming these highly contaminated products. Thus, a risk to the health infants could not be excluded. However, the mean and median contents of AOH in the cereal-based products and the jars were rather low, and the consumption of the infant foods was considered not to pose a risk to the health of infants on average. Although AME and TEN were frequently determined in most of the analyzed infant food products, the contents were rather low. Hence, the intake of these mycotoxins was assumed unlikely to pose a risk to the health of infants. TA was determined frequently in the present cereal- and tomato-based products as well as in the jars. Occasionally, high contents of TA were determined, but none of the TA contents in the cereal-based products exceeded the maximum level of 500 µg/kg as claimed by Rychlik et al. (2016). Thus, the TTC level of 1500 ng/kg body weight per day would not be exceeded due to the consumption of these products. Regarding the toxicological

evaluation of TA, the structural isomer *allo*-TA should also be determined in future investigations. As *allo*-TA is non-cytotoxic, the risk originating from TA might be overestimated, as TA is mainly determined as sum of TA and *allo*-TA (Hickert et al., 2015).

The number of the infant food products analyzed in the present thesis was limited to n = 25. To perform a reliable risk evaluation on a European level on *Alternaria* mycotoxins and to reduce the uncertainty originating from left-censored data, more infant food products should be analyzed and the mycotoxin contents should be quantified precisely utilizing sensitive and reliable methods (EFSA on Contaminants in the Food Chain, 2011; Lorenz et al., 2012; Asam and Rychlik, 2015; Arcella et al., 2016). Thus, the EFSA claimed the commercial availability of analytical standards for the *Alternaria* mycotoxins and the standardization of analytical methods on a national and international level. Besides, reference materials for *Alternaria* mycotoxins should be introduced to perform inter-laboratory validation studies (EFSA on Contaminants in the Food Chain, 2011).

Not only the free forms of mycotoxins pose a risk to the consumer, as the modified forms are possibly cleaved during digestion releasing the aglycon and, therefore, probably contributing to the toxicological effects of the free mycotoxins (Berthiller et al., 2005; Berthiller et al., 2007). Regarding *Alternaria* mycotoxins, especially AOH-9-Glc, AOH-3-S, and AME-3-S should be included in future surveys on occurrence and toxicology, as these modified forms have already been determined in tomato-based products (Walravens et al., 2016; Puntscher et al., 2018).

As the mycotoxins enter the human food chain not only via agricultural products but also via meat, milk, and eggs, the carry-over from feed to food products such as meat, milk, and eggs should be investigated (Lorenz et al., 2012). Furthermore, the potential synergistic effects of mycotoxin cocktails caused by different fungal genera such as *Aspergillus*, *Fusarium*, and *Penicillium* should be investigated (Krska et al., 2017).

6.4 Non-targeted investigations on the *Alternaria* metabolome

6.4.1 Identification of biologically-derived mass signals in non-targeted metabolomics investigations

The key issues in non-targeted metabolomics investigations represent the assignment of elemental compositions to mass signals of biologically-derived metabolites as well as the structural identification of the annotated molecular formulae (Moritz et al., 2013). Biological samples comprise metabolites, which are interconnected via chemical and enzymatic reactions (Breitling et al., 2006a; Breitling et al., 2006b). On this basis, the elemental compositions of biologically-derived mass signals determined at ultra-high mass resolution and superior mass accuracy were assigned unambiguously by applying mass difference network analysis in the present thesis (Tziotis et al., 2011). As a consequence, non-related mass signals as well as background signals and noise were excluded from the mass spectrometric data (Tziotis et al., 2011; Forcisi et al., 2013). The present mass difference network was constructed by starting from already known *Alternaria* metabolites such as dibenzo-α-pyrones and perylene quinones. Biochemical reactions from the primary as well as from the secondary metabolism of *Alternaria* fungi served as mass differences to interconnect the measured *m/z* signals. However, a multitude of secondary metabolites biosynthesized by *Alternaria* fungi are currently still unknown. Besides, biosynthetic pathways of a multitude of secondary metabolites have not been elucidated so far. Thus, not only the starting points of the present network but also the mass difference list need to be considered incomplete hampering the comprehensive annotation of the entire *Alternaria* mycobolome. However, the construction of the mass difference network allowed the annotation of elemental compositions to measured mass signals of currently unknown compounds improving the determination of new fungal metabolites.

Another approach frequently applied for the identification of fungal-derived metabolites constitutes stable isotopic labeling (Bueschl et al., 2017). This technique aims at improving the detection of biologically-derived metabolites by artificially labeling these compounds with stable isotopes. ^{13}C-labeling has become the method of choice over the last years due to the ubiquitous presence of carbon atoms in biological metabolites (Klein and Heinzle, 2012; Bueschl et al., 2017). Microorganisms need to be cultivated in parallel on chemically defined media comprising only native or ^{13}C-enriched carbon sources (Birkemeyer et al., 2005; Bueschl et al., 2017). During parallel cultivation under identical conditions, an equal metabolization of native and ^{13}C-enriched carbon sources by the microorganism is assumed (Klein and Heinzle, 2012; Bueschl et al., 2017). Native and stable isotope-enriched biological samples are mixed and analyzed by mass spectrometry (Winder et al., 2011; Klein and

Heinzle, 2012). The mass spectra comprise mass signals of metabolites exhibiting native carbon isotopic distributions as well as ^{13}C-enriched isotopic distributions (Bueschl et al., 2017). The number of carbons of the respective compounds can be calculated from the mass shifts between the *m*/*z* signals of monoisotopic native and corresponding monoisotopic fully ^{13}C-labeled metabolites (Birkemeyer et al., 2005; Bueschl et al., 2013). As a consequence, stable isotopic labeling not only enhances the detection of biologically-derived metabolites but also improves molecular formula annotation (Kluger et al., 2013; Bueschl et al., 2017). Thus, stable isotope labeling might be a promising technique to improve the detection of fungal derived metabolites in non-targeted metabolomics investigations of *Alternaria* fungi.

6.4.2 Structural identification of metabolites in non-targeted investigations

Once elemental compositions were assigned to measured mass signals in the present thesis, the molecular formulae were searched against databases to obtain provisional structural hints. However, when searching the molecular formulae against two different databases, namely the *Antibase* database and *KEGG*, structural candidates were obtained for only 3% and 18%, respectively, of the elemental compositions. These findings confirm the still limited knowledge of the *Alternaria* mycobolome. Nevertheless, this problem is not only limited to metabolites biosynthesized by *Alternaria* fungi but extends to all existing metabolites. In the area of foodomics, the entirety of all existing metabolites in unprocessed foods, which was estimated to range around 500,000, will be known earliest by around 2025 (Rychlik and Schmitt-Kopplin, 2020). Another problem in non-targeted investigations constitutes the multitude of different databases differing in their accessibility and content. While *PubChem* or *KEGG* are publicly available (Kind and Fiehn, 2006), the access to the *Antibase* database and the *Dictionary of Natural Products* is restricted (Kind et al., 2009; Laatsch, 2017). *KEGG* allows the allocation of metabolites to species-specific pathways of the primary metabolism (Kanehisa et al., 2012), whereas the *Dictionary of Natural Compounds* does not include primary metabolites at all. Besides, cross-database queries are limited and the correctness of the database entries is not guaranteed by peer-reviewed processes (Kind et al., 2009).

A database search frequently results in multiple structural candidates for one elemental composition. Regarding *Alternaria* secondary metabolites, several structural isomers have been reported for one molecular formula such as ATX II/ALTP for $C_{20}H_{14}O_6$ (Podlech et al., 2014), alterlosin I/STTX I for $C_{20}H_{14}O_7$, alterlosin II/7-epi-8-hydroxy-altertoxin I/stemphyriol/6-epi-stemphytriol for $C_{20}H_{16}O_7$ (Stierle et al., 1989; Podlech et al., 2014), altersolanol A/altersolanol D-F for $C_{16}H_{16}O_8$ (Suemitsu et al., 1990; Okamura et al., 1993), or tricycloalternarene 6a/b/bicycloalternarene 2/bicycloalternarene 8/tricycloalternarene E for $C_{21}H_{32}O_5$ (Nussbaum et al., 1999; Liebermann et al., 2000; Yuan et al., 2008). Additionally, yet

unknown structural isomers hamper the correct identification of metabolites. For instance, Zwickel et al. (2018) determined four structural isomers for the molecular formula $C_{20}H_{14}O_6$ utilizing high resolution MS coupled to chromatography, but only ATX II and ALTP could be identified, whereas the other structural isomers remained unknown. However, the mass accuracy of the measurements was limited to 5 ppm (Zwickel et al., 2018).

High resolution mass spectrometers are often coupled to chromatography in non-targeted investigations, as additional information on the number of structural isomers as well as their physico-chemical properties are obtained (Scalbert et al., 2009; Forcisi et al., 2013). By generating at least two independent analytical characteristics for one metabolite during LC-high resolution MS, namely retention time, *m*/*z* signal, and sometimes MS/MS pattern, a metabolite can be clearly identified, if the corresponding reference compound is analyzed under identical conditions (Sumner et al., 2007). In the present FT-ICR-MS measurements, only *m*/*z* signals were determined for the biological metabolites, which is insufficient to clearly identify a compound (Kind and Fiehn, 2006; Sumner et al., 2007). Thus, additional measurements of reference compounds of AOH, AME, TEN, ATX I, ATX II, STTX III, ALTP, and TA by LC-MS/MS were indispensable to clearly prove the presence of these *Alternaria* mycotoxins in the fungal extracts of *A. alternata* and *A. solani* in this thesis. However, authentic reference compounds are very rarely commercially available hampering the unequivocal identification of metabolites in non-targeted investigations (Scalbert et al., 2009). Hence, a multitude of metabolites commonly biosynthesized by *Alternaria* fungi such as bicycloalternarenes, tricycloalternarenes, solanapyrones, and altersolanols could only be identified putatively in the current thesis as no reference compounds were available (Sumner et al., 2007).

Beside the known compounds listed in databases, many unknown metabolites are usually detected in metabolomics investigations hampering a deep understanding of biochemical processes (Kind and Fiehn, 2006). Therefore, unknown metabolites require *de novo* characterizations by isolating and purifying the unknown compounds. Subsequently, the elemental compositions of the purified metabolites need to be determined and their chemical structures need to be identified by MS, MS/MS, ^{1}H, ^{13}C, and 2D NMR, as well as UV measurements (Sumner et al., 2007). In the present thesis, this elaborate procedure was conducted to structurally characterize the metabolite APML only biosynthesized by the *A. alternata* isolates. First, the cultivation of one of the *A. alternata* isolates was optimized. The fungus was cultivated on rice due to the production of higher amounts of APML when cultivated on rice than when cultivated in liquid medium. After the cultivation, the extraction of APML was conducted by utilizing a mixture of ethyl acetate/methanol (1/1; v/v). Subsequently, several purification steps utilizing two columns of silica and one column of C18 material were indispensable to separate APML. Finally, a preparative separation by

HPLC-UV was mandatory to obtain pure APML. The compound was analyzed by ^{1}H, ^{13}C, and 2D-NMR to determine the chemical structure of the fungal metabolite. Furthermore, the concentration of the purified compound was determined by qNMR. Finally, UV measurements were conducted to identify absorption maxima and the respective extinction coefficients. In future targeted investigations, APML will serve as an analytical standard for qualitative and quantitative analyses of food products contaminated with *Alternaria* mycotoxins.

6.4.3 Ultra-high resolution MS as a tool for the segregation of *Alternaria* species

The classification of *Alternaria* fungi has led to great confusion over the past centuries and a systematic revision within the genus *Alternaria* is held necessary, nowadays (Lawrence et al., 2013; Kahl et al., 2015). As *Aspergillus* and *Penicillium* species have been successfully segregated utilizing secondary metabolite profiles (Frisvad et al., 2008), several investigations focused on the mycotoxin profiles of *Alternaria* species for differentiation purposes (Andersen et al., 2002; Andersen et al., 2008; Kahl et al., 2015; Zwickel et al., 2018). However, HPLC-UV/Vis and HPLC-MS/MS measurements of secondary metabolites led to the differentiation of *A. infectoria* from other small-spored *Alternaria* species (Andersen et al., 2002; Zwickel et al., 2018), but a segregation of *A. alternata*, *A. tenuissima*, and *A. arborescence* was impossible (Zwickel et al., 2018). Thus, LC coupled to high resolution MS is increasingly applied, as several hundreds of metabolites are detected simultaneously instead of focussing on several secondary metabolites, only (Klitgaard et al., 2014; Andersen et al., 2015). In the present investigation, the *A. solani* isolate was separated from the three *A. alternata* isolates utilizing the mycotoxin profiles determined by LC-MS/MS. While no unusual *Alternaria* mycotoxin was detected in the liquid medium of the *A. solani* isolate, all *A. alternata* isolates biosynthesized ATX I, ALTP, ATX II, STTX III, and TEN. However, if present, only low amounts of AOH and AME were determined in the liquid media of the *A. alternata* isolates and we concluded that the present cultivation conditions did not favour the biosynthesis of the dibenzo-α-pyrones (Söderhäll et al., 1978). Differences in the biosynthesis of TA were determined between the three *A. alternata* isolates. While two of the *A. alternata* isolates biosynthesized high amounts of TA, the third *A. alternata* isolate did not produce TA at all. This result demonstrates the difficulties in the species segregation of *Alternaria* fungi based on mycotoxin profiles.

Beside the differences determined by the targeted measurements, the non-targeted metabolomics investigations resulted in a clear differentiation of *A. solani* from *A. alternata*. The differences were putatively identified as altechromone A (Könings et al., 2010) and quadrilineatin methylether (Birkinshaw et al., 1957) for *A. solani* and the perylene quinones

ATX III, alterlosins I and II (Stack et al., 1986; Stierle et al., 1989) as well as xanalteric acids I and II (Kjer et al., 2009) for *A. alternata*. While the targeted measurements of the mycotoxin profiles of the *A. alternata* isolates simply revealed differences in the mycotoxin production of TA, the non-targeted analysis of the mycobolome included a multitude of metabolites of the *A. alternata* isolates. Hence, two *A. alternata* isolates clustered together in the PCA plot, whereas the third isolate clustered apart from the other two *A. alternata* isolates. Several discriminating metabolites between the *A. alternata* isolates such as silvaticol (Kawahara et al., 1988), tenuissimasatin (Fang et al., 2012), or isopropyl tetramic acid (Royles, 1995) were statistically determined and putatively identified by performing a database search in *Antibase*. As ultra-high resolution mass spectrometric investigations determine hundreds to thousands of metabolites simultaneously, FT-ICR-MS might be a helpful tool to improve the species segregation of *Alternaria* isolates. However, this hypothesis should be examined by analyzing the metabolite profiles of a multitude of isolates of *Alternaria* fungi belonging to various species groups.

7 Summary

One part of the present thesis reports on the findings obtained by the targeted analysis of *Alternaria* mycotoxins in food products by LC-MS/MS. For the accurate determination of the *Alternaria* mycotoxins AOH, AME, TEN, ATX I, ALTP, and TA in food products, a sensitive LC-MS/MS method was developed. The extraction protocol for the latter mycotoxins was optimized for cereal matrix and required three subsequent extraction steps. To reduce the ion suppression effects during the mass spectrometric measurements due to interfering matrix components, the *Alternaria* mycotoxins were purified using SPE. The protocol for the simultaneous purification of AOH, AME, TEN, ATX I, ALTP, and TA was extensively optimized for DSC-18 cartridges to allow the quantitative determination of all mentioned mycotoxins with good recoveries and precisions. After the method development, the extraction and purification protocols were subjected to a method validation, which included the determination of the LODs and LOQs, the recoveries at three different spiking levels, as well as the inter-injection, the intra-day and inter-day precisions using starch as surrogate model matrix. For the quantification of the analytes, either stable isotopically labeled internal standards ([$^{2}H_{4}$]-AOH, [$^{2}H_{4}$]-AME, [$^{13}C_{6}$,^{15}N]-TA) were utilized or matrix matched calibration (TEN, ATX I, and ALTP) was applied. The newly developed method was also totally applicable to tomato purée and an additional method validation was performed for this matrix. Both method validations resulted in low LODs and LOQs for AOH, AME, TEN, ATX I, ALTP, and TA. The LODs of the analytes ranged from 0.05 to 1.25 µg/kg for starch (as a model for cereals) and from 0.01 to 1.36 µg/kg for fresh tomatoes. The LOQs varied from 0.16 to 4.12 µg/kg in starch and from 0.02 to 5.56 µg/kg in tomato matrix. Besides, good recoveries as well as good precisions were determined for all analytes in both matrices. The

recoveries ranged from 83 to 108% in starch and from 95 to 111% in fresh tomatoes. The inter-injection precisions were below 3% for all analytes. Furthermore, the intra-day and inter-day precisions varied from 1 to 3% as well as from 3 to 8% in starch and in tomato matrix. The newly developed method also allowed the qualitative analysis of ATX II and STTX III, but due to the lack of sufficient amounts of reference compounds, these two mycotoxins were not included in the method validation.

Various cereal- and tomato-based infant food products (n = 25) were obtained from various drugstores and supermarkets in Freising and Munich and were analyzed for their *Alternaria* mycotoxin contamination. All cereal-based infant food products (n = 19) contained at least one of the *Alternaria* mycotoxins and AME, TEN, and TA were determined most frequently. The content of TA ranged from "not detected" to 221 μg/kg, whereas the maximum contents of AOH, AME, TEN, ATX I, and ALTP were 7.17 μg/kg, 0.58 μg/kg, 7.53 μg/kg, 1.52 μg/kg, and 4.47 μg/kg, respectively. Beside the cereal-based products, purée infant foods comprising fruits or vegetables as well as tomato-based products (n = 6) were analyzed. While most of these commodities only contained low amounts of AOH, AME, TEN, and TA, one tomato sauce was highly contaminated with 54.2 μg/kg of AOH, 7.56 μg/kg of AME, and 505 μg/kg TA. Based on the quantitative measurements of the *Alternaria* mycotoxin contamination determined in the present thesis, a risk assessment was conducted. Highly contaminated commodities might pose a risk to the health of infants, as the TTC levels of 1500 ng/kg body weight per day for TEN and TA and 2.5 ng/kg body weight per day for AOH and AME were exceeded many times over after an oral consumption of this specific food product. However, if the mean or median was considered for the risk evaluation, the consumption of the contaminated food products was unlikely to pose a risk to the health of infants.

Beside the targeted analyses of *Alternaria* mycotoxins, the second part of the present thesis covers the results of the non-targeted measurements of primary and secondary metabolites biosynthesized by *Alternaria* fungi by FT-ICR-MS. For the non-targeted investigations, *A. alternata* and *A. solani* isolates were isolated from various parts of tomato and potato plants. The pure isolates were cultivated in a synthetic chemically defined liquid medium comprising different salts and glucose. During the cultivation of the fungi, samples of the liquid medium containing *Alternaria* secondary metabolites were taken, sterilely. The liquid medium was desalted prior to analysis by solid phase extraction utilizing DSC-8 cartridges. After the cultivation, the mycelia were separated from the liquid medium. The cells of the fungal mycelia were disrupted utilizing a Precellys homogenizer and the extracts were desalted by solid phase extraction to ensure a comparable sample preparation protocol for the cell extracts and the liquid medium. The eluates of the liquid media and the cell extracts

were analyzed by ultra-high resolution FT-ICR-MS and, additionally, the *Alternaria* mycotoxins present in the extracts were measured by targeted LC-MS/MS.

The ultra-high resolution of the FT-ICR-MS instrument allowed the assignment of molecular formulae to the measured mass signals. Utilizing the *NetCalc* algorithm, the elemental compositions of the *m/z* signals were generated by constructing a network interconnecting known *Alternaria* secondary metabolites from the literature with the measured mass signals via mass differences. The mass differences corresponded to common biochemical reactions. In total, 2883 molecular formulae comprising C, H, N, O, P, and S were assigned to *m/z* signals determined as $[M-H]^-$ ions within an annotation error of ± 0.2 ppm. To obtain possible structural candidates for the molecular formulae, the latter were matched to the entries of the database *Antibase*. The molecular formulae of the *A. alternata* isolates were putatively assigned to various perylene quinones, such as alterlosin I and II, stemphyltoxin I, III, and IV, and altertoxins, as well as bicycloalternarenes, tricycloalternarenes, and AF toxins. The elemental compositions produced by *A. solani* were putatively matched to altersolanols and solanapyrones. The *KEGG* database allowed the allocation of the elemental compositions to metabolites of the primary metabolism of the fungi. However, searching the *Antibase* database and *KEGG*, only 3 and 18% of the elemental compositions were assigned to a possible structural candidate. This result demonstrated the limited knowledge on the metabolites biosynthesized by *Alternaria* fungi.

Various *Alternaria* isolates were analyzed in the present study and the differences in their metabolite profile were determined, statistically. Although three isolates of the species group *A. alternata* were investigated, one isolate was clearly separated from the other two isolates in the PCA plots. The discriminating metabolites between two *A. alternata* isolates were determined by volcano plots and were putatively identified by the *Antibase* database. The differences between the *A. alternata* isolates were also obvious in the targeted LC-MS/MS measurements of the *Alternaria* mycotoxins AOH, AME, TEN, ATX I, ATX II, ALTP, STTX III, and TA. While one *A. alternata* isolate did not biosynthesize TA, the other two *A. alternata* isolates showed a similar mycotoxin profile regarding TEN, ATX I, ATX II, ALPT, and STTX III. Differences were also determined in the biosynthesis of the dibenzo-α-pyrones.

Beside the variations between the three *A. alternata* isolates, the discriminating metabolites between *A. solani* and *A. alternata* were specified. The *A. solani* isolate did not produce any of the common *Alternaria* mycotoxins determined by LC-MS/MS, whereas all the *A. alternata* isolates produced TEN and the perylene quinones. The FT-ICR-MS measurements allowed the detection of further metabolic differences between the *A. solani* and *A. alternata* isolates. While *A. solani* putatively biosynthesized altechromone A and quadrilineatin

methyl ether, the *A. alternata* isolates putatively produced xanalteric acids I and II as well as alterlosin I and II. Due to the lack of reference compounds of these metabolites, their unequivocal identification was not possible. A multitude of metabolites significantly discriminated between *A. solani* and *A. alternata*, but no structural candidates were provided by databases. However, one of the discriminating metabolites only biosynthesized by *A. alternata* was identified as APML by MS and NMR after isolating and purifying the compound.

To obtain sufficient amounts of APML for identification and characterization purposes, rice was inoculated with *A. alternata*. After the cultivation, the overgrown rice was extracted using organic solvents and the extract was laboriously purified applying several consecutive columns of silica gel and one column of C18 material. The fractions containing the metabolite APML were combined and further purified, respectively. Subsequently, APML was obtained by preparative HPLC on a C-18 column under acidic conditions. Finally, the pure compound was characterized by ^{1}H, ^{13}C, and 2D-NMR measurements and the chemical structure was identified as APML.

8 Outlook

The results of the targeted analyses of *Alternaria* mycotoxins in infant food products demonstrated the frequent contamination of agricultural commodities with the fungal secondary metabolites. However, the need for future investigations on the secondary metabolites of *Alternaria* fungi was obvious. Generally, not only AOH, AME, TEN, ATX I, ALTP, and TA, should be analyzed in products for human consumption but also the modified forms of AOH and AME should be included in analyses, once their reference compounds become available. Since the modification is potentially cleaved during digestion resulting in a release of the free forms, the consumer might be endangered by additional adverse effects. As the presence of ATX II has been reported in a naturally infected apple sample just recently (Puntscher et al., 2020), reference compounds of ATX II and STTX III should be provided either by chemical synthesis or by the purification of fungal extracts to allow not only toxicological investigations but also quantitative studies on these metabolites in food commodities.

The analysis of 25 infant food products in the present thesis provided first insights into the contamination situation of agricultural goods with *Alternaria* mycotoxins, yet more and different food products should be analyzed in future investigations to obtain a more holistic view. Furthermore, these investigations should be extended to several consecutive years to also cover the fluctuations in the contents of *Alternaria* mycotoxins due to different environmental conditions. Only comprehensive quantitative investigations on the *Alternaria* mycotoxin contamination of food products allow the EFSA to perform sound risk assessments. As partly high *Alternaria* mycotoxin contents were determined in the infant foods of the

present investigation, the manufacturers would be well advised to include *Alternaria* mycotoxins into their quality controls to ensure the safety of their products.

The non-targeted studies of the present thesis allowed deeper insights into the mycobolome of *Alternaria* fungi. However, many metabolites biosynthesized by the genus *Alternaria* remained unknown. Thus, future investigations should focus on *de novo* characterizations of secondary metabolites aiming at unraveling biosynthetic pathways of the secondary metabolites as well as the identification of yet unknown modified forms of mycotoxins. Comprehensive characterizations of the chemical structure of *Alternaria* secondary metabolites should include exact mass, elemental composition, fragmentation pattern, ^{1}H, ^{13}C, and 2D NMR, and UV measurements. These data should be added to public databases to improve the identification of unknown compounds. Although the chemical structure of the metabolite APML was characterized in the present thesis, its biochemical function in the secondary metabolism of the fungus remains unclear. Apart from that, the presence in food products as well as the toxicological capabilities of APML should be included in future investigations. The altertoxins I-III exhibited mutagenic and genotoxic effects *in vitro* (Fleck et al., 2012; Lorenz et al., 2012). As APML reveals structural analogy to these altertoxins, it can be speculated, that this mycotoxin exhibits mutagenic and genotoxic effects *in vitro* as well. Beside the toxicological effects, the intestinal absorption of APML should be examined. The permeability of APML into the portal blood might be relatively low due to the much higher polarity of APML compared to the other perylene quinones (Lüllmann et al., 2003). Additionally, it can be assumed, that the permeability of APML into the portal blood might be reduced by covalent binding of the molecule to cell components caused by the epoxide group (Fleck et al., 2014).

As the non-targeted measurements identified differences in the mycobolome of different *A. alternata* isolates and the *A. solani* isolate, additional isolates belonging to the species group of *A. alternata* and other species groups should be analyzed by FT-ICR-MS to evaluate the suitability of the methodology for species segregation purposes. In the present thesis, the fungi were cultivated in a chemically defined liquid medium. However, *Alternaria* fungi colonize on host plants, thus, inducing biochemical interactions of the plant with the pathogen. FT-ICR-MS investigations of plant-fungus interactions might contribute to the identification and understanding of biochemical processes induced by the colonization of fungi on plants.

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I List of tables

II List of figures

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IV Scientific contributions

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V Appendix

A Quantitation of Six *Alternaria* Toxins in Infant Foods Applying Stable Isotope Labeled Standards

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ORIGINAL RESEARCH
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Quantitation of Six *Alternaria* Toxins in Infant Foods Applying Stable Isotope Labeled Standards

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Alternaria fungi are widely distributed saprophytes and plant pathogens. As pathogens, *Alternaria* fungi infect crops and vegetables and cause losses in the fields and during postharvest storage. While farmers suffer from declining yields, consumers are endangered by the formation of secondary metabolites, because some of these exhibit a pronounced toxicological potential. The evaluation of the toxicological capabilities is still ongoing and will contribute to a valid risk assessment. Additionally, data on the incidence and the quantity of *Alternaria* mycotoxins found in food products is necessary for dietary exposure evaluations. A sensitive LC-MS/MS method for the determination of the *Alternaria* mycotoxins alternariol (AOH), alternariol monomethylether (AME), tentoxin (TEN), altertoxin I (ATX I), alterperylenol (ALTP), and tenuazonic acid (TA) was developed. AOH, AME, and TA were quantified using stable-isotopically labeled standards. TEN, ATX I, and ALTP were determined using matrix matched calibration. The developed method was validated by using starch and fresh tomato matrix and resulted in limits of detection ranging from 0.05 to 1.25 μg/kg for starch (as a model for cereals) and from 0.01 to 1.36 μg/kg for fresh tomatoes. Limits of quantification were determined between 0.16 and 4.13 μg/kg for starch and between 0.02 and 5.56 μg/kg for tomatoes. Recoveries varied between 83 and 108% for starch and between 95 and 111% for tomatoes. Intra-day precisions were below 4% and inter-day precisions varied from 3 to 8% in both matrices. Various cereal based infant foods, jars containing vegetables and fruits as well as tomato products for infants were analyzed for *Alternaria* mycotoxin contamination (n = 25). TA was the most frequently determined mycotoxin and was detected in much higher contents than the other toxins. AME and TEN were quantified in many samples, but in low concentrations, whereas AOH, ATX I, and ALTP were determined rarely, among which AOH had higher concentration. Some infant food products were highly contaminated with *Alternaria* mycotoxins and the consumption of these individual products might pose a risk to the health of infants. However, when the mean or median is considered, no toxicological risk was obvious.

Keywords: *Alternaria* mycotoxins, stable isotope dilution assay (SIDA), LC-MS/MS, cereal based infant food, tomato products

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INTRODUCTION

Fungi of the genus *Alternaria* are ubiquitous microorganisms growing on a wide range of substrates including soil, wall papers, decaying organic material and, most important from both toxicological and economical aspects, agricultural crops used for human and animal nutrition (Ostry, 2008). Infection of plants with *Alternaria* is commonly believed to occur on the field and many *Alternaria* species are well-known plant pathogens responsible for a series of plant diseases, e.g., black rot of tomatoes, black and gray rot of citrus fruits and black point of cereals (Logrieco et al., 2009). However, some *Alternaria* species are also able to grow at low temperature and are responsible for the postharvest decay of fruits and vegetables even at refrigerated storage or transport (Ozcelik et al., 1990). Unrevealing the taxonomy of the genus *Alternaria* is still a matter of ongoing research. Species differentiation by molecular biology seems to be more promising than the traditional morphologic approach (Zwickel et al., 2018).

The number of fungal secondary metabolites with toxic impact, the so-called mycotoxins, isolated from *Alternaria* fungi has reached at least 70 compounds up to now (Arcella et al., 2016). They exhibit great structural divergence and are commonly divided into five groups (**Figure 1**):

- Dibenzo-α-pyrones (e.g., alternariol, AOH; alternariol-monomethylether, AME),
- Tetramic acid derivatives (e.g., tenuazonic acid, TA),
- Perylene quinones (e.g., altertoxins I – III, ATX I – III),
- Specific toxins produced by *Alternaria alternata* subspecies *lycopersici* (AAL-toxins),
- Miscellaneous structures (e.g., tentoxin, TEN).

The variety of secondary fungal metabolites is further increased by the so-called modified mycotoxins (Rychlik et al., 2014). In case of *Alternaria* toxins the sulfate (S) conjugates AOH-3-S, AOH-9-S, and AME-3-S were identified as metabolites produced by the fungus itself, whereas the glucose (Glc) conjugates AOH-3-Glc, AOH-9-Glc, AME-3-Glc, and AME-7-Glc are regarded as plant metabolites (Soukup et al., 2016). Recently, AOH-9-Glc, AOH-3-S, and AME-3-S have been found for the first time in naturally contaminated tomato sauce for human consumption (Puntscher et al., 2018).

Analytical methods capable to determine this variety of analytes in food commodities are nowadays exclusively based on liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). In the recent years, methods have been developed to analyze three (Rodríguez-Carrasco et al., 2016), four (Berardis et al., 2018), five (Prelle et al., 2013), six (Noser et al., 2011), eight (Malachová et al., 2014), and even twelve (Zwickel et al., 2016) *Alternaria* toxins simultaneously, sometimes also including their modified forms (Walravens et al., 2014, 2016; Puntscher et al., 2018).

However, precise quantitation of these different substances still remains challenging, especially in multi-analyte approaches. Stable isotope dilution assays are meanwhile regarded as the gold standard of quantitative analytical methods and are more and more applied also in mycotoxin analysis (Asam and Rychlik, 2015). LC-MS/MS is severely affected by matrix interferences that can manifest themselves either as signal suppression or signal enhancement. By using stable isotope labeled internal standards, these interferences can be optimally minimized. Moreover, losses during sample preparation are also completely compensated for, because respective labeled standards and analytes are chemically indistinguishable and, therefore, show the same recovery throughout all steps of the sample preparation. In case of *Alternaria* mycotoxins the availability of labeled standards is still limited. So far, only the chemical syntheses of labeled AOH and AME (Asam et al., 2009; Liu and Rychlik, 2015), TEN (Liu and Rychlik, 2013), and TA (Asam et al., 2011b; Lohrey et al., 2013) and the biochemical isolation of labeled AOH, AME, altenuene (ALT), altenuisol (AS), ATX I – III, ALTP, and stemphyltoxin III (STTX III) (Liu and Rychlik, 2015) have been described.

The toxicity of *Alternaria* toxins has not yet been clarified in detail for all substances and is still a matter of ongoing research. AOH and AME are believed to be mutagenic because of their genotoxic effects *in vitro* (Brugger et al., 2006). On a molecular basis, AOH has been shown to interact with topoisomerases I and II with effects that have been described as "poisoning" (Fehr et al., 2009). Further research revealed that besides AOH, also AME, ATX I, ATX II, and ALTP are able to provoke these effects (Jarolim et al., 2016). Mutagenicity of ATX I, II, and III has long been known (Stack and Prival, 1986), but recently ATX II was identified as the major mutagen produced by *Alternaria* (Fleck et al., 2012; Schwarz et al., 2012), although the mode of mutagenic action of the latter is not clear yet (Fleck et al., 2016). TA is an acutely toxic substance with oral LD_{50} values between 81 and 225 mg/kg body weight for mice (Miller et al., 1963; Smith et al., 1968). TA inhibits protein biosynthesis by suppressing the release of new proteins from the ribosome (Shigeura and Gordon, 1963). Potentially, it is produced as virulence factor to facilitate colonization of the fungus on plants (Kang et al., 2017). TEN is considered as a phytotoxin, inhibiting photophosphorylation, and inducing chlorosis (Arntzen, 1972).

The Panel on Contaminants in the Food Chain (CONTAM) of the European Food Safety Authority (EFSA) evaluated the risks for public health related to *Alternaria* toxins in food in the year 2011 (EFSA, 2011). Their assessment was based on the threshold of toxicological concern (TTC) concept (Kroes et al., 2004) due to limited toxicity data for the *Alternaria* toxins. Although the panel also faced limited occurrence data, they estimated the critical TTC values to be exceeded by AOH and AME, but not by TEN and TA. In 2016, the EFSA reported a dietary exposure assessment of *Alternaria* toxins (Arcella et al., 2016). Compared to 2011, similar (AOH) or higher (AME, TEN, TA) exposures were estimated. Cereals and tomato based products were the main origin of *Alternaria* toxin intake and it was noted that infants were the population group with the highest dietary exposure.

In the year 2012, we conducted a survey about the contamination of infant food with the *Alternaria* mycotoxin TA. At this time, we found extremely high contamination of infant food samples based on millet (Asam and Rychlik, 2013).

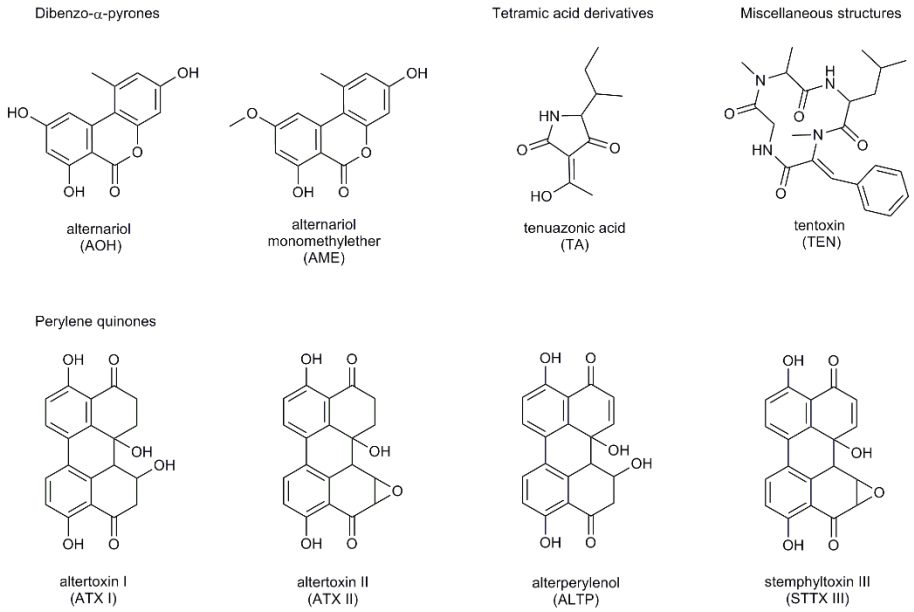

FIGURE 1 | Structure of the *Alternaria* mycotoxins AOH, AME, TA, TEN, ATX I and II, ALTP, and STTX III.

After toxicological evaluation, infant foods were regarded as a potential health hazard if a warning limit of 500 μg/kg of TA was exceeded (Rychlik et al., 2016). Few other studies dealt with this topic so far. In one study from Canada, AOH and AME were detected in 27 out of 30 samples of cereal-based infant food with maximum values of 4.4 μg/kg (AOH) and 9.0 μg/kg (AME) (Scott et al., 2012).

However, in our study in 2012 we were not able to include the other *Alternaria* toxins into the analytical scope due to limited sensitivity of the LC-MS equipment. Therefore, the aim of the present study was the development of a new method for the six *Alternaria* toxins AOH, AME, TA, TEN, ATX I, and ALTP and to apply this method to infant food in order to gain further insight in the actual contamination situation of infant food with *Alternaria* toxins.

MATERIALS AND METHODS

Chemicals and Reagents

Analytical standards of AOH, AME, TA, and TEN were obtained from Sigma-Aldrich (Steinheim, Germany). ATX I, ATX II, ALTP, and STTX III were biosynthesized, purified by preparative HPLC and characterized by mass spectrometry (MS) and nuclear resonance spectroscopy (NMR) as reported earlier (Liu and Rychlik, 2013). TA was released from its commercial copper salt as described in the literature (Shephard et al., 1991; Siegel et al., 2009). The stable isotopically labeled standards [$^2H_4$]-AOH and [$^2H_4$]-AME were synthesized according to the literature (Asam et al., 2009). [$^{13}C_6$,^{15}N]-TA was prepared in our laboratory as published (Asam et al., 2011b). The labeled standards were also chromatographically purified and characterized by MS and NMR studies.

Formic acid (≥98%) was obtained from Sigma-Aldrich (Steinheim, Germany). Ammonium formate for mass spectrometry (≥99.0%) was purchased from Sigma-Aldrich (Bellefonte, PA, United States). Ammonia solution (25%, for LC-MS) and starch (high purity) were obtained from Merck KGaA (Darmstadt, Germany). Acetonitrile (analytical grade), methanol (analytical grade), water (analytical and LC-MS grade), and isopropanol (LC-MS grade) were received from VWR International GmbH (Ismaning, Germany). Acetonitrile (LC-MS grade) was obtained from Carl Roth GmbH & Co., KG (Karlsruhe, Germany). Methanol (LC-MS grade) was purchased from Honeywell^{TM} Riedel-de Häen^{TM} (Seelze, Germany).

Preparation of Stock Solutions and Calibration Standards

Stock solutions of unlabeled and labeled *Alternaria* mycotoxins were prepared in concentrations ranging from 10 to 100 μg/mL in acetonitrile (AOH, AME, and TEN) or methanol (ATX I, ALTP, and TA). The stock solutions were further diluted for sample preparations and method validations. All solutions were stored at −20°C in the dark. The concentrations of solutions were confirmed by UV spectrometry (Genesys, 10S, UV–Vis spectrophotometer, Thermo Fisher Scientific, Madison, WI, United States) using published extinction coefficients (Zwickel et al., 2016). However, ATX II and STTX III were available only in amounts that were not detectable by UV–VIS and, therefore, were only qualitatively included in the method.

LC-MS/MS Analysis

The chromatographic separation of the analytes was performed on a Shimadzu Nexera X2 UHPLC system (Shimadzu, Kyoto, Japan). The LC-parameters for AOH, AME, TEN, ATX I, ATX II, STTX III, and ALTP were adopted from Liu and Rychlik (2013, 2015). A HyperClone BDS-C18 column (150 × 3.2 mm, 3 μm, 130 Å, Phenomenex, Aschaffenburg, Germany) protected by a C18-guard column (4 × 2.0 mm ID, Phenomenex, Aschaffenburg, Germany) served as stationary phase. The column was tempered to 30°C. The flow rate was set to 0.2 mL/min. The binary gradient system consisted of acetonitrile/2-propanol/water (solvent A, 17.5/17.5/65, *v/v/v*) and methanol (solvent B). The mobile phase was held at 0% B for the first 3 min. The gradient raised linearly from 0 to 100% B in the following 19 min and remained at 100% B for 1 further min. The mobile phase returned to 0% B within the next 2 min and the column was equilibrated for 5 min. 10 μL were used as injection volume. Using an additional valve after the column, the flow was introduced into the mass spectrometer not until 6.9 min after injection to prevent the instrument from matrix contamination.

Due to the different polarity of TA, the mycotoxin had to be analyzed in an additional LC-MS/MS run. The LC-parameters for TA were based on Asam et al. (2013). A Gemini-NX C18 column (150 × 4.6 mm, 3 μm, 110 Å, Phenomenex, Aschaffenburg, Germany) protected by a Gemini-NX C18-guard column (4 × 3.0 mm ID, Phenomenex, Aschaffenburg, Germany) served as stationary phase. The column was tempered to 40°C. The flow rate was set to 0.5 mL/min. The binary gradient system consisted of 5 mM ammonium formate (solvent A, pH 9) and methanol (solvent B). The mobile phase was held at 5% B for 3 min, raised linearly from 5 to 100% B in 5 min and remained at 100% B for 2 min. Thereafter, the gradient returned to 5% B within 3 min and the column was equilibrated for 10 min. The injection volume was 10 μL. Using an additional valve after the column, the flow was introduced into the mass spectrometer not until 6.5 min after injection to prevent the instrument from matrix contamination.

Using automated column switching and fourfold solvent selection provided by the instrument, both methods could be run in the same sequence.

The LC was coupled to a triple quadrupole ion trap mass spectrometer (LCMS-8050, Shimadzu Corporation, Kyoto, Japan). The instrument operated in the negative electrospray ionization (ESI) mode for all analytes. Parameters for the interface were adjusted as follows: heating gas flow 10 L/min, nebulizing gas flow 3 L/min, drying gas flow 10 L/min, heat block temperature 400°C, desolvation line temperature 250°C, interface temperature 300°C, interface voltage 4 kV and collision-induced dissociation gas pressure 270 kPa. The mass spectrometer worked in the scheduled multiple reaction monitoring (MRM) mode for MS/MS measurements. The voltages for the fragmentation of each analyte were optimized via direct infusion of standard solutions of AOH, AME, TEN, ATX I, ATX II, ALTP, STTX III, and TA at a concentration of 1 μg/mL. Two mass transitions were chosen, one as quantifier and one as qualifier for confirmation. Optimized voltages and collision energies, the retention time as well as the quantifier/qualifier ratio of each analyte are listed in **Table 1**. The LabSolutions software (Shimadzu, Kyoto, Japan) was utilized for data acquisition and data analysis.

Sample Preparation

In preliminary experiments, different solvent mixtures consisting of acetonitrile, methanol, and water were tested for ideal mycotoxin extraction from grain matrix. The extraction was optimized by adding different amounts of formic acid and acetic acid as well as the adjustment of the extraction duration. Various cartridges with different sorbent material (hydrophilic-lipophilic-balanced (HLB, Waters Corporation, Milford, MA, United States), C18 (Discovery® DSC-18, Supelco, Bellefonte, PA, United States), polyamide (PA, CHROMABOND®, Machery-Nagel GmbH & Co., KG, Düren, Germany), NH_2 (CHROMABOND®, Machery-Nagel GmbH & Co., KG, Düren, Germany), and Strata-X (Polymeric reversed phase, Phenomenex, Aschaffenburg, Germany) were tested for the simultaneous purification of *Alternaria* mycotoxins with special regards to TA. From the results of these experiments the optimized sample preparation method was developed as follows:

One gram of ground and homogenized sample was weighed into a 50 mL centrifuge tube and the isotopically labeled internal standards were added to the sample (100 μL of [$^2H_4$]-AOH (0.1 μg/mL), 50 μL of [$^2H_4$]-AME (0.1 μg/mL) and 100 μL of [$^{13}C_6$,^{15}N]-TA (1.0 μg/mL)). The mycotoxins were extracted by adding 15 mL of a mixture of acetonitrile/water (84/16; *v/v*) and 0.2 mL of formic acid and horizontal shaking for 30 min (175 rpm). The sample was centrifuged at room temperature at 1690 × g for 5 min and the residue was extracted again as described above. The third extraction step was performed by adding 15 mL of a mixture of acetonitrile/methanol/water (50/25/25; *v/v/v*) and 0.3 mL of formic acid. After 30 min of horizontal shaking (175 rpm), the sample was filtered. The combined extracts and the filtrate were rotary evaporated to dryness at 40°C. The residue was reconstituted in 12 mL of water (pH 5.5). The mycotoxins were purified by solid phase extraction (Discovery® DSC-C18, 500 mg, 6 mL, Sigma-Aldrich, Bellefonte, PA, United States). The C18 material was washed with 6 mL of methanol and conditioned with 6 mL of water (pH 5.5). The sample was completely loaded onto the column. After washing the column with 12 mL of water, the column was dried under vacuum. The mycotoxins were eluted with 6 mL of methanol. For TA, an additional elution step with 9 mL of methanol + 2% ammonium hydroxide was performed. The eluate was rotary evaporated to dryness at 40°C. The residue was reconstituted in 1 mL of methanol/water (1/1; *v/v*). Remaining matrix precipitated during night at −20°C. The sample was membrane filtered (0.22 μm) and analyzed by LC-MS/MS.

Twenty-five infant food samples were collected from various supermarkets and drugstores in Germany. Cereal based infant food samples were either single grain or multi-grain products, containing spelt, oat, millet, rice, and wheat. Jars, containing either vegetables or fruits as well as two tomato products were purchased.

TABLE 1 | Precursor ions and product ions of the unlabeled and labeled *Alternaria* mycotoxins, optimized fragmentation conditions, retention times, and quantifier/qualifier ratio.

Analyte	Precursor ion *m/z*	Product ion *m/z*	Q1 Pre-bias [V]	CE [V]	Q3 Pre-bias [V]	Retention time [min]	Ratio quantifier/qualifier*
ATX I	351.20	297.20	25	28	19	9.6	1.15
		314.20	27	32	21		
ALTP	349.30	261.25	18	28	15	10.2	0.74
		303.00	18	19	18		
AOH	257.30	213.00	15	23	12	11.4	1.27
		215.05	14	24	13		
[$^2H_4$]-AOH	261.30	217.00	15	23	12	11.4	2.09
		218.05	14	24	13		
TEN	413.30	141.05	15	23	23	12.5	1.46
		271.30	15	17	17		
ATX II	349.40	313.25	16	25	12	13.6	0.45
		331.30	20	15	20		
STTX III	347.20	329.20	17	22	21	15.4	1.16
		301.10	17	35	18		
AME	271.30	256.20	20	23	25	18.2	3.63
		228.20	19	30	13		
[$^2H_4$]-AME	275.30	260.20	20	23	25	18.2	3.47
		232.20	19	30	13		
TA	196.30	112.05	22	26	20	8.3	1.01
		139.00	14	22	11		
[$^{13}C_{6,15}N$]-TA	203.25	113.05	22	26	20	8.3	0.91
		142.00	14	22	11		

**Mean of five injections. The quantifier/qualifier ratios were determined in pure solvents.*

Calibration and Quantitation

AOH, AME, and TA were quantified using isotopically labeled internal standards, whereas TEN, ATX I, and ALTP were quantified using matrix matched calibration.

For AOH, AME, and TA, calibration functions were obtained by mixing various amounts of analyte (A) with constant amounts of internal standard (IS). The calibration functions ranged from molar ratios [n(A)/n(IS)] of 0.01 to 100 (1:100, 1:50, 1:10, 1:5, 1:2, 1:1, 2:1, 5:1, 10:1, 100:1). The internal standards for AOH and AME were [$^2H_4$]-labeled. For TA, the [$^{13}C_6$,^{15}N]-labeled isotopologue served as internal standard. The calibration points were measured by LC-MS/MS. The calibration functions were calculated using linear regression after plotting molar ratios [n(A)/n(IS)] against peak area ratios [A(A)/A(IS)]. Linearity was confirmed by Mandel's fitting test.

Matrix matched calibrations for TEN, ATX I, and ALTP were performed on two different matrices. Potato starch was spiked with various amounts of TEN (0.1–100 μg/kg), ATX I (0.4–20 μg/kg), and ALTP (0.6–20 μg/kg). Blank tomato puree was spiked with varying amounts of TEN (0.1–100 μg/kg), ATX I (0.4–20 μg/kg), and ALTP (0.4–20 μg/kg). After sample preparation, the calibration points were analyzed by LC-MS/MS. The calibration curve was constructed from peak area [A(A)] against spiked contents of the analyte [w(A)] and the calibration function was received via linear regression. Linearity of the matrix matched calibration functions was confirmed by Mandel's fitting test.

Mycotoxin contamination in cereal based infant food samples or jars was either calculated by using the respective calibration function (AOH, AME, and TA) or by using the matrix-matched calibration function (TEN, ATX I, and ALTP).

Method Validation

The sample preparation and the LC-MS/MS method were validated according to Vogelgesang and Hädrich (1998). Potato starch was chosen as surrogate blank matrix for the method validation, because all analyzed grain flours contained *Alternaria* mycotoxins. To obtain a blank tomato matrix, fresh and sound whole tomatoes were pureed and analyzed by LC-MS/MS to confirm the absence of *Alternaria* mycotoxins.

For the determination of limits of detection (LODs) and quantitation (LOQs), AOH, AME, TEN, ATX I, ALTP, and TA were spiked to the blank matrices at four different levels, respectively. Potato starch was spiked with AOH (1.0, 3.0, 7.0, and 10 μg/kg), AME (0.1, 0.3, 0.7, and 1.0 μg/kg), TEN (0.1, 0.4, 0.7, and 1.0 μg/kg), ATX I (0.8, 2.0, 5.0, and 8.0 μg/kg), ALTP (0.4, 1.6, 2.8, and 4.0 μg/kg), and TA (2.5, 8.0, 16, and 25 μg/kg). Fresh tomatoes were spiked with AOH (0.15, 0.6, 1.0, and 1.5 μg/kg), AME (0.015, 0.06, 0.1, and 0.15 μg/kg), TEN (0.15, 0.6, 1.0, and 1.5 μg/kg), ATX I (0.5, 2.0, 3.5, and 5.0 μg/kg), ALTP (0.4, 1.6, 2.8, and 4.0 μg/kg), and TA (1.5, 6.0, 10, and 15 μg/kg). Each level was prepared in triplicate. The sample preparation was performed as described above and samples were analyzed by LC-MS/MS.

The recoveries for each mycotoxin were determined in starch and tomato matrix at three different levels, respectively. Potato

starch was spiked with AOH (3.0, 7.0, and 10 μg/kg), AME (0.3, 0.7, and 1.0 μg/kg), TEN (0.4, 0.7, and 1.0 μg/kg), ATX I (2.0, 5.0, and 8.0 μg/kg), ALTP (1.6, 2.8, and 4.0 μg/kg), and TA (15, 100, and 200 μg/kg). Tomato puree was spiked with AOH (0.6, 1.0, and 1.5 μg/kg), AME (0.06, 0.1, and 0.15 μg/kg), TEN (0.6, 1.0, and 1.5 μg/kg), ATX I (2.0, 3.5, and 5.0 μg/kg), ALTP (1.6, 2.8, and 4.0 μg/kg), and TA (50, 100, and 200 μg/kg). Each level was prepared in triplicate. The samples were prepared as described above and analyzed by LC-MS/MS. The content of toxins in the samples were calculated either using SIDA or matrix matched calibration and the recovery was calculated for each toxin as follows: R = found amount [μg/kg]/spiked amount [μg/kg].

Infant food samples containing *Alternaria* mycotoxins were analyzed to determine the inter-injection precisions, intra- and inter-day precisions. A seven-grain infant food product contained TEN (23 μg/kg), ALTP (6 μg/kg), and TA (114 μg/kg) and was spiked with AOH (10 μg/kg), AME (1.5 μg/kg), and ATX I (6 μg/kg). An organic tomato sauce contained AOH (75 μg/kg), AME (9 μg/kg), and TA (490 μg/kg) and was spiked with TEN (8 μg/kg), ATX I (5 μg/kg), and ALTP (6 μg/kg).

The inter-injection precision was determined by multiple injections of one sample (n = 5). The intra-day precision was calculated after injecting three samples in triplicate into the LC-MS/MS. For the inter-day precision, the samples were prepared in triplicate in 3 weeks and injected in triplicate into the LC-MS/MS. The mycotoxin contamination was either calculated by using the respective calibration function (AOH, AME, and TA) or by using the matrix-matched calibration function (TEN, ATX I, and ALTP).

RESULTS

Method Development

The *Alternaria* mycotoxins AOH, AME, TEN, ATX I, ALTP, and TA were extracted from food samples, purified by solid phase extraction and analyzed by LC-MS/MS. To optimize the extraction of *Alternaria* mycotoxins from grain matrix, different mixtures of acetonitrile, methanol, and water were tested. Applying only one extraction step resulted in insufficient recoveries of the mycotoxins. Therefore, two additional extraction steps were indispensable to extract the mycotoxins sufficiently. For the first and second extraction, a mixture of acetonitrile/water (84/16; *v/v*) and the addition of formic acid showed best extraction sufficiency for most of the mycotoxins. The volume of the extraction solvents was set to 15 mL, on the one hand, to obtain good recoveries and, on the other hand, to save time during evaporation. Especially the extraction of the perylene quinones required a third extraction step. The addition of methanol to the extraction solvent increased the recoveries of the perylene quinones and the solvent mixture was optimized to acetonitrile/methanol/water (50/25/25; *v/v/v*). The addition of formic acid to the extraction solvent improved in particular the extraction efficiency of the perylene quinones. The mycotoxin extracts were then purified by solid phase extraction. Different solid phases were tested for their matrix reduction and their mycotoxin retention, in particular for their simultaneous retention of TA together with the other *Alternaria* mycotoxins. Promising results provided only C18, Strata-X and HLB cartridges as all tested mycotoxins were retained sufficiently by these sorbent materials. Due to economic reasons, C18 material was finally chosen as sorbent material for the sample preparation. The *Alternaria* mycotoxins show different polarity and, therefore, the pH during the conditioning, sample loading, and elution was optimized with special regards to TA. For optimal retention of TA on C18 during sample loading, the pH of the sample had to be adjusted to 5.5. Elution of the mycotoxins was performed using 6 mL of methanol. The recovery of TA was improved by applying a second elution step with methanol containing 2% of ammonium hydroxide. The amount of sample needed for analysis was also adjusted. Different amounts of sample were weighed into centrifuge tubes and the recovery of mycotoxins was determined. Best recoveries were obtained for 1 g of sample, as 1.5 or 2 g of sample needed to be extracted with more solvent. The usage of more solvent would also increase duration of evaporation and, therefore, sample preparation time. Less than one gram of sample might not reflect inhomogeneity of the sample sufficiently. The developed sample preparation method for grains was totally applicable to tomato matrix.

LC-MS/MS Analysis

The LC-parameters for AOH, AME, TEN, ATX I, ATX II, STTX III, and ALTP were adopted from Liu and Rychlik (2013, 2015). **Figure 2** displays LC-MS/MS chromatograms of ATX I, ALTP, AOH, TEN, and AME **(A)**, of the internal standards [$^2H_4$]-AOH and [$^2H_4$]-AME **(B)**, and of ATX II and STTX III **(C)**. A naturally contaminated infant food in jar, consisting of apple, pear, and cherry, is shown in chromatogram **(D)**. TA was added to the existing chromatographic run but due to different polarity, the retention was unsatisfyingly low. Therefore, TA had to be analyzed in a separate LC-MS/MS run. The LC-parameters for TA were based on Asam et al. (2013). Chromatograms of TA **(A)** and [$^{13}C_6$,^{15}N]-TA **(B)** are displayed in **Figure 3**. Chromatogram **(C)** shows a naturally contaminated seven-grain infant food sample. In **Figure 4**, AOH, AME, and TA are depicted in contents of 1.00, 0.10, and 2.50 μg/kg. The labeled internal standards [$^2H_4$]-AOH, [$^2H_4$]-AME, and [$^{13}C_6$,^{15}N]-TA are shown at contents of 10.0, 5.00, and 10.0 μg/kg. Due to the isotope effect, slight retention time shifts of the deuterated internal standards [$^2H_4$]-AOH and [$^2H_4$]-AME compared to the unlabeled analytes AOH and AME are observed during the chromatographic separation. The mass difference between [^{13}C] and [^{12}C] is proportionally much lower than between [^2H] and [^1H] and, therefore, the retention time shift between [$^{13}C_6$,^{15}N]-TA and TA is not as pronounced as for the deuterated standards (Rychlik and Asam, 2008).

The fragmentation of AOH, AME, TEN, ATX I, ATX II, STTX III, ALTP, and TA was optimized by injecting solutions of reference compounds (1.0 μg/mL). Mass transitions of the quantifiers and qualifiers of the analytes were checked for interfering matrix compounds. As the matrix did not overlap with the mass transitions of the analytes, specificity of AOH, AME, TEN, ATX I, ALTP, and TA was assumed during analysis, but constantly monitored through the quantifier/qualifier ratio.

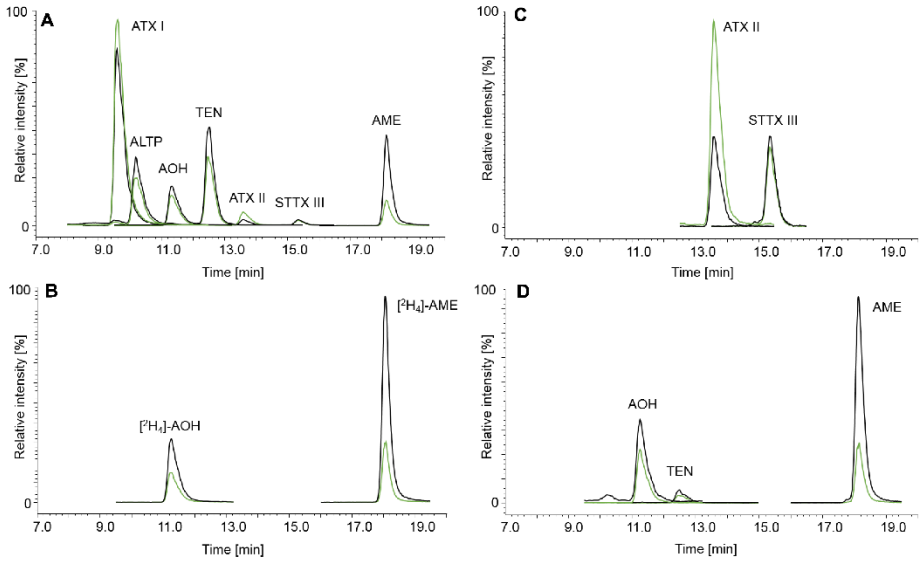

FIGURE 2 | Chromatograms of the *Alternaria* mycotoxins ATX I (130 μg/kg), ALTP (20.0 μg/kg), AOH (20.0 μg/kg), TEN (25.0 μg/kg), ATX II (approximately 2.00 μg/kg, see Section "Preparation of Stock Solutions and Calibration Standards"), STTX III (approximately 3.00 μg/kg, see Section "Preparation of Stock Solutions and Calibration Standards"), and AME (5.00 μg/kg) **(A)** and the isotopically labeled internals standards [$^2H_4$]-AOH (20.0 μg/kg) and [$^2H_4$]-AME (5.00 μg/kg) **(B)**. Chromatogram **(C)** shows the standards ATX II (approximately 2.00 μg/kg, see Section "Preparation of Stock Solutions and Calibration Standards") and STTX III (approximately 3.00 μg/kg, see Section "Preparation of Stock Solutions and Calibration Standards") in more detail. The mycotoxin contamination of an infant food in jar containing apple, pear, and cherry is depicted in chromatogram **(D)**, containing AOH (2.86 μg/kg), TEN (0.93 μg/kg), and AME (1.42 μg/kg). The black and green mass transitions represent the quantifier and qualifier of the analyte and labeled internal standard, respectively.

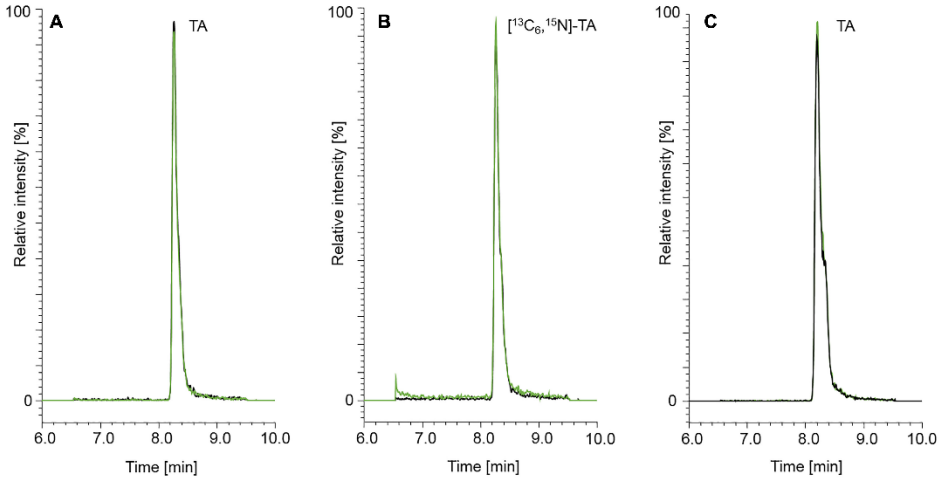

FIGURE 3 | Chromatograms of TA (50.0 μg/kg) **(A)** and the isotopically labeled internal standard [$^{13}C_6$,^{15}N]-TA (50.0 μg/kg) **(B)**. A naturally contaminated seven-grains infant food sample is shown in chromatogram **(C)**. The content of TA determined in the seven-grains infant food was 149 μg/kg. The black and green mass transitions represent the quantifier and qualifier of the analyte and labeled internal standard, respectively.

Calibration and Quantification

Calibration functions of AOH, AME, and TA were obtained by analyzing mixtures of analyte with the isotopically labeled internal standards. Linearity was confirmed between molar ratios of analyte (A) and internal standard (IS) [n(A)/n(IS)] of 0.01 to 100, respectively.

Matrix matched calibrations for TEN, ATX I, and ALTP were performed for two different matrices to examine the range

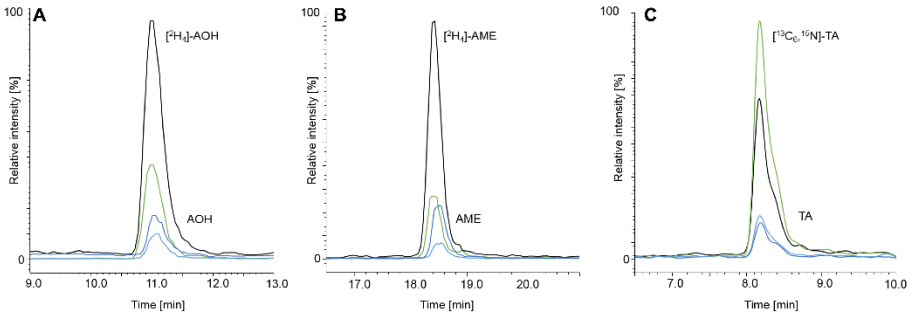

FIGURE 4 | Chromatograms at LOQ level of AOH, [$^2H_4$]-AOH **(A)**, AME, [$^2H_4$]-AME **(B)**, TA and [$^{13}C_6$,^{15}N]-TA **(C)**. AOH, AME, and TA are depicted in spiked starch matrix at contents of 1.00, 0.10, and 2.50 μg/kg, respectively. The labeled internal standards were added to the matrix at contents of 10.0 μg/kg for [$^2H_4$]-AOH, 5.00 μg/kg for [$^2H_4$]-AME, and 10.0 μg/kg for [$^{13}C_6$,^{15}N]-TA. The black and the green mass transitions represent the quantifiers and the qualifiers of the labeled standards, whereas the dark blue and light blue mass transitions represent the quantifiers and qualifiers of the analytes, respectively.

of linearity. The linearity of the matrix matched calibration functions was confirmed from 0.1 to 100 μg/kg for TEN, from 0.4 to 20 μg/kg for ATX I, and from 0.6 to 20 μg/kg for ALTP in starch matrix. In tomato puree, the linearity of the calibration functions was confirmed from 0.1 to 100 μg/kg for TEN, from 0.4 to 20 μg/kg for ATX I, and from 0.4 to 20 μg/kg for ALTP.

Method Validation

Limits of detection (LOD) and limits of quantification (LOQ) were determined according to Vogelgesang and Hädrich (1998). As no cereal flour from the supermarket was free from *Alternaria* mycotoxins, potato starch was chosen as blank matrix for the method validation. To represent the jar's matrix and the tomato products, fresh tomato puree was chosen as blank matrix for the method validation. AOH, AME, TEN, ATX I, ALTP, and TA were spiked to the blank matrices in triplicate at four different spiking levels. LODs ranged from 0.05 to 1.25 μg/kg in starch matrix (**Table 2**), and from 0.01 to 1.36 μg/kg in tomato matrix (**Table 3**). LOQs ranged from 0.16 to 4.13 μg/kg in starch and from 0.02 to 5.56 μg/kg in tomato puree (**Tables 2,3**).

Recoveries were determined by spiking AOH, AME, TEN, ATX I, ALTP, and TA to the respective blank matrices. The level of spiking complied with contents of the mycotoxins determined in naturally contaminated food products. For the starch matrix, the recoveries ranged from 83 to 108% (**Table 2**). For tomato matrix, the recoveries ranged from 95 to 111% (**Table 3**).

Various precisions (relative standard deviations) of the analytes were calculated (**Tables 2,3**). Inter-injection precisions were determined by repetitive injecting (n = 5) a cereal based infant food sample or the tomato soup into the LC-MS/MS instrument. Relative standard deviations ranged from 2 to 3% in the cereal based product and from 1 to 2% in the tomato soup. The intra-day and inter-day precisions were determined by preparing one sample in triplicate and in 3 weeks. The calculated intra-day precisions lay between 1 and 3% in cereals and between 1 and 3% in the tomato matrix. Inter-day precisions varied from 3 to 7% in the cereal based infant food and from 3 to 8% in the tomato soup.

Analysis of Infant Food Samples

Various cereal based infant food samples (n = 19) as well as jars (n = 6) were purchased from local drugstores and supermarkets and analyzed for their content of AOH, AME, TEN, ATX I, ALTP, and TA (**Table 4**). Within the cereal based infant foods, single grain as well as multi-grain products were analyzed. ATX II and STTX III were not detected in any of the infant food samples. The other *Alternaria* mycotoxins were detected in cereal based infant food above the limit of quantification in 17 out of 19 samples. TA was determined in 89% of the products with contents above the limit of quantification. The contamination of the latter ranged from 5.66 to 221 μg/kg and exceeded the contents of the other mycotoxins. Single grain products with partially high contamination of TA were millet (221 μg/kg), spelt (102 μg/kg), rice (109 μg/kg), and two multi-grain products (104 and 149 μg/kg). The median contamination of TA in the cereal based products was 22.0 μg/kg. AOH was determined in one spelt and one multi-grain product with contents of 7.17 and 4.73 μg/kg. AME was determined in 53% of all cereal based infant products with contents above the limit of quantification. The contamination of AME ranged from 0.23 to 0.58 μg/kg with a median of 0.23 μg/kg, which can be considered as low contamination. Eighty-four percent of all products contained TEN in amounts from 0.18 to 7.53 μg/kg. The median was 0.43 μg/kg. ATX I was detected in only one wheat infant food sample with a content of 1.52 μg/kg. ALTP was detected in one multi-grain product above the limit of quantification with a content of 4.47 μg/kg.

Four different purees in jars as well as one tomato sauce and one tomato soup were analyzed for AOH, AME, TEN, ATX I, ALTP, and TA contamination (**Table 5**). Five out of six samples contained *Alternaria* mycotoxins above the limit of quantification. AOH and TEN were determined in one jar

TABLE 2 | Limits of detection, limits of quantification, recoveries, and precisions determined for *Alternaria* mycotoxins in starch.

Analyte	LOD [µg/kg]	LOQ [µg/kg]	Recovery [%]			Precision (RSD) [%]		
			Level 1	Level 2	Level 3	Inter-injection (n = 5)	Intra-day (n = 3)	Inter-day (n = 9)
AOH	0.50	1.81	101 ± 6	97 ± 2	92 ± 2	2	2	5
AME	0.05	0.23	94 ± 8	103 ± 1	108 ± 5	2	2	3
TEN	0.05	0.16	91 ± 7	83 ± 1	83 ± 2	3	3	7
ATX I	0.42	1.49	101 ± 6	94 ± 5	91 ± 3	2	3	6
ALTP	0.31	1.03	105 ± 5	94 ± 2	97 ± 4	2	1	5
TA	1.25	4.13	95 ± 2	92 ± 5	106 ± 4	2	1	5

TABLE 3 | Limits of detection, limits of quantification, recoveries, and precisions determined for *Alternaria* mycotoxins in fresh tomatoes.

Analyte	LOD [µg/kg]	LOQ [µg/kg]	Recovery [%]			Precision (RSD) [%]		
			Level 1	Level 2	Level 3	Inter-injection (n = 5)	Intra-day (n = 3)	Inter-day (n = 9)
AOH	0.16	0.55	103 ± 3	95 ± 9	100 ± 2	1	1	3
AME	0.01	0.02	111 ± 1	97 ± 1	96 ± 2	1	1	4
TEN	0.05	0.22	101 ± 3	107 ± 2	109 ± 1	1	1	6
ATX I	0.19	0.76	107 ± 4	101 ± 2	103 ± 2	2	1	4
ALTP	0.21	0.74	107 ± 4	97 ± 2	100 ± 1	1	3	5
TA	1.36	5.56	107 ± 2	99 ± 1	96 ± 3	2	1	8

TABLE 4 | Contents of *Alternaria* mycotoxins in infant foods based on the cereals indicated.

Variety	AOH [µg/kg]	AME [µg/kg]	TEN [µg/kg]	ATX I [µg/kg]	ALTP [µg/kg]	TA [µg/kg]
Wheat 1	–	(0.15) [a]	–	–	–	(2.62) [a]
Wheat 2	(0.76) [a]	0.37 ± 0.13	0.60 ± 0.06	1.52 ± 0.11	–	8.38 ± 0.28
Wheat 3	–	0.33 ± 0.11	1.29 ± 0.09	–	–	10.2 ± 0.1
Wheat 4	–	–	(0.06)[a]	–	–	–
Oat 1	–	(0.08)[a]	(0.10)[a]	–	–	22.0 ± 0.5
Oat 2	–	0.25 ± 0.01	0.18 ± 0.02	–	–	8.54 ± 0.75
Millet 1	–	(0.06) [a]	0.33 ± 0.04	–	–	43.6 ± 0.2
Millet 2	(1.01) [a]	0.35 ± 0.05	0.86 ± 0.03	–	–	221 ± 2
Spelt 1	–	(0.05)[a]	0.43 ± 0.01	–	–	102 ± 1
Spelt 2	7.17 ± 0.65	0.27 ± 0.07	0.41 ± 0.15	(0.67) [a]	–	49.6 ± 3.0
Spelt 3	(0.67) [a]	(0.05)[a]	0.72 ± 0.08	–	–	23.7 ± 0.5
Rice 1	–	0.58 ± 0.24	2.22 ± 0.36	–	–	109 ± 1
rice 2	(0.67) [a]	(0.13)[a]	0.30 ± 0.04	–	–	10.1 ± 0.1
Multi-grain 1	4.73 ± 0.04	0.55 ± 0.21	0.41 ± 0.03	–	–	12.4 ± 0.1
Multi-grain 2	–	–	0.21 ± 0.02	–	–	5.66 ± 0.31
Multi-grain 3	–	0.23 ± 0.06	0.62 ± 0.17	–	–	66.0 ± 0.9
Multi-grain 4	(1.37) [a]	0.56 ± 0.13	0.60 ± 0.06	–	–	6.15 ± 0.49
Multi-grain 5	–	0.46 ± 0.08	2.08 ± 0.55	(0.81) [a]	–	104 ± 1
Multi-grain 6	–	0.07 [a]	7.53 ± 0.97	–	4.47 ± 0.31	149 ± 1
Mean[b]	0.89	0.24	1.00	0.17	0.24	50.2
Median[b]	0	0.23	0.43	0	0	22.0

– : not detected, [a]: value between LOD and LOQ, [b]: calculation of mean and median: 0 µg/kg were used for "–", the twofold value of the respective LODs were used for values below the LOQs.

with contents of 2.86 and 0.44 µg/kg. AME was determined in contents ranging from 0.04 to 7.56 µg/kg with a median of 0.14 µg/kg. TA was detected in two of the purees at a mean of 4.95 µg/kg. ATX I, ATX II, ALTP, and STTX III were not detected in any of the puree products. 8.73 µg/kg of TA were quantified in the tomato soup, whereas the tomato sauce contained high amounts of AOH (54.2 µg/kg), AME (7.56 µg/kg), and TA (505 µg/kg).

TABLE 5 | Contents of *Alternaria* mycotoxins in puree infant food consisting of vegetables and fruits, and in tomato products.

Variety	AOH [μg/kg]	AME [μg/kg]	TEN [μg/kg]	ATX I [μg/kg]	ALTP [μg/kg]	TA [μg/kg]
Tomato sauce	54.2 ± 2.8	7.56 ± 0.72	0.44 ± 0.09	–	–	505 ± 26
Apple-pear-cherry	2.86 ± 0.99	1.42 ± 0.07	0.93 ± 0.02	–	–	(5.38) [a]
Pumpkin and potato	(0.32) [a]	–	–	–	–	–
Cherry and banana	(0.17) [a]	0.14 ± 0.07	–	–	–	(4.51) [a]
Vegetable	–	0.04 ± 0.02	–	–	–	–
Tomato soup	–	0.04 ± 0.01	–	–	–	8.73 ± 0.03
Mean[b]	9.62	1.53	0.23	–	–	86.5
Median[b]	0.32	0.14	0	–	–	2.51

–: not detected, [a]: value between LOD and LOQ, [b]: calculation of mean and median: 0 μg/kg were used for "–", the twofold value of the respective LODs were used for values below the LOQs.

DISCUSSION

Method Validation

LODs and LOQs determined in our method validation process are in accordance with the LODs and LOQs of methods recently reported in the literature. Liu et al. determined LODs and LOQs in starch matrix of 0.088 to 0.36 μg/kg and 0.27 to 1.1 μg/kg for AOH, AME, ATX I, and ALTP (Liu and Rychlik, 2015). For TA, low LODs and LOQs were achieved for both matrices. As the sample preparation and sample clean-up was extensively optimized for TA, derivatization with 2,4-dinitrophenylhydrazine (Siegel et al., 2009; Asam et al., 2011b) was not necessary to obtain low LODs and LOQs.

Zwickel et al. calculated LOQs of AOH, AME, TEN, ATX I, and TA of 0.4 up to 0.9 μg/kg in tomato juice (Zwickel et al., 2016). Puntscher et al. recently achieved LODs and LOQs in tomato sauce of 0.05–6 and 0.1–12 μg/kg with a dilute and shoot approach (Puntscher et al., 2018).

In our method validation process, recoveries ranged from 83 to 111% for AOH, AME, TEN, ATX I, ALTP, and TA in both matrices. To achieve these good recoveries, the samples needed to be extracted three times with different solvent mixtures. Performing only one extraction step resulted in losses of analytes between 19 and 42%. AOH, AME, and TA were quantified using stable isotopically labeled internal standards and the recoveries ranged from 92 to 111%. Quantification of TEN, ATX I, and ALTP by matrix matched calibrations resulted in a slightly lower recovery rate for TEN at two spiking levels. For ATX I and ALTP, matrix matched calibrations compensated for analyte losses during sample preparation and recoveries varied between 91 and 109%.

Inter-injection, intra- and inter-day precisions were determined for cereal and tomato based products and resulted in precisions below 4% for inter-injection and intra-day precisions and below 9% for inter-day precisions. Zwickel et al. calculated intra- and inter-day precisions varying from 3.6 to 9.2% and 4.0 to 10.7% in tomato juice (Zwickel et al., 2016). In contrast to this, a recently published dilute and shoot approach revealed intra-day precisions varying from 9 to 34% at low concentrations and from 3 to 16% at high concentrations in tomato sauce. In wheat flour, intra-day precisions ranged from 11 to 83% at low concentrations and from 3 to 11% at high concentrations (Puntscher et al., 2018).

Analysis of Infant Food Samples

In our survey, cereal based infant products were frequently contaminated with *Alternaria* mycotoxins, especially AME, TEN, and TA. While the level of contamination of AME and TEN is mainly rather low, high variations in the content of TA were observed with a mean content of 50.2 μg/kg and a median content of 22.0 μg/kg. The variations were detected not only among different grain varieties but also within one grain variety. Grain products such as oat flakes, wheat flour, rye flour, and maize grit have been analyzed already by Asam et al., who found a median content of TA of 16 μg/kg (Asam et al., 2012). In a subsequent study, Asam et al. quantified the toxin in various infant cereal products (Asam and Rychlik, 2013). The products comprising of wheat, oats, rye, spelt, maize, and barley contained TA in amounts of 8–30 μg/kg. Four different millet products were contaminated with high amounts of TA ranging from 130 to 1200 μg/kg (Asam and Rychlik, 2013). In our present survey, two millet infant cereals were analyzed and contained 43.6 and 221 μg/kg of TA. Two of the multi-grain products contained millet flour and were contaminated with 66.0 and 140 μg/kg of TA. In total, none of the analyzed infant food samples in our survey reached the high contamination of millet with TA of up to 1200 μg/kg, which were determined by Asam and Rychlik (2013). It has to be noted that we undertook all effort to collect more millet based infant food samples, but in contrast to 2012 the availability of this product group was very limited. However, as far as it can be deduced from the analyzed samples, the manufactures obviously optimized their quality control and none of the products exceeded the warning limit of 500 μg/kg, which is beneficial for the consumer's health. As the multi-grain products contained various grains, millet cannot be identified as the sole source of TA contamination.

In 2012, Scott et al. also quantified AOH and AME in various infant foods from Canada. In their survey, single grain as well as

multi-grain products were analyzed and revealed 0.5–4.4 μg/kg of AOH and 0.5–2.0 μg/kg of AME (Scott et al., 2012). For Europe, the EFSA assessed the dietary exposure of the European population to *Alternaria* mycotoxins. The mean contamination of wheat grain, spelt grain, and oats were 0.3–39.7 μg/kg of AOH, 0.03–7.1 μg/kg of AME, 0.03–3.8 μg/kg of TEN, and 2.6–168.7 μg/kg of TA (Arcella et al., 2016). In a recently published survey, nine wheat flours from the Austrian market were analyzed for free and modified *Alternaria* mycotoxins. No *Alternaria* mycotoxin could be detected above the respective limits of quantification (Puntscher et al., 2018). In contrast to this, AOH, AME, TEN, ATX I, and TA were detected in two out of four wheat based cereal samples in our study.

A previous survey by our group analyzed various cereal flours for their content of ATX I, ALTP, ATX II, AOH, AME, and TEN (Liu and Rychlik, 2015). ATX I was determined in three different flours with contents of 2.4–4.7 μg/kg. ALTP was determined in one sample (0.87 μg/kg). AOH, AME, and TEN were quantified in contents ranging from 1.1 to 23, 0.31 to 0.34, and 1.6 to 6.0 μg/kg.

Apart from the cereal products we analyzed four different purees in jars, one tomato sauce and one tomato soup. The results of the tomato sauce were striking, because it contained up to 54.2 μg/kg AOH, 7.56 μg/kg AME, and 505 μg/kg TA. When comparing these unexpectedly high contents of mycotoxins to data from the literature, similarly high concentrations amounting to 25 μg/kg of AOH and 5.3 μg/kg of AME have been reported in a triple concentrated tomato paste (Asam et al., 2011a). Analyzing TA in various tomato products, Asam et al. detected 363 to 909 μg/kg of TA in tomato paste (Asam et al., 2011b). The tomato paste was triple concentrated and, therefore, the raw material could be estimated to have contained 120 to 300 μg/kg. The tomato sauce in our survey is ready to use and consists of 65% of tomatoes as well as 13% of concentrated tomato paste. Therefore, it can be assumed that the concentrated tomato paste might be the reason for the high mycotoxin contamination in the final tomato sauce product. In a recent assessment, the EFSA estimated the chronic dietary exposure of humans to *Alternaria* mycotoxins (Arcella et al., 2016). Lower and upper bound mean concentrations ranged from 0.0 to 5.7 μg/kg of AOH, from 0.0 to 1.0 μg/kg of AME, from 0.0 to 3.9 μg/kg of TEN, and from 34 to 54 μg/kg of TA. Tomato puree contained up to 6.5 μg/kg AOH, 1.9 μg/kg AME, 3.3 μg/kg TEN, and 113 μg/kg TA (EFSA, 2011). These data are well in line with those of our previously analyzed puree infant food in jars that contained TA in amounts ranging from 1.0 up to 78 μg/kg. Tomato soup contained 25 μg/kg of TA (Asam and Rychlik, 2013). The TA content of 78 μg/kg in banana and cherry jars from 2012 (Asam and Rychlik, 2013) was not reached in this study, as the TA content in our banana and cherry jar was only 4.51 μg/kg.

In a recently published survey (Puntscher et al., 2018), various tomato sauces (n = 12) were analyzed for free and modified *Alternaria* mycotoxins. AOH was detected in two samples above the LOQ (1.4 and 20.2 μg/kg). AME and TEN were determined in one sample and the content was 4.0 and 0.6 μg/kg, respectively. TA was quantified in eight samples with contents ranging from 42 to 323 μg/kg (Puntscher et al., 2018). Although the AOH content of 20.2 μg/kg in one of the tomato sauces was high, the tomato sauce for children in our survey exceeded this value by a factor of two.

From the Swiss market, Noser et al. analyzed 85 tomatoes and tomato products and reported even higher toxin contents. One tomato puree contained 790 μg/kg TA, 30 μg/kg AOH, 8 μg/kg AME, and 2 μg/kg TEN. In tomato sauces and soups 4–144 μg/kg of TA, 4–10 μg/kg of AOH, and 1–4 μg/kg of AME were determined. However, the group did not analyze tomato products which are intended for infant consumption (Noser et al., 2011).

Walravens et al. analyzed 28 tomato sauces and determined AOH in contents up to 41.6 μg/kg, AME up to 3.8 μg/kg, and TA up to 330.6 μg/kg, which is well in line with our findings. Tomato concentrate contained up to 31.0 μg/kg AOH, 6.10 μg/kg AME, and 174.3 μg/kg TA (Walravens et al., 2016).

Risk Evaluation

A solid risk assessment on *Alternaria* mycotoxins is still not feasible, as toxicological and occurrence data on the toxins are insufficient. Therefore, the TTC approach was applied for AOH, AME, TEN, and TA. For TEN and TA, the TTC level was set to 1500 ng/kg body weight per day (EFSA, 2011). In 2016, Rychlik et al. evaluated the exposure and risk to infants based on the consumption of millet based infant food (Rychlik et al., 2016). In their study, millet based infant food products were contaminated with TA with contents up to 1200 μg/kg. Rychlik et al. calculated an intake of TA of 3670 ng/kg body weight upon consumption of the highly contaminated millet product. A maximum limit of TA in infant food products of 500 μg/kg was claimed so that the daily exposure of infants would fall below the TTC of 1500 ng/kg body weight per day for TA (EFSA, 2011; Rychlik et al., 2016). In the study presented here, all cereal based infant food samples contained TA below 500 μg/kg and, therefore, the products comply with this limit set by Rychlik et al. (2016).

Due to their genotoxicity, the TTC levels for AOH and AME were set to 2.5 ng/kg body weight per day (EFSA, 2011). One spelt product in our survey contained 7.17 μg/kg AOH. Considering an infant's weight of 7 kg and the size of a portion of 18 g, as suggested by the manufacturer, the intake would be 18.4 ng/kg body weight. This exceeds the TTC value by a factor of around seven. Therefore, a risk to the health of the infant cannot be excluded. One millet, and two multi-grain samples contained 1.01, 3.40, and 1.37 μg/kg of AOH, respectively. Calculating the intake of AOH based on the portions size of 20 to 25 g, the intakes also exceed the TTC value of 2.5 ng/kg. Considering the mean content of AOH of 0.89 μg/kg from our limited survey, the respective intake would not exceed the TTC level of 2.5 ng/kg

body weight per day (EFSA, 2011). Moreover, the determined contents of AME and TEN in the cereal based infant food products were rather low and cannot be considered as a risk to the health of infants.

As further infant foods, four purees in jars and two tomato products were analyzed for mycotoxin contamination. Especially the tomato sauce contained high amounts of AOH, AME, TEN, and TA. Considering the size of a portion of 50 g and an infant's weight of 10 kg at the age of 12 months, the intake exceeds the TTC levels of AOH and AME many times over. Therefore, for this individual product, a risk to the health of infants cannot be excluded (EFSA, 2011) and we called the manufacturer's attention to this contamination. The median contents of the purees and tomato products were 0.32 μg/kg for AOH, 0.14 μg/kg for AME and 2.51 μg/kg for TA. On the basis of these calculations, the consumption of the infant food products do not constitute a risk to the health of the infants on average (EFSA, 2011).

Not only the free forms of the *Alternaria* mycotoxins attract the interest in quantitative analysis, but also the modified forms of AOH and AME are more frequently analyzed. Puntscher et al. quantitated the AOH-9-glucoside and the AOH-3-sulfate and AME-3-sulfate in food samples above the respective limits of quantification for the first time. Due to these modified forms of *Alternaria* mycotoxins, human health might be much more endangered than assumed and should be considered for tolerable daily intake determination (Puntscher et al., 2018). Further studies are underway to include also these toxins into our validated method.

CONCLUSION

A LC-MS/MS method for the simultaneous determination of six *Alternaria* mycotoxins was developed and the extraction and purification of mycotoxins was optimized. The method was validated successfully according to Vogelgesang and Hädrich (1998). The method validation was performed in starch and tomato matrix and resulted in low LODs and LOQs and good recoveries for all analytes. Good precisions confirmed stability and robustness of the method.

Various cereal based infant foods, jars containing vegetables and fruits and tomato based products for infants were analyzed for *Alternaria* mycotoxins. Studies on infant foods are rare and so far were limited to certain toxins (Scott et al., 2012; Asam and Rychlik, 2013). Our study included six toxins in the analysis of infant foods for the first time. The analyses resulted in partly high contaminations of the infant food products and risks to the health of infants cannot be excluded. These new and unexpected results show that more infant food products should be analyzed to determine mycotoxin contamination and to perform a proper risk assessment with special regards to infants. Moreover, the manufacturers should be made aware of the necessity to screen their products more thoroughly for these toxins and take the appropriate measures to reduce their contents.

AUTHOR CONTRIBUTIONS

SA, MG, and MR designed the experiments. KG optimized the mycotoxin extraction and purification and performed the method validation for starch matrix. RK optimized analyte purification by solid phase extraction. AM performed the method validation for tomato matrix. EB analyzed the infant food samples. MG and SA wrote the manuscript. MR revised the manuscript. All authors contributed to the revision of the manuscript.

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B Comprehensive Analysis of the *Alternaria* Mycobolome Using Mass Spectrometry Based Metabolomics

RESEARCH ARTICLE

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Comprehensive Analysis of the *Alternaria* Mycobolome Using Mass Spectrometry Based Metabolomics

Marina Gotthardt, Basem Kanawati, Frank Schmidt, Stefan Asam, Richard Hammerl, Oliver Frank, Thomas Hofmann, Philippe Schmitt-Kopplin, and Michael Rychlik**

Scope: ***Alternaria*** **fungi are widely distributed plant pathogens infecting grains and vegetables and causing major harvest losses in the field and during postharvest storage. Besides, consumers are endangered by the formation of toxic secondary metabolites. Some of these secondary metabolites are chemically characterized as mycotoxins, but the majority of the** ***Alternaria*** **mycobolome still remains unknown.**
Methods and results: Fourier-transform ion cyclotron resonance mass spectrometry (FTICR-MS) and LC-MS/MS are combined for the non-targeted and targeted analysis of the metabolome of three ***A. alternata*** **isolates and one** ***A. solani*** **isolate. Due to the ultra-high resolution of FTICR-MS, unique molecular formulae are assigned to measured** ***m/z*** **signals. The molecular formulae are matched to entries of the databases Antibase and Kyoto Encyclopedia of Genes and Genomes. The non-targeted analysis of the fungal extracts reveals variations in the secondary metabolite profile of** ***A. alternata*** **and** ***A. solani*****. Differences in the biosynthesis of dibenzo-α-pyrones, perylene quinones, tentoxin, and tenuazonic acid of the** ***A. alternata*** **and** ***A. solani*** **isolates are determined applying targeted LC-MS/MS.**
Conclusion: FTICR-MS analyses reveal clear differences in the metabolic profile of the ***A. solani*** **and the** ***A. alternata*** **isolates.**

1. Introduction

Fungi of the genus *Alternaria* are widely distributed on seeds, plants, animals, in the soil, and in the atmosphere.[1] Growing in various regions and during different seasons, *Alternaria* species exhibit endophytic, saprophytic, or pathogenic growth.[1,2] The fungi decompose natural as well as artificial substrates[3] and cause plant diseases such as black rot of tomato, olive, and carrots and black and grey rot of citrus fruits.[1,2] Besides, cereals such as wheat, barley, oats, and sorghum are frequently infected by *Alternaria* fungi[4] resulting in losses of agricultural products in the field and during postharvest storage.[5] Common *Alternaria* species are *Alternaria (A.) alternata*, *A. tenuissima*, *A. arborescence*, *A. radicina*, *A. brassicae*, *A. brassicicola*, and *A. infectoria*.[6]

In addition to losses of agricultural goods, *Alternaria* fungi endanger consumers by the production of mycotoxins and secondary metabolites with partly unknown toxicological potential.[7] The mycotoxins accumulate in agricultural products leading to a decline of the food quality.[8] Over 70 different secondary metabolites are described in the literature and more than 30 exhibit toxicological potential.[9]

Common *Alternaria* mycotoxins belong to five different structural classes, namely 1) tetramic acid derivatives (tenuazonic acid [TA]), 2) dibenzo-α-pyrones (alternariol [AOH], alternariol monomethyl ether [AME], and altenuene), 3) perylene derivatives (altertoxin I [ATX I], altertoxin II [ATX II], alterperylenol [ALTP], and stemphyltoxin III [STTX III]),[2] 4) miscellaneous structures (tentoxin [TEN]), and 5) *A. alternata* f. sp. *lycopersici* toxins (AAL-toxins).[9] In previous studies, the benzopyrones exhibited genotoxic, cytotoxic, and mutagenic effects in vitro[2,5] and caused DNA damages in human colon carcinoma cells.[10] Additionally, AOH and AME were described to act as topoisomerase poison and to inhibit the catalytic activity of topoisomerase IIα.[5] While the acute toxicity of AOH and AME is rather low,[4] TA is acutely toxic to mice, chicken, and dogs.[2] No mutagenicity was observed for the tetramic acid derivative in bacterial systems,[5,11] whereas the altertoxins were mutagenic in the *Salmonella* Ames Test [11,12] and strongly genotoxic in mammalian and human cells.[13,14] Contrarily to the other mycotoxins, TEN is

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characterized as phytotoxin and inhibits the cyclic photophosphorylation in chloroplasts.[15]

As *Alternaria* mycotoxins are neither legislatively controlled nor routinely analyzed,[16] they are considered as "emerging mycotoxins."[17] Besides, the modifications of AOH and AME with sulfates and glucosides attract increasing attention.[18] These so-called "modified mycotoxins" are either produced by the fungi themselves or the mycotoxins are metabolized by plants for detoxification purposes.[19–21] After oral consumption, the modification is potentially hydrolyzed during digestion, which releases the aglycon.[22–24] In a recently developed LC-MS/MS method, AOH-9-glucoside and AME-3-sulfate were detected in naturally contaminated foods demonstrating the necessity to routinely analyze also the chemically modified mycotoxins in agricultural products.[17] Furthermore, the modified forms should be included in future risk evaluations[19] and also added to mass spectrometric databases.

In 2011, the Panel on Contaminants in the Food Chain of the European Food Safety Authority (EFSA) assessed the risk to human health originating from *Alternaria* mycotoxins in agricultural products. The estimated chronic dietary exposure of AOH and AME exceeded the threshold of toxicological concern (TTC) of 2.5 ng kg^{-1} body weight per day and, therefore, additional toxicity data are indispensable for further risk evaluations. TA and TEN are non-genotoxic and the estimated chronic dietary exposure did not reach the TTC value of 1500 ng kg^{-1} body weight per day.[9] However, recent studies, particularly on infant foods, highlighted that these TTC values were exceeded[25] and required the establishment of a legal limit for TA in food products for infants.[26]

To perform reliable risk assessments, the metabolic capabilities of *Alternaria* fungi need to be investigated and various species need to be identified correctly. Traditionally, the systematics of *Alternaria* fungi was based on morphological characteristics,[3,8] but morphology alone was insufficient due to overlapping traits of closely related species.[2] In addition to 3D sporulation patterns on agar plates,[8,27,28] *Alternaria* species were also classified based on the sequence variation in the translation elongation factor 1-α.[8] The analysis of further genes resulted in distinct *Alternaria* species clusters.[3] As DNA-based studies did not always match the species-groups defined in morphological surveys,[1] excreted metabolites produced by the fungi were analyzed by HPLC-UV and LC-MS/MS and used for their differentiation.[29–31] The species group of *A. infectoria* was separated from *A. arborescence*, *A. alternata*, and *A. tenuissima*, but the latter were indistinguishable based on the mycotoxin profile obtained by LC-MS/MS.[30,31] As high-resolution mass spectrometers offer the simultaneous detection of hundreds to thousands of metabolites, these instruments are increasingly in use for the differentiation of fungal species.[32,33]

In addition to the species segregation, high-resolution mass spectrometers improve the holistic characterization of the fungal mycobolome. The comprehensive detection of low-molecular-weight metabolites of an organism is called metabolomics.[34] As the metabolome of organisms varies depending on genotype, cell cycle stage, or environment,[35] metabolomics approaches focus on the detection of a wide range of possibly produced compounds.[34] It has to be kept in mind that sample preparation and the selection of the ionization mode in the electrospray ionization (ESI) source will significantly influence the detected metabolite profile.[34,36] Metabolomics approaches commonly use time-of-flight (TOF), Orbitrap, and Fourier transform ion cyclotron resonance (FT-ICR) mass analyzers.[34] As FTICR-MS combines ultra-high mass resolution and superior mass accuracy,[34,37,38] unique elemental compositions can be assigned clearly to measured m/z signals. Although the allocation of molecular formulae to metabolites is possible,[39] the structural identification of molecular formulae remains the bottleneck in mass spectrometric metabolomics studies.[36] FTICR-MS analysis was utilized to investigate alterations between *Chlamydia pneumoniae*-infected and non-infected Hep-2 cells[38] and to identify differences in the growth stages of bacteria.[39] Applying FTICR-MS to foods is called "foodomics" aiming to analyze the functionality, the nutritional value, and the safety of agricultural products.[24] Regarding mycotoxins and related food contaminants, foodomics investigations can support the identification of new or fungal metabolites on foods and can provide further data for proper risk assessments.[24]

In the present study, various fungal isolates of *A. alternata* and *A. solani* were cultivated in a chemically defined liquid medium and the extracts were analyzed by direct infusion FTICR-MS. Due to the determination of hundreds of metabolites, we aim at obtaining a more holistic insight into the fungal mycobolome. Assigned molecular formulae are intended to be allocated to metabolites using the Antibase and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases. Complementing this non-targeted FTICR-MS approach with targeted LC-MS/MS analysis[25] will help to clearly identify *Alternaria* mycotoxins, as LC-MS/MS offers advantages in selectivity[40] and the possibility to chromatographically separate isomers such as ATX II and ALTP. Additionally, the LC-MS/MS measurements are intended to provide quantitative results on intra- and extracellular mycotoxin contents.

2. Results and Discussion

2.1. Comprehensive Insights into the Fungal Mycobolome

2.1.1. Selection of Fungal Isolates

A. alternata isolates were analyzed by FTICR-MS to obtain a comprehensive insight into the fungal mycobolome. To cover a "general" *A. alternata* metabolome irrespective of the origin of the fungi, *A. alternata* was isolated from different sources such as potato leaves, tomato leaves, and moldy tomatoes. Apart from the three small-spored *A. alternata* isolates 1, 2, and 3, one *A. solani* isolate was analyzed to reveal interspecies variations. The fungi were cultivated in a chemically defined liquid medium consisting of salts and glucose, facilitating the detection of metabolites solely biosynthesized by *Alternaria*. As most of the metabolites produced by the fungi are excreted into the media,[29] the cultivation in liquid medium circumvented the extensive extraction of metabolites after growing the isolates on solid medium. In the literature, metabolic profiling is mainly performed by growing the fungi on solid media due to a higher quantity and a higher number of produced metabolites.[29] In 2018, Zwickel et al. performed a study on the metabolic profiles of different *Alternaria* species

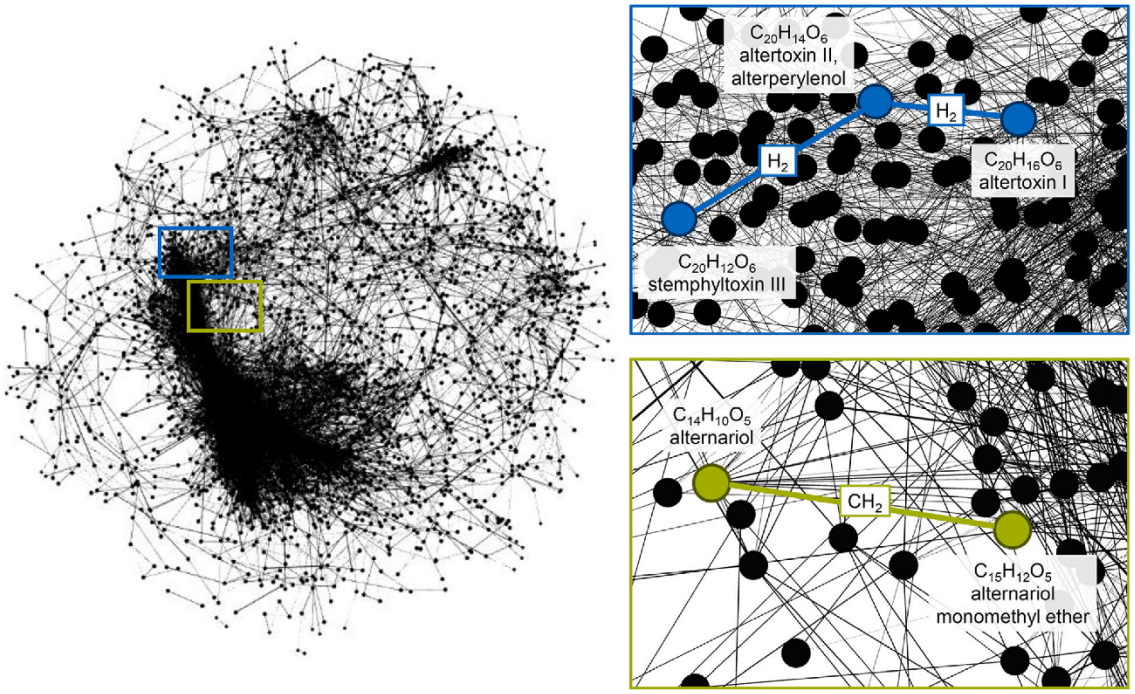

Figure 1. Network generated for annotation purpose. The blue box shows the connections between STTX III, ATX II/ALTP, and ATX I, and the green box displays the computational connection between AOH and AME.

grown on rice.[31] After the cultivation, the mycotoxins were laboriously extracted using a mixture of acetonitrile/water/acetic acid.[41]

2.1.2. Molecular Formula Annotations

The network established during the annotation process is shown in **Figure 1**. The black nodes in the network represent assigned molecular formulae, which are linked via edges representing biochemical reactions.[42] The smaller sections of the network illustrate the biochemical connections between the molecular formulae of AOH and AME, and between the molecular formulae of ATX I, ATX II/ALTP, and STTX III. The elemental compositions of AOH and AME are linked via the accurate mass difference of CH_2, whereas the accurate mass difference of H_2 interconnects the molecular formulae of the perylene quinones ATX I, ATX II/ALTP, and STTX III. In addition to ATX I, ATX II/ALTP, and STTX III, molecular formulae of other perylene quinones described in the literature were detected in the data. The molecular formulae of ATX III ($C_{20}H_{12}O_6$),[43] STTX IV ($C_{20}H_{12}O_7$),[43] alterlosin I/STTX I ($C_{20}H_{14}O_7$),[43,44] stemphyperylenol ($C_{20}H_{16}O_6$),[43] and alterlosin II/7-*epi*-8-hydroxyaltertoxin I/stemphytriol/6-*epi*-stemphytriol ($C_{20}H_{16}O_7$)[43,44] were determined and **Figure 2** displays their elemental compositions as well as their chemical structures. Applying LC-Orbitrap, Zwickel et al. determined ATX I, ATX II, STTX III, as well as hydrated and dehydrated forms of these mycotoxins corresponding to further perylene quinones.[31] Besides, the latter authors detected additional peaks in the ion chromatograms of ATX I, ATX II, and STTX III, sharing the same elemental compositions as the respective mycotoxins within the mass error range (± 5 ppm) of the applied LC-Orbitrap MS instrument.

Apart from the determination of mycotoxins, a comprehensive analysis of the fungal mycobolome also includes the detection of precursors of metabolites. Previous studies on the biosynthesis of perylene quinones of fungi postulated a dinaphthyl intermediate as a precursor.[45,46] The dinaphthyl intermediate is supposed to originate from two tetralone derivatives biosynthesized from one acetate and six malonyl units.[46,47] Molecular formulae of 1,3,6,8-tetrahydroxynapthalene ($C_{10}H_8O_4$), syctalone ($C_{10}H_{10}O_4$), 1,3,8-trihydroxynaphtalene ($C_{10}H_8O_3$), vermelone ($C_{10}H_{10}O_3$),[48] and perylene quinone ($C_{20}H_{10}O_4$)[43] were detected in our data and might represent precursors of the respective perylene quinones. Biosynthetic pathways of fungal secondary metabolites are often not clarified yet and reference compounds for the precursors are often not available. Therefore, the identification of the precursors could not be performed in the present study.

Modified forms of mycotoxins attract increasing attention in fungal investigations and sulfo-conjugations were frequently detected by different research groups.[19,31,49] During FTICR-MS measurements, the molecular formulae of the sulfo-conjugated forms of the mycotoxins AOH, AME, altenuisol, and altenuene[31] were not detected, which is not surprising as the intensities of the *m/z* signals of AOH, AME, and altenuisol in the mass spectra were already low.

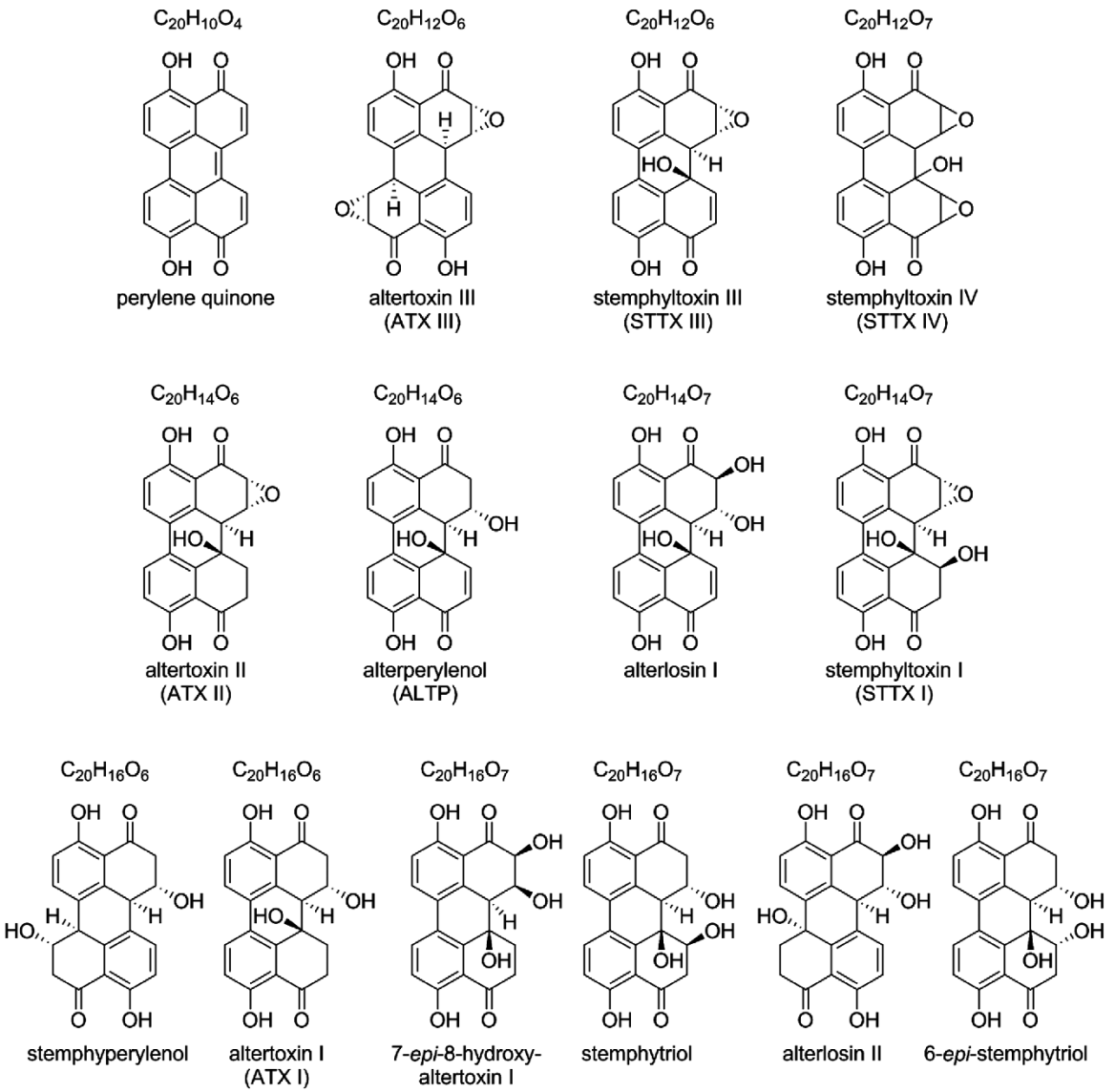

Figure 2. Molecular formulae and structures of perylene quinone derivatives in the literature possibly detected in the FTICR-MS data.[43,44,47]

The number of possible structural suggestions and the information on the biological context were extended by matching the molecular formulae to entries of two different databases. For the annotation of secondary metabolites, the elemental compositions were checked against the subset *Alternaria* of the database Antibase.[50] In total, 86 of the 2883 molecular formulae were assigned to metabolites, which equals 3 % annotation rate. 97 % of the molecular formulae could not be assigned to metabolites using Antibase. Applying only the subsets *A. alternata* and *A. solani* of Antibase to the data, mainly solanapyrones and altersolanols were annotated for *A. solani*, whereas bicyclo- (BCA) and tricycloalternarenes (TCA) were assigned for *A. alternata*. Detailed information on the detected experimental masses, the theoretical neutral masses, the molecular formulae as well as the annotated metabolites using the subsets *A. alternata* and *A. solani* are depicted in **Table 1**.

Besides the secondary metabolites, molecules of the primary metabolism can be detected by FTICR-MS analysis. The assigned molecular formulae were compared to the entries of the KEGG database and subsequently allocated to species-specific pathways.[69] Comparing the assigned molecular formulae to the entries of the KEGG database resulted in 527 annotations (18 %) and, due to molecules with the same molecular formula, in 1820 metabolite annotations. The database assignments of less than 20 % illustrate the extent of the still unknown metabolism of *Alternaria* fungi and demonstrate the low coverage of the total

Table 1. Detected experimental masses, theoretical neutral masses, annotation errors, annotated molecular formulae, and metabolite candidates of the database match in Antibase for the subsets *A. alternata* and *A. solani*.[50]

Experimental mass	Theoretical neutral mass	Mass error of annotation [ppm]	Subset in Antibase	Annotated molecular formula	Assignments during database search for *A. solani* and *A. alternata*
301.14452	302.15181	−0.044	*A. solani*	$C_{18}H_{22}O_4$	Solanapyrone A,[51] solanapyrone E,[52] prosolanapyrone III [53]
330.17112	331.17836	0.103	*A. solani*	$C_{19}H_{25}NO_4$	Solanapyrone C [54]
333.09796	334.10526	−0.059	*A. solani*	$C_{17}H_{18}O_7$	Altersolanol G [55]
335.07725	336.08452	0.016	*A. solani*	$C_{16}H_{16}O_8$	Altersolanol A,[56] Altersolanol D–F[57]
271.06118	272.06848	−0.064	*A. alternata*	$C_{15}H_{12}O_5$	AME[58]
319.15511	320.16238	0.030	*A. alternata*	$C_{18}H_{24}O_5$	TCA A[59]
323.15002	324.15729	0.022	*A. alternata*	$C_{17}H_{24}O_6$	AF toxin II, AF toxin IIA, AF toxin IIC[60]
345.20716	346.21441	0.083	*A. alternata*	$C_{21}H_{30}O_4$	ACTG Toxin D,[61] TCA 2a/b, TCA 8a,[62] ACTG Toxin E[63]
347.22279	348.23006	0.028	*A. alternata*	$C_{21}H_{32}O_4$	TCA 1a/b,[64] BCA 3, BCA 9[65]
361.20204	362.20933	−0.015	*A. alternata*	$C_{21}H_{30}O_5$	TCA C[59]
361.23843	362.24571	−0.006	*A. alternata*	$C_{22}H_{34}O_4$	TCA 11a/b,[62] BCA 4, BCA 5[65]
363.21769	364.22498	−0.015	*A. alternata*	$C_{21}H_{32}O_5$	TCA 6a/b,[62] BCA 2, BCA 8[65] TCA E[59]
365.23334	366.24063	−0.017	*A. alternata*	$C_{21}H_{34}O_5$	BCA 1[65]
367.08232	368.08961	−0.032	*A. alternata*	$C_{20}H_{16}O_7$	Alterlosin II [44]
377.23334	378.24063	−0.034	*A. alternata*	$C_{22}H_{34}O_5$	TCA 7a/b,[62] BCA 10[65]
379.24899	380.25628	−0.027	*A. alternata*	$C_{22}H_{36}O_5$	BCA 11[65]
381.22825	382.23554	−0.029	*A. alternata*	$C_{21}H_{34}O_6$	BCA 6[65]
387.21768	388.22498	−0.047	*A. alternata*	$C_{23}H_{32}O_5$	TCA B[59]
389.23332	390.24063	−0.085	*A. alternata*	$C_{23}H_{34}O_5$	TCA D[59]
395.24390	396.25119	−0.045	*A. alternata*	$C_{22}H_{36}O_6$	BCA 7[65]
413.21944	414.22671	0.030	*A. alternata*	$C_{22}H_{30}N_4O_4$	TEN[66]
415.23507	416.24236	−0.018	*A. alternata*	$C_{22}H_{32}N_4O_4$	Dihydrotentoxin[67]
423.20244	424.20972	−0.013	*A. alternata*	$C_{22}H_{32}O_8$	AF toxin III, AF toxin 3A[68]
439.19734	440.20464	−0.033	*A. alternata*	$C_{22}H_{32}O_9$	AF toxin 1, AF toxin A1[68]

diversity of all existing metabolites.[70] Besides, great attention should be paid to multiple annotations to only one molecular formula also hampering the identification of the metabolites.[38] This problem was addressed by Nielsen et al., as the researchers determined multiple entries of Antibase 2008 exhibiting identical molecular formulae. For example, the elemental composition of $C_{15}H_{22}O_3$ resulted in 113 metabolite candidates.[71] Another example of multiple assignments was given by Zwickel et al.[31] ATX II and ALTP share the same molecular formula and, therefore, exhibit identical *m/z* values in the mass spectrum. In the survey of Zwickel et al., various *Alternaria* isolates were analyzed by high-resolution mass spectrometry and four chromatographically separated peaks in the ion chromatogram of ATX II were assigned to the same molecular formula. Only ATX II and ALTP were identified, whereas the other two peaks could not be allocated to metabolites.[31] The results of Nielsen et al. and Zwickel et al. demonstrate the difficulty in dealing with multiple assignments of molecular formulae to metabolites.[31,71]

To support the identification of metabolites, MS/MS spectra, specific UV–vis spectra, and authentic reference compounds are indispensable.[30,71,72] In our survey, the lack of reference compounds allowed solely the hypothetical identification of the database assignments. The unambiguous identification of the metabolites was only performed for AOH, AME, ATX I, ATX II, ALTP, STTX III, TEN, and TA using targeted LC-MS/MS analysis (see Section 3.2).

Additionally, the metabolites were allocated to metabolic pathways listed in the KEGG database.[69] **Figure 3** displays various pathways of amino acids, carbohydrates, and lipids related to *A. alternata*. The black bars represent the number of annotated metabolites belonging to one specific pathway. Contrarily, the shaded bars show the number of molecules, which belong to the respective pathway, but the molecular formulae of which were not detected in the data. The percentage of detected molecular formulae with regard to the number of all metabolites belonging to the pathways shown in Figure 3 was mainly below 50%. This

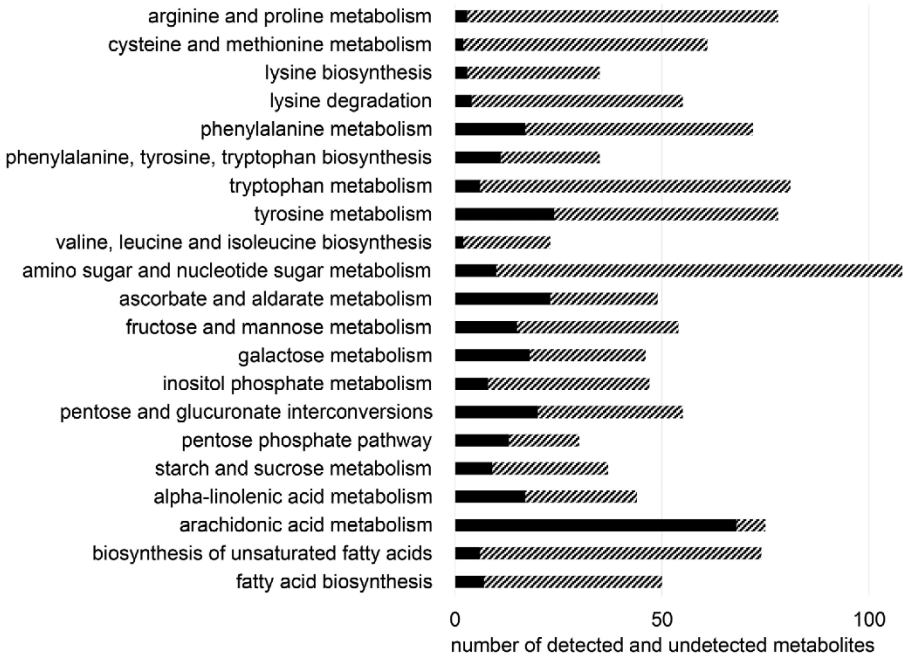

Figure 3. The illustration displays the number of detected molecular formulae (black bars) of various metabolic and biosynthetic pathways in the KEGG database.[69] The shaded bars represent metabolites belonging to the respective pathways, but the molecular formulae were not detected in the FTICR-MS data.

may be due to the restrictions of the applied methodology, for example, a too small or large molecule size (beyond the detection limit of the MS analyzer), the presence of other elements than CHNOSP in the elemental composition, the SPE conditions during sample preparation, the ionization mode during MS measurements, or simply due to low concentrations of the molecules.

After the annotation of *m/z* signals to molecular formulae, the hydrogen to carbon ratios (H/C) and the oxygen to carbon ratios (O/C) were calculated from the elemental compositions.[38] The H/C ratios of the molecular formulae were plotted against the O/C ratios[73] and each *Alternaria* isolate was displayed separately on such a van Krevelen diagram. In the van Krevelen plot, different metabolite classes have their specific position based on different elemental compositions (**Figure 4**).[36] The positions of the metabolite classes of fatty acids, amino acids and peptides, carbohydrates, and polyphenols are displayed in Figure 4a. For all *Alternaria* isolates, 72% of the molecular formulae corresponded to a CHO composition, followed by 17% of a CHNO composition, and 11% of CHOS and CHNOS compositions. Comparing the plots of Figure 4a–d with each other, the profiles of the mycobolome differ. In the *A. solani* samples, fatty acids and condensed terpenoids are displayed, whereas in the *A. alternata* samples, the polyphenols were dominant. All investigated *Alternaria* fungi share the presence of amino acids and peptides.

As the molecular formulae of the *Alternaria* mycotoxins AOH, AME, ATX I, ATX II, ALTP, STTX III, and TA, of further perylene quinones, possible precursors and other secondary metabolites were detected in the FTICR-MS data, we assumed a representative coverage of the *Alternaria* mycobolome under the given conditions. Besides, the suitability of the liquid medium for the cultivation of *Alternaria* isolates was confirmed and the sample preparation protocol as well as the conditions during the measurements allowed the detection of a wide range of fungal secondary metabolites.

2.2. LC-MS/MS Detection of *Alternaria* Mycotoxins

In addition to the non-targeted analysis of fungal extracts, the identification and quantification of the mycotoxins AOH, AME, ATX I, ATX II, ALTP, STTX III, TEN, and TA were complemented by targeted LC-MS/MS analysis. The mycotoxins were compared to reference compounds and were identified based on retention times and mass transitions.[25] A chromatogram of AOH, AME, ATX I, ATX II, ALTP, STTX III, and TEN is displayed in Figure S1a, Supporting Information. An additional chromatographic run had to be performed for TA due to different polarity (Figure S1b, Supporting Information). As adequate amounts of stock solutions were not available for ATX II and STTX III, quantitative values could not be calculated for these mycotoxins. The sample preparation and LC-MS/MS analysis were not fully validated for the fungal cultures in this study, and therefore, no limits of detection and quantification were calculated. However, to precisely identify the mycotoxin signals in the LC-MS/MS run, a minimum peak area unit of 10^5 was stated. The peak areas of the mycotoxins are displayed as mean values of areas of the five biological replicates (**Figure 5**). If one or two of the five replicates

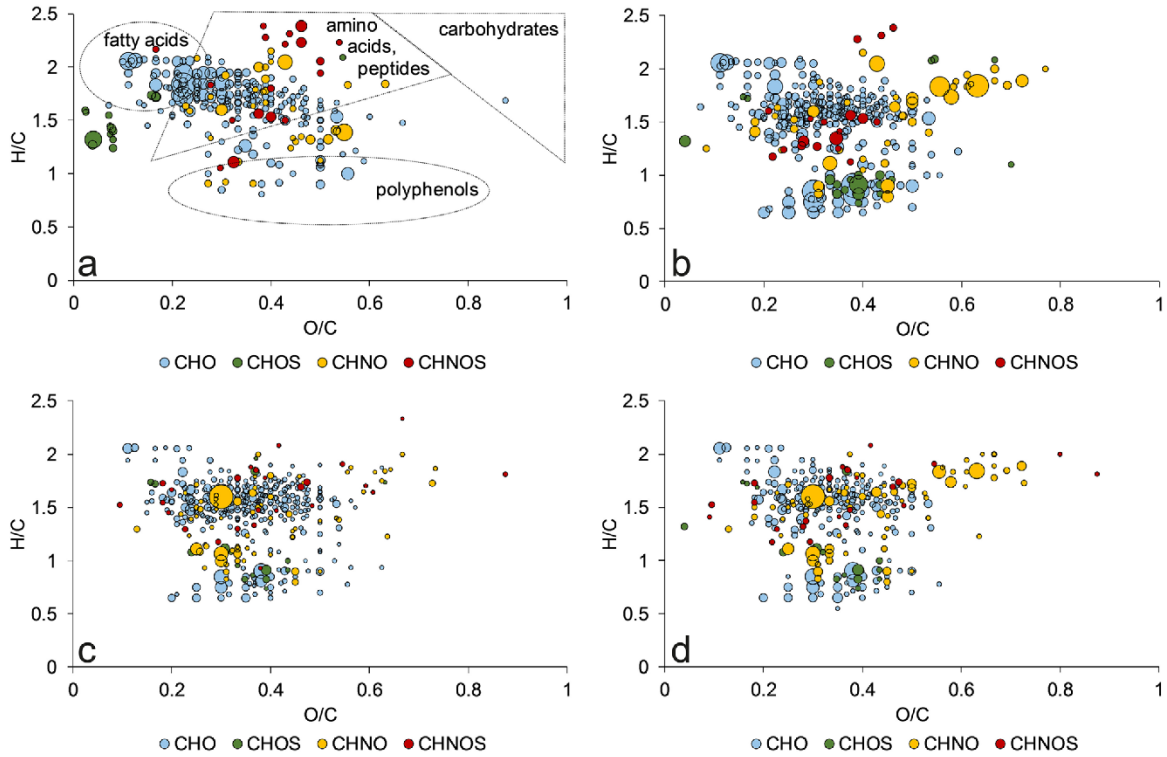

Figure 4. Van Krevelen diagrams of a) *A. solani*, b) *A. alternata* isolate 1, c) *A. alternata* isolate 2, and d) *A. alternata* isolate 3. The labeling of the chemical groups was performed according to Roullier-Gall et al.,[36] Liu et al.,[74] and Schmitt-Kopplin et al.[75]

showed peak areas below the area cut off, a peak area of 1×10^5 was used for the calculation of mean values. Outliers were detected by applying Dixon's Q testing.

The *A. solani* isolate did not produce any of the targeted *Alternaria* mycotoxins above the peak area cut off, neither on the first day of sampling nor on the other days of cultivation. In the literature, *A. solani* is reported to produce AOH and AME.[76,77] AOH was consistently detected in the medium of the *A. alternata* isolate 2, whereas the *A. alternata* isolate 1 and the *A. solani* isolate did not produce AOH at all. AME was not detected in the media of any isolate. As the benzopyrones were neither detected in the extracts of *A. solani* nor often analyzed in the samples of *A. alternata*, the excretion of AOH and AME into the liquid medium might be low. The three *A. alternata* isolates produced various mycotoxins exceeding the minimum peak area (Figure 5a1–d1). The most frequently detected mycotoxins were the perylene quinones ATX I, ATX II, ALTP, and STTX III. These mycotoxins were produced by all *A. alternata* isolates and were detected at each day of sampling. During the cultivation, the peak area of ATX II and STTX III decreased to less than one tenth for all *A. alternata* isolates when comparing the peak area of the 4th to the 11th day of cultivation. Contrarily, a decrease of the peak area of ATX I and ALTP could not be observed. ATX II and STTX III structurally share an epoxy group,[43] probably sensitive to chemical degradation during the cultivation process. The *A. alternata* isolates 1, 2, and 3 produced TEN, which was detected from the 7th day of cultivation on. TA was produced by the isolates 2 and 3, while the isolate 1 did not produce TA at all. Due to the different sensitivity of the analytes in the mass spectrometer, the peak areas of the mycotoxins were transferred into contents via one-point calibration (Figure 5a2–d2). Quantitative results could not be calculated for ATX II and STTX III due to the lack of adequate amounts of stock solutions. The determined contents of AOH were below 5 µg kg^{-1} for the isolates 2 and 3. ATX I and ALTP were detected in all *A. alternata* isolates in contents ranging from 28 to 233 µg kg^{-1} and from 40 to 182 µg kg^{-1}. TA was produced by the isolates 2 and 3 and the calculated amounts varied from 5400 to 20 000 µg kg^{-1}. The highest content of TEN of 37 µg kg^{-1} was produced by the isolate 1. However, it has to be mentioned that the calculated contents were obtained using only one-point calibration and were not quantified by matrix matched calibration or by using isotopically labeled internal standards. Therefore, the given contents should only be classified as semi-quantitative.

The biosynthetic capabilities of diverse *Alternaria* species has been reported by Andersen et al. [30] and Zwickel et al.,[31] who both have been growing their isolates on solid media. Anderson et al. investigated 87 *Alternaria* isolates and allocated 22 isolates to the *A. arborescence*, *A. infectoria*, *A. tenuissima*, and *A. alternata* species groups.[30] Similarly, Zwickel et al.[31] performed studies on 93 isolates of *A. alternata*, *A. arborescence*, *A. tenuissima*, and *A. infectoria*. In the former study, the *A. alternata*, *A. arborescence*, and *A. tenuissima* isolates frequently produced AOH, AME,

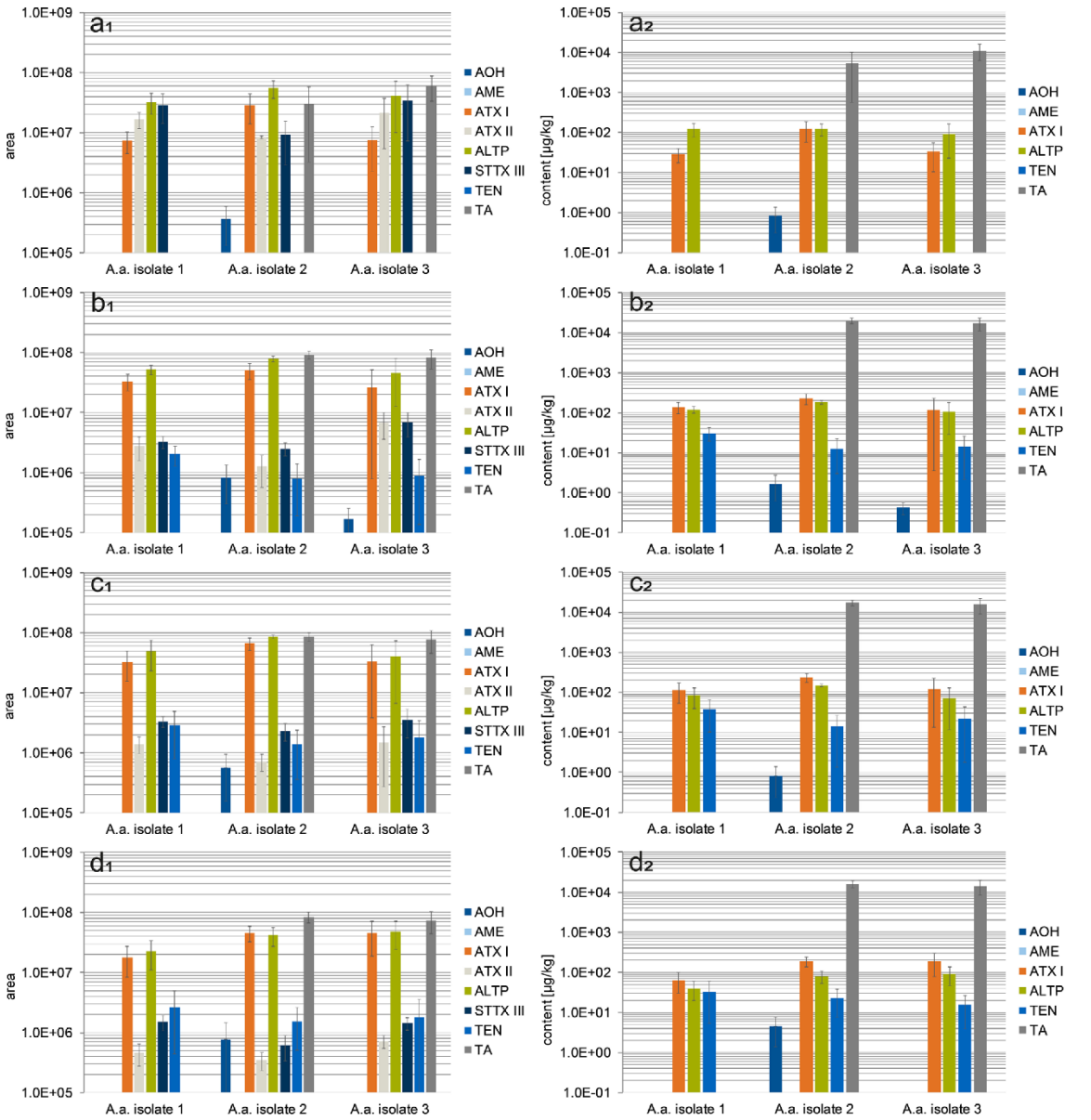

Figure 5. The peak areas of AOH, AME, ATX I, ATX II, ALTP, STTX III, TEN, and TA are displayed for the 4th day (a1), 7th day (b1), 9th day (c1), and 11th day (d1) of cultivation. The peak areas are shown as mean values and standard deviations of the five replicates. The *A. solani* isolate is not displayed, as this isolate did not produce any mycotoxin above the peak area limit. a2–d2) Contents of the mycotoxins AOH, AME, ATX I, ALTP, TEN, and TA in the liquid medium of the 4th, 7th, 9th, and 11th day of cultivation calculated via one-point calibration. Note the logarithmic axis of the peak areas and of the mycotoxin contents.

and altenuene, whereas TEN and TA were biosynthesized less frequently. In the study of Zwickel et al., 21 isolates belonged to the *A. alternata* species group and 90% of the isolates produced ATX I, 81% produced STTX III, and 76% ATX II and ALTP. AOH and AME as a group as well as TA were biosynthesized by 81% and 76% of the *A. alternata* isolates, respectively. Interestingly, five out of the 93 *Alternaria* isolates did not biosynthesize any of the analyzed mycotoxins. These results are partly different to ours, as the benzopyrones were not detected in the liquid medium of our *A. alternata* isolate 1 and were rarely determined in the samples of the *A. alternata* isolate 3. One explanation for this discrepancy could be given by the study of Söderhäll et al.,[78] who investigated the mycotoxin production of *A. alternata* under the exposure of white light. Depending on the growth phase of

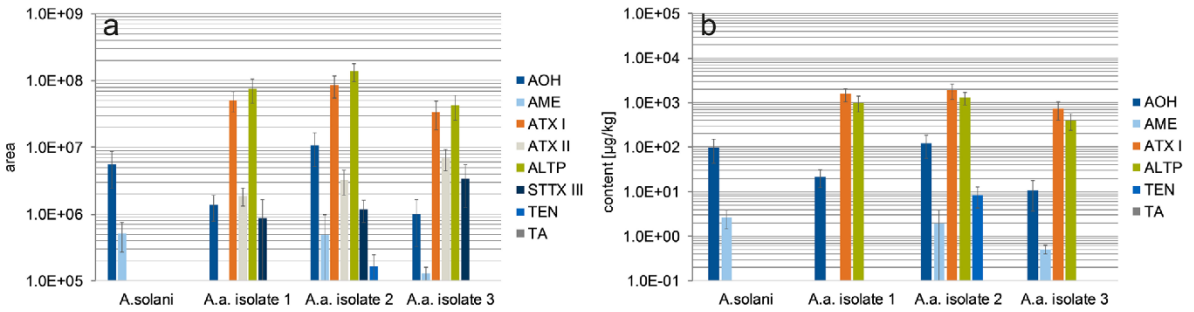

Figure 6. a) The peak areas of AOH, AME, ATX I, ATX II, ALTP, STTX III, TEN, and TA in the disrupted cells form the mycelium. The peak areas are shown as mean values and standard deviations of the five replicates. b) The contents of the mycotoxins AOH, AME, ATX I, ALTP, and TEN in the fungal cells calculated via one-point calibration. Note the logarithmic axis of the peak areas and of the mycotoxin contents.

the fungus, the biosynthesis of the benzopyrones was almost completely inhibited after the light exposure,[78] which may have been also the case for our cultures. The perylene quinones ATX I, ATX II, ALTP, and STTX III were produced by all *A. alternata* isolates, which is in good agreement with the results of Zwickel et al.[31] Alike in the study of Zwickel et al.,[31] TEN was produced by all *A. alternata* isolates but was detected above the peak area limit only from the second day of sampling on. The results of Zwickel et al. that some of the *A. alternata* isolates did not produce any *Alternaria* mycotoxins clearly demonstrate the differences in the metabolic capabilities of isolates belonging to the same species group.[30,31] Due to the differences in the TA production of the three *A. alternata* isolates in our survey, large numbers of reliable isolates of the same taxon should be used for differentiation investigations based on mycotoxin profiles.[79]

The mycotoxins AOH, AME, ATX I, ATX II, ALTP, STTX III, TEN, and TA were also determined in the extracts of fungal cells from the mycelium (**Figure 6**a). Contrarily to the liquid medium, AOH was detected in the cells of all investigated *Alternaria* isolates and AME was determined in the cells of *A. solani* and of the *A. alternata* isolates 2 and 3. This confirmed the ability of the *A. solani* isolate to produce these two mycotoxins. The perylene quinones were verified in the cells of all *A. alternata* isolates. Interestingly, TEN was only determined in the cells of the isolate 2, whereas TA was not detected in any of the *Alternaria* cells. Figure 6b displays the contents of AOH, AME, ATX I, ALTP, and TEN calculated via one-point calibration. The highest contents were determined for ATX I and ALTP, ranging from 730 to 1900 µg kg^{-1} and from 400 to 1300 µg kg^{-1}, respectively. AOH was detected in contents from 11 to 120 µg kg^{-1} and AME from 0.5 to 2.7 µg kg^{-1}. Again, the contents have to be considered as semi-quantitative. Although the benzopyrones were detected frequently in the extracts of the disrupted cells, AOH and AME were only rarely analyzed in the liquid medium. As the benzopyrones were also detected in almost all *A. alternata* cultures in the study of Zwickel et al.,[31] this suggests that these compounds are generally formed, but only excreted under certain conditions to the medium. Contrarily, the perylene quinones ATX I, ATX II, ALTP, and STTX III were determined in the extracts of the cells and were excreted into the liquid medium. A different tendency was observed for TA as this mycotoxin was fully excreted into the liquid medium and was not detectable inside the fungal cells. The transport mechanisms to export these metabolites are still largely unknown. It can be hypothesized that extracellular vesicles reported to carry virulence factors[80] may be involved.

2.3. Differentiation between *Alternaria* Species

Apart from the comprehensive description of the *Alternaria* mycobolome, similarities and differences between samples can be detected by FTICR-MS and subsequent Principal Component Analysis (PCA). Before performing the PCA, the matrix was filtered by keeping only *m/z* signals that occurred in at least four out of five biological replicates ensuring the biological importance of the remaining signals. The PCA was performed on the whole data set and each day of sampling is displayed as an individual PCA plot (**Figure 7**). In the scores plot of the 4th day of cultivation (Figure 7a), the replicates of *A. solani* and the controls revealed distinct clusters displaying differences in the second component. For the *A. alternata* isolate 1, four of the five replicates clustered together while the fifth replicate was determined in the cluster of the *A. solani* isolate. The replicates of the *A. alternata* isolates 2 and 3 showed high variation and did not form distinct clusters. In the PCA model, 26.9% of the total variance are explained in the PC1 and 8.6% are explained in the PC2. On the 7th day of cultivation (Figure 7b), clear clusters are formed by the replicates of the isolate 1, the *A. solani* isolate, and the controls. The clusters of the isolates 2 and 3 are partly overlapping and, therefore, the two isolates cannot be separated clearly. A similar cluster formation to the 7th day of cultivation is obtained on the 9th day of cultivation (Figure 7c). However, the replicates of the *A. solani* isolate and the controls are overlapping. At the 11th day of cultivation (Figure 7d), the *A. solani* replicates and the controls form distinct clusters, which are clearly separated in the second component. The isolate 1 forms a distinct cluster and the replicates of the isolates 2 and 3 are overlapping. Again, the highest variation was determined between the five replicates of the isolate 3. Due to the formation of clusters in the PCA, differences in the mycobolome of the different *Alternaria* isolates are obvious. The distinction of the *A. alternata* and *A. solani* isolates in the PCA was based on more than 3000 *m/z* values, whereas the differentiation by LC-MS/MS was performed using eight mycotoxins. The FTICR-MS measurements confirmed the LC-MS/MS results according to which the *A. solani* isolate differs from the *A. alternata*

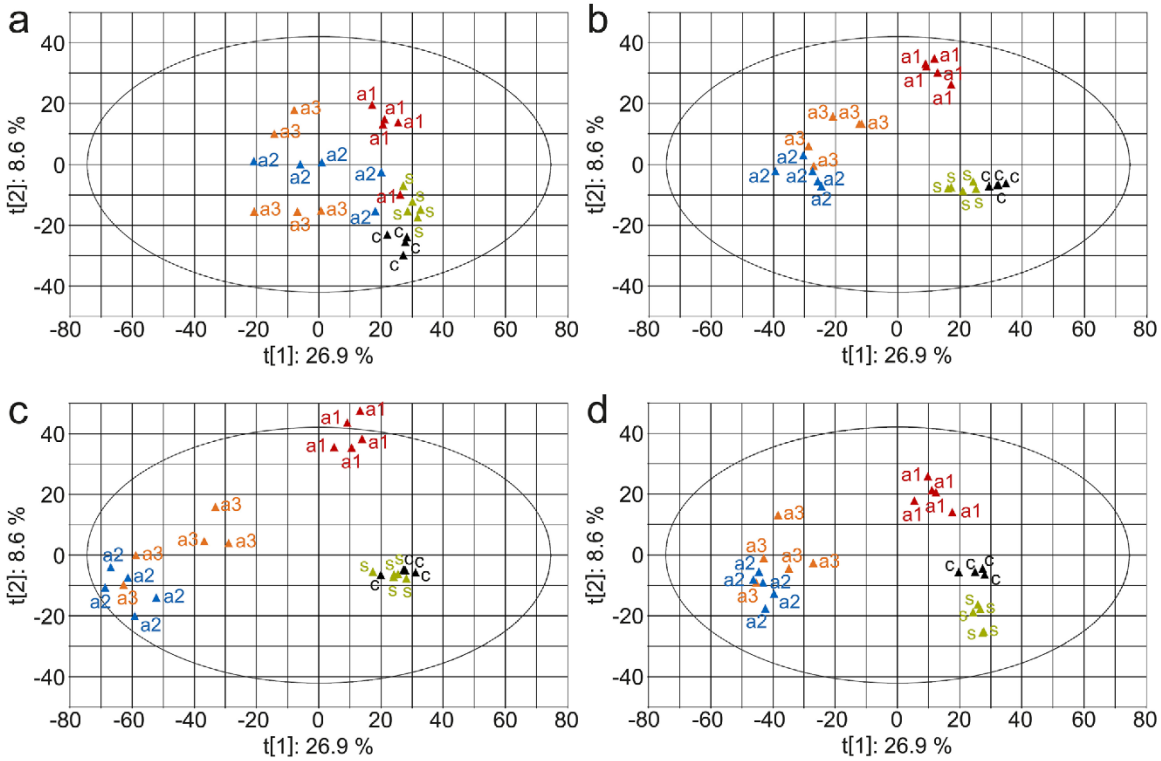

Figure 7. Scatter plots of the principal component analysis (PCA) of the FTICR-MS data. The plots display *A. solani* (▲,s), the three *A. alternata* isolates 1 (▲,a1), 2 (▲,a2), and 3 (▲,a3) and the control samples (▲,c) on the 4th day (a), the 7th day (b), the 9th day (c), and the 11th day (d) of cultivation.

isolates in the mycotoxin production. Besides, the *A. alternata* isolate 1 was proven to vary from the other two *A. alternata* isolates.

In the literature, different approaches were applied to differentiate between various *Alternaria* species. Zwickel et al. compared the metabolic capabilities of various *Alternaria* isolates belonging to *A. alternata*, *A. arborescence*, *A. tenuissima*, and *A. infectoria*.[31] Based on the metabolic profile and overall low mycotoxin production, the *A. infectoria* isolates were segregated from *A. alternata*, *A. arborescence*, and *A. tenuissima*. Contrarily, the analysis of various mycotoxins by HPLC-MS/MS could not separate the isolates of *A. alternata*, *A. arborescence*, and *A. tenuissima*.[31] Andersen et al. obtained identical results when analyzing the mycotoxin profiles of *A. infectoria*, *A. alternata*, *A. arborescence*, and *A. tenuissima*.[30] Besides the analysis of mycotoxin profiles, Andersen et al. performed a metabolic differentiation of *A. alternata*, *A. gaisen*, *A. limoniasperae*, *A. longipes*, *A. tangelonis*, and *A. turkisafria* based on direct infusion MS. In the mass spectra, 100 to 400 ions were detected, respectively, and the clustering of the isolates resulted in a separation of four of the six *Alternaria* species. *A. gaisen*, *A. turkisafria*, *A. tangelonis*, and *A. alternata* clustered in four separated clusters, whereas one isolate of *A. limoniasperae* and *A. longipes* clustered apart from the other isolates of the related species-group, respectively.[32] In our survey, we analyzed three different *A. alternata* isolates by FTICR-MS and the *A. alternata* isolate 1 clustered apart from the other two isolates in the PCA plots. Accordingly, Andersen et al. did not obtain distinct clusters for the *A. limoniasperae* and *A. longipes* isolates as one isolate clustered apart from the other isolates belonging to the same species-group.[32]

2.3.1. Determination of Discriminating Metabolites

To identify metabolites, which are responsible for the grouping in the PCA plots, volcano plots were created. Only *m/z* values that were assigned to molecular formulae were considered to be relevant metabolite candidates.[81] The volcano plots were created by plotting the $-\log_{10}$ *p*-value against the $\log_2$ fold change of the MS signal intensities of the *A. solani* and the *A. alternata* replicates.[82] A volcano plot was created for the *A. solani* and the *A. alternata* isolate 1 at the 11th day of cultivation (**Figure 8**a). The horizontal line in the plot represents the significance value of 0.01. The higher the *y* value of one molecular formula is, the more significant is the difference. Interesting molecular formulae are located on the upper left part and upper right part of the plot.[82]

To allocate the discriminating molecular formulae to metabolites, all discriminating elemental compositions were checked against Antibase.[50] The assignments are displayed as blue triangles for discriminating molecular formulae of *A. solani* and as green hashes for *A. alternata*. If one allocated molecular formula was discriminating in the *A. solani*–*A. alternata* isolate 1 comparison, as well as in the *A. solani*–*A. alternata* isolate 2

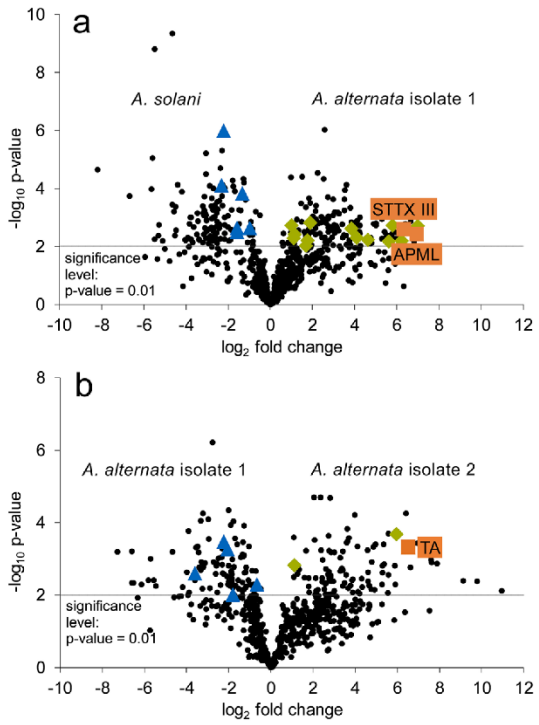

Figure 8. The volcano plot (a) displays the discriminant masses between *A. solani* and the *A. alternata* isolate 1 at the 11th day of cultivation. The volcano plot (b) depicts the discriminant masses between the *A. alternata* isolate 1 and 2 at the 11th day of cultivation. The triangles and hashes represent molecular formulae that were assigned to metabolites by the Antibase database.[50] Due to the lack of reference compounds, the identification of these metabolites could only be performed for STTX III, APML, and TA (displayed as squares) using targeted LC-MS/MS analysis. Details on the extraction and purification, on the structure elucidation, on the mass spectrometric fragmentation pattern, and UV–vis absorption spectrum of APML are displayed in Supporting Information.

and *A. solani*–*A. alternata* isolate 3 comparison (Figure S2, Supporting Information) its *m/z* value, the theoretical neutral mass, the error of annotation, the molecular formula as well as the metabolite assignment were listed in **Table 2.** For *A. solani*, four molecular formulae were significantly different in all comparisons and were allocated to the metabolites altechromone A,[83] quadrilineatinmethylether,[84] 2,4-dihydroxy-6-acetonylbenzoic acid,[85] and (8R,9S)-9,10-epoxy-8-hydroxy-9-methyldeca-(2E,4Z,6E)-trienoic acid[86] (Table 2). For *A. alternata*, seven molecular formulae were assigned to metabolites (Table 2), for example, ATX III[12]/STTX III,[87] xanalteric acid I/II,[88] alterlosin I, and alterlosin II.[44] One of the orange marked squares in the volcano plot a (Figure 8) represents the molecular formula of the mycotoxin STTX III, identified by targeted LC-MS/MS analysis.[89] In the LC-MS/MS studies, the perylene quinones ATX I, ATX II, ALTP, and STTX III were only produced by the *A. alternata* isolates. In the literature, the production of the perylene quinones by *A. alternata* and, additionally, by *A. arborescence* and *A. tenuissima* is reported. Contrarily, the biosynthesis of these mycotoxins by *A. solani* is not mentioned.[31] Interestingly, the molecular formulae of ATX I ($C_{20}H_{16}O_6$) and ATX II/ALTP ($C_{20}H_{14}O_6$) were also detected in the samples of *A. solani* in our FTICR-MS measurements but were not identified as ATX I, ATX II, and ALTP by LC-MS/MS analysis using reference compounds. As these molecular formulae were also detected in the samples of *A. solani*, these elemental compositions are not identified as discriminant masses by the volcano plots. Obviously, our *A. solani* isolate produces different compounds with the same molecular formulae as the perylene quinones, which points to the need of using these complementary methods for differentiating the metabolomes. The second orange marked square in Figure 8a represents the molecular formula ($C_{23}H_{20}O_9S$) of a discriminating metabolite named alterperylenepoxide A-9-mercaptolactate (APML). In this study, APML was extracted from overgrown rice, purified by various stationary and mobile phases, and characterized by ^{1}H and ^{13}C NMR analysis as well as ^{1}H–^{1}H correlated spectroscopy (COSY), Heteronuclear Single Quantum Coherence (HSQC), and Heteronuclear Multiple Bond Correlation (HMBC) 2D NMR analysis (Tables S2–S4 and Figures S3–S11, Supporting Information).

Due to the lack of reference compounds, the other assigned elemental compositions were only tentatively allocated to metabolites. The remaining molecular formulae that were also significantly different among *A. solani* and *A. alternata* could not be assigned by Antibase.[50] This reveals the still unknown metabolites produced by *Alternaria* fungi and the necessity for additional studies on the comprehensive analysis of the *Alternaria* mycobolome.

Apart from the differences in the mycobolome of *A. solani* and *A. alternata*, variations between different *A. alternata* isolates can be determined. The volcano plot b in Figure 8 displays the differences in the mycobolome of the *A. alternata* isolates 1 and 2. The discriminating elemental compositions were checked against the entries of Antibase[50] and five (three) molecular formulae were assigned to metabolites of the isolate 1 (isolate 2) (**Table 3**). The orange marked square (Figure 8b) represents the molecular formula of the mycotoxin TA, which was already proven to be discriminant by targeted LC-MS/MS analysis. Although the two isolates belong to the same species group and were cultivated under the same conditions, some metabolites differ in averaged signal intensities or are only produced by one of the two isolates. To confirm and improve the results, more isolates from the species groups of *A. alternata* and *A. solani* need to be analyzed by FTICR-MS and also fungal isolates from different origins should be included. As it is not possible to fully characterize the fungal mycobolome using only one type of instrument,[72] different approaches and various analytical techniques are required to receive a more holistic picture of the mycobolome of *Alternaria* fungi.[93] A more holistic knowledge on the secondary metabolism of the food contaminating fungus *Alternaria* enables proper risk evaluations on food and feed. After isolating and characterizing known *Alternaria* mycotoxins as well as new secondary metabolites, their toxicological capabilities should be investigated. Besides, accurate quantitative analytical methods are needed to obtain more data on the occurrence and contents of *Alternaria* metabolites in agricultural commodities. A sound knowledge on the toxicology as well as on the occurrence of the fungal food contaminants is essential to allow the EFSA to perform proper risk evaluations on food and feed products.

Table 2. Discriminant molecular formulae between *A. alternata* and *A. solani* assigned to metabolites by Antibase.[50]

Species	Experimental mass	Theoretical neutral mass	Mass error of annotation [ppm]	Molecular formula	Metabolite assignments by Antibase[50]
A. solani	189.05572	190.06300	0.007	$C_{11}H_{10}O_3$	Altechromone A[83]
A. solani	207.06628	208.07356	−0.008	$C_{11}H_{12}O_4$	Quadrilineatin methylether[84]
A. solani	209.04554	210.05283	−0.026	$C_{10}H_{10}O_5$	2,4-Dihydroxy-6-acetonyl-benzoic acid[85]
A. solani	209.08194	210.08921	0.005	$C_{11}H_{14}O_4$	(8R,9S)-9,10-epoxy-8-hydroxy-9-methyldeca-(2E,4Z,6E)-trienoic acid[86]
A. alternata	207.02990	208.03718	−0.009	$C_{10}H_8O_5$	Iso-ochracinic acid[90]
A. alternata	221.08193	222.08921	−0.014	$C_{12}H_{14}O_4$	3-Epideoxyradicinol[91]
A. alternata	267.08741	268.09469	−0.002	$C_{13}H_{16}O_6$	9,10-Epoxy-3-methoxy-3-epiradicinol[92]
A. alternata	347.05612	348.06339	0.009	$C_{20}H_{12}O_6$	ATX III,[12] STTX III[87]
A. alternata	363.05102	364.05831	−0.020	$C_{20}H_{12}O_7$	Xanalteric acid I/xanalteric acid II[88]
A. alternata	365.06667	366.07396	−0.026	$C_{20}H_{14}O_7$	Alterlosin I[44]
A. alternata	367.08232	368.08961	−0.032	$C_{20}H_{16}O_7$	Alterlosin II[44]

For *A. solani*, four discriminant molecular formulae were annotated to metabolites, whereas for *A. alternata*, seven molecular formulae were assigned to metabolite candidates. The identification of STTX III was performed using targeted LC-MS/MS analysis, while the identification of the other assigned metabolites could not be performed due to the lack of reference compounds.

Table 3. Discriminant elemental compositions of the *A. alternata* isolates 1 and 2 detected by FTICR-MS and assigned to metabolite candidates by Antibase.[50]

A. alternata isolate	Experimental mass	Theoretical neutral mass	Mass error of annotation [ppm]	Molecular formula	Metabolite assignments by Antibase [50]	Log_2 fold change	$-Log_{10}$ *p*-value
Isolate 1	193.05063	194.05791	−0.002	$C_{10}H_{10}O_4$	Silvaticol,[94] porriolide [95]	−2.23	3.48
Isolate 1	211.09758	212.10486	−0.027	$C_{11}H_{16}O_4$	3-Carboxy-2-methylene-4-pentenyl-4-butenolide,[96] methylenolactocin,[97] depudecin[98]	−0.67	2.29
Isolate 1	221.04554	222.05283	−0.031	$C_{11}H_{10}O_5$	Tenuissimasatin [99]	−3.59	2.61
Isolate 1	251.16526	252.17255	−0.024	$C_{15}H_{24}O_3$	Deoxyuvidin B [100]	−2.05	3.27
Isolate 1	415.23507	416.24236	−0.018	$C_{22}H_{32}N_4O_4$	Dihydrotentoxin/cyclo(L-leucyl-*N*-methyl-L-phenylalanylglycyl-*N*-methyl-L-alanyl) [67]	−1.80	2.01
Isolate 2	182.08227	183.08954	−0.014	$C_9H_{13}NO_3$	Isopropyl tetramic acid[101]	5.96	3.69
Isolate 2	196.09792	197.10519	−0.001	$C_{10}H_{15}NO_3$	L-TA,[102,103] isobutyl tetramic acid[101]	6.52	3.32
Isolate 2	363.05102	364.05831	−0.020	$C_{20}H_{12}O_7$	Xanalteric acid I/xanalteric acid II [88]	1.12	2.82

The discriminant molecular formulae could not be identified by LC-MS/MS due to the lack of reference compounds. The only exception was TA, which was identified by targeted LC-MS/MS analysis and was already identified as discriminant mycotoxin between the *A. alternata* isolates 1 and 2.

3. Concluding Remarks

The mycobolome of various *Alternaria* isolates was analyzed by FTICR-MS and complemented with LC-MS/MS analyses. From the total number of detected *m/z* signals of the FTICR-MS, 35% could be assigned to unequivocal molecular formulae of potential metabolites. Of these formulae, only 3% could be verified as specific fungal metabolites using the Antibase database, which particularly focuses on fungi. Performing an additional database search against the KEGG database resulted in only 18% of assignments of the 2883 molecular formulae to general cellular metabolites. This result indicates that only about one fifth of the metabolome signals are potentially known. However, this estimation of the current database knowledge becomes even worse, when we consider that only a small number of the metabolites, whose molecular formulae were effectively detected by FTICR-MS, could be assigned by targeted LC-MS/MS using authentic reference compounds as revealed by the missing perylene

quinones in the *A. solani* extracts in the LC-MS/MS measurements. On the other hand, complementing the FTICR-MS data with targeted LC-MS/MS analyses is necessary, as the molecular formulae of the direct infusion FTICR-MS measurements generally can be assigned to several metabolites, due to lack of retention time information in direct infusion FTICR-MS. Another striking result from the pathway analyses was the missing detection of over 50 % of expected metabolites in both LC-MS/MS and FTICR-MS measurements, which also indicates that our coverage of the metabolome is still very low. The low percentage of database allocations demonstrates the need for further comprehensive investigations of the *Alternaria* mycobolome. The clear identification of interesting metabolites remains the bottleneck of metabolomics and authentic reference compounds of fungal metabolites are necessarily needed for identification purposes. The non-targeted analysis of the fungal mycobolome by FTICR-MS unraveled variations in the metabolome of *A. solani* and *A. alternata* and enabled the detection of discriminating metabolites. One of these discriminating metabolites was identified as alterperylenepoxide A-9-mercaptolactate by ^{1}H, ^{13}C, and 2D NMR analysis after isolating the metabolite. In addition to the interspecies variations, differences within the *A. alternata* species were determined. Further isolates from the species group of *A. alternata* and *A. solani* need to be analyzed by FTICR-MS to confirm the intra- and inter-variation of the mycobolome. Additionally, isolates from different small spored *Alternaria* species such as *A. tenuissima* and *A. arborescence* should be included in future investigations. Combined to additional analytical tools, FTICR-MS is a promising tool for the chemotaxonomic differentiation of fungal isolates. In future mycobolome investigations, FTICR-MS should also be applied to *Alternaria* fungi-plant interactions or in the field of food contaminants related to *Alternaria* mycotoxins.

4. Experimental Section

Chemicals and Reagents: Reference compounds of AOH, AME, TA, and TEN were purchased from Sigma-Aldrich (Steinheim, Germany). TA was released from its commercial copper salt according to the literature.[104,105] ATX I, ATX II, ALTP, and STTX III were biosynthesized as described previously.[106] After biosynthesis, the analytical standards were purified by preparative HPLC and characterized by nuclear resonance spectroscopy (NMR) as reported earlier.[106]

Agar, ammonium formate (≥99.0 %, for mass spectrometry), ammonium sulfate, arginine, potassium dihydrogen phosphate, sodium acetate trihydrate, sodium nitrate, and sucrose were obtained from Sigma-Aldrich (Steinheim, Germany). Ammonia solution (25%, for LC-MS), formic acid (>98%), glucose, iron sulfate heptahydrate, potassium chloride, potassium nitrate, and sodium hydroxide were received from Merck KGaA (Darmstadt, Germany). Sodium hypochlorite and Tween 20 were purchased from AppliChem GmbH (Darmstadt, Germany). Calcium nitrate tetrahydrate, dichloromethane (technical grade), ethyl acetate (technical grade), formic acid (>99%, for mass spectrometry), magnesium sulfate heptahydrate, acetonitrile and water (HPLC grade, LC-MS grade), isopropanol (technical grade, HPLC grade, LC-MS grade), and methanol (HPLC grade) were obtained from VWR (Ismaning, Germany). Methanol (LC-MS grade) was purchased from Honeywell International Inc. (Seelze, Germany). Water was purified using a Milli-Q system (Millipore, Darmstadt, Germany).

Preparation of Stock Solutions: Stock solutions of *Alternaria* mycotoxins were prepared in acetonitrile (AOH, AME, TEN) or methanol (ATX I, ALTP, TA) in concentrations ranging from 10 to 100 μg mL^{-1}. For quantitative measurements, the stock solutions were further diluted. All solutions were stored in the dark at −20 °C. The absorptions of the solutions were measured by a Genesys, 10S, UV–vis spectrophotometer (Thermo Fisher Scientific, Madison, Wisconsin, USA) and the concentrations were confirmed by applying the published extinction coefficients.[107] However, ATX II and STTX III were only qualitatively included in the method, as the available amounts of these reference compounds were not detectable by UV–vis.

Preparation of Synthetic Nutrient-Poor Agar: Glucose (0.2 g), magnesium sulfate heptahydrate (0.5 g), potassium chloride (0.5 g), potassium dihydrogen phosphate (1 g), potassium nitrate (1 g), and sucrose (0.2 g) were dissolved in 100 mL of water. To adjust the pH to 5.5, 600 μL of sodium hydroxide (1 mol L^{-1}) were added. 22 g of agar were solved in 900 mL of water and, after the unification of both solutions, the medium was autoclaved for 20 min at 121 °C.[108]

Isolation of Alternaria Fungi: *A. alternata* was isolated from potato leaves (Uelzen, Germany, isolate 1), tomato leaves (Aitrang, Germany, isolate 2), and tomatoes (Aitrang, Germany, isolate 3). The *A. solani* isolate originated from potato leaves (Kirchheim, Germany). After harvesting, the plant leaves were dried. The surface of the dried leaves and fresh tomato was sterilized using 3% of sodium hypochlorite. Subsequently, small pieces of plant tissue showing typical symptoms of infection were placed on SN agar and cultivated at 22 °C and 65% relative humidity for 1 week. Alternately, the isolates were exposed to black light for 12 h and subsequently cultivated in the dark for 12 h. Single spores were isolated from the overgrown agar plates and cultivated on synthetic nutrient-poor agar at 22 °C and 65% relative humidity for 2 weeks. Again, 12 h of black light exposure was followed by the cultivation in the dark. The overgrown agar plates with pure isolates were used for further experiments. All fungi were obtained in 2015.

Cultivation of Fungal Isolates and Metabolite Extraction for Analysis: To obtain samples for FTICR-MS measurements, the *Alternaria* isolates were cultivated in a synthetic liquid medium. The liquid medium contained ammonium sulfate (0.2 g L^{-1}), calcium nitrate tetrahydrate (0.3 g L^{-1}), glucose (4.0 g L^{-1}), iron sulfate heptahydrate (0.02 g L^{-1}), magnesium sulfate heptahydrate (0.25 g L^{-1}), potassium chloride (0.25 g L^{-1}), potassium dihydrogen phosphate (0.5 g L^{-1}), sodium acetate trihydrate (0.66 g L^{-1}), and sodium nitrate (2.0 g L^{-1}). After adjusting the pH to 5.5 using formic acid, 35 mL of the liquid medium were transferred into polycarbonate Erlenmeyer flasks and autoclaved at 121 °C for 20 min.

The sterile liquid medium was inoculated with defined spore suspensions. For the preparation of the spore suspensions, 3 mL of detergent solution (0.5% Tween 20) was pipetted on the overgrown agar plates and the mycelium and spores were scratched. The spores were counted using a Thoma chamber and the spore suspensions were diluted to 8.75×10^5 spores per milliliter for *A. alternata* and to 2×10^5 spores per milliliter for *A. solani*.[108] 25 μL of the spore suspensions of the *A. alternata* isolates and 100 μL of the spore suspension of the *A. solani* isolate were added to the liquid medium to receive equal amounts of total spores. During FTICR-MS measurements, contaminations originating from chemicals, solvents, plastic, and glass surfaces were detected, and these contaminations were compensated for by the analysis of control samples. The control samples were obtained by adding 25 μL of pure detergent solution to the liquid medium. All samples were prepared in replicates of five. The Erlenmeyer flasks were sealed with septa allowing sterile sampling during the cultivation process after 4, 7, 9, and 11 days using cannulas and syringes. The fungi were cultivated in the dark (26 °C, 110 rpm)[109] and the isolates were exposed to artificial daylight for half an hour a day.

The liquid medium was analyzed by FTICR-MS after 4, 7, 9, and 11 days of cultivation. 3 mL of the medium was sterilely taken from the Erlenmeyer flasks and centrifuged (15 000 × *g*, 10 min) to separate the mycelium. The pH of the supernatant was adjusted to pH 2 using formic acid. To protect the ESI source of the instrument from contamination, salts of the liquid medium were removed by solid phase extraction (Discovery DSC-8, Supelco, Bellefonte, PA, USA). The C8 material was washed with 1 mL of methanol and conditioned with 1 mL of water (pH 2). After the sample loading (2 mL), the column was washed with 5 mL of water (adjusted to pH 2 using formic acid). The elution of the analytes was performed with 1 mL of methanol.

Besides, the mycelia of the fungi after eleven days of cultivation were also analyzed by FTICR-MS and LC-MS/MS. After separating the liquid medium from the mycelium by centrifugation, the latter was thoroughly washed with water to remove remaining liquid medium. 200 mg of the mycelium were weighed into a nucleo spin bead tube (type A, 0.6–0.8 mm, Machery Nagel, Düren, Germany), previously cleaned with 5 mL of water and 5 mL of methanol. After adding 1 mL of ice-cold methanol/water (90/10, v/v), the disruption of the cells was performed at 6800 rpm (4 × 30 s) utilizing a Precellys homogenizer (Bertin instruments, Montigny-le-Bretonneux, France). The homogenizer operated at −10 °C using liquid nitrogen. After the disruption, the cell suspension was centrifuged at 21 000 × *g* for 10 min, the supernatant was dried under nitrogen and the residue was resolved in 2 mL of water (adjusted to pH 2 using formic acid). To ensure comparability of medium and mycelium samples, the aqueous solutions were desalted by solid phase extraction (Discovery DSC-8, Supelco, Bellefonte, PA, USA). The C8 material was washed with 1 mL of methanol and conditioned with 1 mL of water (adjusted to pH 2 using formic acid). After loading the sample onto the column, the latter was washed with 5 mL of water (adjusted to pH 2 using formic acid). The elution of the analytes was performed with 1 mL of methanol.

FTICR-MS Analysis: The acquisition of ultra-high-resolution mass spectra was performed on a Bruker Solarix Fourier transform ion cyclotron resonance mass spectrometer (FTICR-MS) (Bruker Daltonics GmbH, Bremen, Germany) coupled to a 12 Tesla superconducting magnet (Magnex Scientific Inc., Yarnton, GB). The direct infusion of samples was performed with an APOLO II ESI source (Bruker Daltonics GmbH, Bremen, Germany) which operated in the negative ionization mode. The samples were diluted with methanol (1/10; v/v) prior to injection and were introduced into the ESI source at a syringe flow rate of 120 µL h^{-1} by a Gilson autosampler (Gilson, Inc., Meddleton, WI, USA). The spectra were externally calibrated by using ion clusters of arginine (10 mg L^{-1} in methanol) and were acquired with a time domain transient of four mega words in size. Measured masses ranged from *m/z* 150 to *m/z* 1000. For each sample, 300 scans were accumulated. This was equivalent to 13 min of analysis time. The capillary voltage was set to 3600 V and the spray shield voltage was −500 V. The drying gas flow rate and the drying gas temperature were adjusted to 4.0 L min^{-1} and 200 °C. The ion accumulation time was 0.3 s. A resolving power of 600000 at *m/z* 300 was achieved. Subsequently, internal calibration was carried out on each mass spectrum by using a calibration list of *Alternaria* metabolites described in the literature. The calibration list covered an *m/z* range of 160 to 730 Da. The Data Analysis Version 4.2 (Bruker Daltonics GmbH, Bremen, Germany) was used to process raw spectra. The *m/z* values with a signal to noise ratio of 7 and a relative intensity threshold of 0.01% were exported as mass lists. The mass lists were de-noised from the well-known Gibbs sidelobes (wiggles) by the use of a special program of denoising[110] and the clean mass lists were subsequently aligned using an in-house written program (peak alignment window width: ± 1 ppm).[39,111] In total 120 spectra were measured.

Molecular Formula Annotation and Database Assignments of FTICR-MS Data: The *m/z* signals that occurred in at least two out of five biological replicates were assigned unequivocally to molecular formulae by an in-house written software tool named NetCalc. The annotation of the molecular formulae is based on a mass difference network consisting of nodes and edges. The nodes represent *m/z* values (metabolite candidates) and edges constitute biochemical reactions.[42] The biochemical reactions can be expressed as mass differences between substrates and products and are predefined in a mass difference list covering 191 reaction-equivalent mass differences such as oxidation, reduction, hydroxylation, methylation, and the loss of CO_2.[34,42] As starting points of the network, 41 reference masses (*Alternaria* metabolites) with exact deprotonated mass and molecular formula were specified. Originating from the references, measured *m/z* values were assigned to molecular formulae by comparing the mass differences of all signals of a mass spectrum to the mass difference list.[34,42] Signals of isotopes and masses with an unusual mass defect were not included in the network.[42,81] The assignments of the elemental compositions contained only C, H, N, O, S, and P. The molecular formula allocation was performed on 8139 *m/z* values resulting in 4467 monoisotopic elemental compositions (55%) with 3285 molecular formulae exhibiting an annotation error within ± 0.2 ppm (74%).[81,112] During FTICR-MS measurements, adduct ions such as chloride adducts are formed for some metabolites. 402 adducts were removed and 2883 annotated elemental compositions were used for further investigations. The calculated mass difference network was constituted using Gephi 0.9.2 software.[113]

To assign the experimental *m/z* values to metabolite candidates, the annotated molecular formulae were matched to the entries of Antibase.[50] For *Alternaria* and other genera of fungi, 95–98 % of the metabolites described in the literature are included in the database.[71] Additionally, the molecular formulae were matched to the entries of the KEGG database (http://www.genome.jp/kegg/compound/).[69]

The annotated elemental compositions were used to calculate the H/C and O/C ratios. Subsequently, the ratios were displayed in a 2D van Krevelen diagram to visualize the variations within the metabolic profile of the fungal isolates.[36]

Statistical evaluations were only performed on *m/z* values that occurred in at least four out of five biological replicates. PCA, an unsupervised statistical method, was applied for reducing the complexity of the data. After z-score normalization, the variation in the data was displayed as a set of new independent variables called the principal components. It was used for providing an overview of the complex multivariate data and for detecting outliers and relations between samples.[114,115] The PCA was performed using Simca-P 9.0 software (Umetrics, Sweden).

To determine the most discriminative molecular formulae between the *A. alternata* and the *A. solani* isolates, volcano plots were created. Volcano plots display the $\log_2$ fold change (ratio of averaged intensities of measured *m/z* signals) on the *x*-axis. The *y*-axis shows the $-\log_{10}$ *p*-value of measured intensities to significantly determine discriminating molecular formulae between the *A. alternata* and the *A. solani* isolates.[82]

LC-MS/MS Analysis of Alternaria Mycotoxins: AOH, AME, TEN, ATX I, ATX II, STTX III, and ALTP were chromatographically separated on a Shimadzu Nexera X2 UHPLC system (Shimadzu, Kyoto, Japan). As stationary phase, a HyperClone BDS-C18 column (150 • 3.2 mm, 3 µm, 130 Å, Phenomenex, Aschaffenburg, Germany) was utilized. Further details on the instrument conditions, the solvent mixtures, and the gradient were published recently.[25] TA had to be analyzed in an additional LC-MS/MS run, due to the more polar character of the molecule. A Gemini-NX C18 column (150 × 4.6 mm, 3 µm, 110 Å, Phenomenex, Aschaffenburg, Germany) served as stationary phase. The solvent mixtures, the gradient, and further details on the chromatographic separation of TA are listed in the literature.[25] The LC was connected to a triple quadrupole mass spectrometer (LCMS-8050, Shimadzu Corporation, Kyoto, Japan). All analytes were detected in the negative ESI mode. Details on the mass spectrometric conditions were published recently[25] and are listed in Table S1, Supporting Information. The LabSolutions software (Shimadzu, Kyoto, Japan) was used for data acquisition and data analysis.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

M.G., M.R., and P.S.-K. designed the experiments. M.G. performed the cultivation of fungi and the salting of samples. M.G. and B.K. analyzed the samples by using FTICR-MS and M.G. performed the LC-MS/MS measurements. F.S. accomplished the extraction and purification of APML and F.S., R.H., O.F., and T.H. confirmed the structure of APML by NMR analyses. M.G. wrote the manuscript. M.R., B.K., and P.S.K. revised the manuscript. All authors contributed to the revision of the manuscript.

Keywords

Alternaria alternata, alterperylenepoxide A-9-mercaptolactate, Fourier transform ion cyclotron resonance mass spectrometry, non-targeted metabolomics, ultra-high resolution

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C Supporting Information: Comprehensive Analysis of the *Alternaria* Mycobolome Using Mass Spectrometry Based Metabolomics

Molecular Nutrition & Food Research

Supporting Information

Comprehensive analysis of the *Alternaria* mycobolome using mass spectrometry based metabolomics

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1 Optimization of the sample preparation for FTICR-MS measurements

1.1 Desalting of the liquid medium

Due to the chemical diversity of secondary metabolites, the sample preparation clearly affects the type of metabolites detected in the mass spectrometer.[1] The extraction of metabolites is usually performed using organic solvents such as chloroform,[2] ethyl acetate,[3] or dichloromethane.[1] In our study, the samples needed to be desalted prior to direct infusion into the ESI source either by the extraction of metabolites using organic solvents or by applying solid phase extraction. The extraction of fungal metabolites from the aqueous medium by dichloromethane led to contaminations of the

samples. Therefore, solid phase extraction was preferred for desalting purposes despite possible losses of fungal metabolites.[4] Before desalting, adjusting the pH of the liquid medium to 2 rather than to basic conditions was preferred as 50 % of all secondary metabolites exhibit an acidic moiety, while around 10 % show basic attributes.[1] The desalting step was optimized by analyzing solid phase eluates of the *A. alternata* isolates 1 and 2 and subsequent FTICR-MS and LC-MS/MS measurements. As reversed phases retain analytes with a wide range of different polarities,[5] we focused on reversed phase materials for the desalting step. In the literature, C18 [6] and C8 cartridges [3] are commonly used for the analysis of fungal metabolites. In our study, retention capabilities of C18 (Discovery® DSC-18, Supelco, Bellefonte, PA, USA) and C2 material (Bond Elut C2, Agilent Technologies Inc., Santa Clara, CA, United States) were compared. The utilization of the C18 material resulted in a slightly higher number of detected signals in the FTICR-MS spectra than the usage of the C2 material. The determination of the *Alternaria* mycotoxins by LC-MS/MS did not reveal clear differences between the C18 and C2 phases. As neither distinct differences in the number of signals nor explicit variations in the mycotoxin contents were detected, C8 material was chosen as a compromise between C18 and C2 polarity.

1.2 Metabolite extraction from fungal cells for FTICR-MS measurements

Apart from the exometabolome, the metabolites located in the cells were analyzed to extend the metabolic picture of *Alternaria* fungi. To enable the detection of these metabolites, the disruption of the cells was optimized. Precellys glass beads (Precellys lysing kit, tough micro-organism lysing, VK05; Bertin Corp., Rockville, MD, USA) and Nucleo spin bead tubes (type A, 0.6–0.8 mm, Machery Nagel, Düren, Germany) were tested for their cell disruption efficiency. The extracts obtained by Precellys glass beads were highly contaminated with signals originating from the surface of either the plastic tubes or the glass beads. The contamination after the usage of Nucleo spin bead tubes was much lower and, therefore, these bead tubes were chosen for the cell disruption. Additionally, the composition of the extraction solvent was optimized and a methanol/water mixture (90/10, v/v) gave best results. While performing the disruption of the cells, the homogenizer was cooled to -10 °C using liquid nitrogen. After the destruction, the samples still contained high amounts of salts, which resulted in salt-clusters during FTICR-MS measurements. Therefore, the extracts were evaporated to dryness, reconstituted in 2 mL of water (adjusted to pH 2 using formic acid), and desalted by solid phase extraction following the protocol of the liquid medium.

2 Biosynthesis, extraction, purification, and characterization of the fungal metabolite alterperylenepoxide A-9-mercaptolactate (APML)

Using volcano plots, significantly different molecular formulae between *A. alternata* and *A. solani* were determined. One of the molecular formulae discriminating for *A. alternata* was extracted from rice medium. The metabolite was purified using various stationary and mobile phases and subsequently characterized by ^{1}H, ^{13}C, and 2D NMR.[7]

2.1 MS/MS spectra of APML

The ultra-high resolution of FTICR-MS allowed the annotation of the experimental *m/z* signal of 471.07550 to the molecular formula $C_{23}H_{20}O_9S$ (theoretical $[M-H]^-$: 471.07553; mass error of

annotation: -0.064 ppm). Due to the high signal intensity of the discriminating *m/z*, MS/MS spectra were acquired on the FTICR mass spectrometer (Bruker Daltonics GmbH, Bremen, Germany). The ion accumulation time was adjusted to 1.5 s. The precursor was fragmented at a collision energy of 20 eV and the fragments are displayed in Figure S3. The obtained fragments (Fig. S3) were compared to LC-MS/MS measurements of known *Alternaria* mycotoxins and were similar to precursor and fragment ions of ATX II and ALTP (Table S1). Therefore, a chemical structure similar to perylene quinones was suggested for the metabolite with the molecular formula of $C_{23}H_{20}O_9S$.

2.2 Cultivation of the *A. alternata* isolate 1 on rice

25 g of parboiled rice were weighed in polycarbonate Erlenmeyer flasks and 15 mL of water were added. The rice medium was autoclaved (121 °C, 20 min) and subsequently inoculated with defined spore suspensions of the *A. alternata* isolate 1. For the preparation of the spore suspensions, 3 mL of detergent solution (0.5 % tween 20) were pipetted on the overgrown agar plate and mycelium and spores were scratched. The spore suspension was diluted to $1.25 \cdot 10^6$ spores/mL [8] and 25 µL of the suspension were added to the rice medium. The fungi were cultivated at 26 °C (110 rpm) in the dark for seven days [9] and were exposed to artificial light for half an hour a day. In total, 56 Erlenmeyer flasks (25 g of rice each) were cultivated.

2.3 Extraction and purification of the metabolite APML

The overgrown rice was thoroughly mashed and the fungal metabolites were extracted three times (1 h, 250 rpm) with 1 L of ethyl acetate/methanol (1/1; v/v). The extracts were decanted, combined, and evaporated to dryness using a rotary evaporator at 30 °C. The residue was dry loaded onto a column of silica gel (125 g, Mesh 70-230, pore size 60 Å, particle size 63–200 µm, Sigma-Aldrich, Steinheim, Germany) equilibrated with dichloromethane/methanol (9/1; v/v). The metabolites were gradually eluted with 200 mL of dichloromethane/methanol (9/1; 7/1; 3/1; 1/1; 1/3; 1/7; 0/1; v/v) and 30 fractions (50 mL each) were collected. Fractions containing the metabolite $C_{23}H_{20}O_9S$ were identified by LC-MS/MS analysis and the fractions 20-29 were further purified using a column of silica gel (125 g). The column was equilibrated with dichloromethane/methanol (9/1; v/v) and the metabolites were gradually eluted with 400 mL of dichloromethane/methanol (9/1; 3/1; 1/1; 0/1; v/v). Again, 30 fractions were collected (50 mL each) and the fractions containing the discriminative metabolite (18–29) were combined and further purified using a column of C18 material (50 g, end-capped, Chromabond, Machery-Nagel GmbH & Co. KG, Düren, Germany). The column was equilibrated with acetonitrile/water (10/90; v/v) and the metabolites were gradually eluted using 50 mL of acetonitrile/water (10/90; 15/85; 20/80; 25/75; 30/70; 40/60; 60/40; 80/20; 100/0; v/v). 90 fractions (5 mL each) were collected and fraction 47 was further purified using a reversed-phase, semi-preparative HPLC column. The preparative separation was performed on a Merck-Hitachi LaChrom HPLC System (Tokyo, Japan). A YMC-Pack Pro C18 column (150 · 10 mm, S-5 µm, 12 nm, YMC Europe GmbH, Dinslaken, Germany) protected by a C18-guard column (4 · 2.0 mm ID, Phenomenex, Aschaffenburg, Germany) served as stationary phase. The flow rate was set to 1.25 mL/min. The binary gradient system consisted of water + 0.1 % formic acid (solvent A) and acetonitrile (solvent B). The mobile phase was held at 25 % B for the first 3 min. The gradient raised linearly from 25 % B to 65 % B in the following 15.5 min and finally raised to 100 % B during

1 min. 100 % B was held for 3 min and the mobile phase returned to 25 % B within the next 1.5 min. Subsequently, the column was equilibrated for 3 min. 100 µL were used as injection volume. The UV absorption was measured at 254 nm. The eluting peak at 17.2 min was collected (Figure S4). After evaporation of the solvents at 30 °C, a yellow, amorphous solid was obtained.

2.4 Qualitative NMR analysis of the metabolite alterperylenepoxide A-9-mercaptolactate (APML)

The purified compound was characterized by ^{1}H (400 MHz), ^{13}C (100 MHz), and 2D NMR (^{1}H–^{1}H COSY, HSQC, HMBC) spectroscopy in acetonitrile-d_3 on a Bruker AV III system (298 K, Bruker, Rheinstetten, Germany). The spectrum was referenced to the residual signal of the acetonitrile-d_3 solvent.

The ^{1}H and ^{13}C chemical shifts of our discriminating metabolite (Table S2, Fig. S6, and Fig. S7) were partially congruent to the ^{1}H and ^{13}C chemical shifts of the metabolite alterperylenepoxide A described earlier in the literature.[10] The ^{13}C NMR data of the isolated metabolite (Table S2 and Fig. S7) disclosed the existence of one carboxylic acid carbon (δ_C 174.1 ppm) and one keto group (δ_C 204.6 ppm). In addition, H,H- and H,C-correlated 2D-NMR experiments presented in Figures S8–S10 enabled the unequivocal structure elucidation. Six quaternary carbons (δ_C 137.8, 128.9, 126.2, 125.4, 123.2, 114.9 ppm), four aromatic protons (δ_H 6.87, 7.04, 7.58, 8.04 ppm) as well as two methylene groups (δ_H 3.00–3.18 and 3.71/3.00–3.18 ppm) adjacently to CH groups (δ_H 4.19, 4.41 ppm) were identified. The connection of a mercaptolactic acid side chain to the alterperylenepoxide A [10] moiety was achieved via HMBC optimized for $^{2,3}J_{C,H}$ couplings by a correlation signal of C9 resonating at 49.1 ppm to the methylene group 3'.

No ^{1}H–^{1}H COSY coupling was observed between H-9 (δ_H 4.19 ppm (t, J = 3.3 Hz, 1H)) and H-3' (δ_H 3.00–3.18 ppm (m, 1H), δ_H 3.09–3.17 (m, 1H)) (Fig. S8). Therefore, a heteroatom was expected between C-9 and C-3'. The MS/MS experiments by FTICR-MS confirmed the cleavage of a mercaptolactic acid side chain ($C_3H_6O_3S$) from the precursor ion (*m/z* 471.07506). Therefore, a sulfur heteroatom connected the C-9 of alterperylenepoxide A to the C-3' of mercaptolactic acid. The metabolite was named, in accordance to the literature, "alterperylenepoxide A-9-mercaptolactate" (APML).[10] The chemical structure of APML is displayed in Figure S5 and the chemical shifts of the carbons and the protons are depicted in Table S2.

The chemical structure of APML was already determined as "thioperylenol" by Kurz et al. and the ^{1}H and ^{13}C shifts of APML in our NMR measurements were in accordance to those of Kurz et al..[7] In future evaluations, various agricultural products need to be tested for the occurrence of APML.

2.5 Quantitative NMR of APML

The absolute concentration of APML was calculated by quantitative ^{1}H nuclear magnetic resonance spectroscopy (qNMR) according to Frank et al..[11] The purified APML was dissolved in 600 µL of acetonitrile-d_3 in NMR tubes (5 · 178 mm, USC-Tubes, Bruker, Faellanden, Switzerland) and measured on the Bruker AV III. Tyrosine (purity ≥ 99 %, Sigma-Aldrich, Steinheim, Germany) was used as external standard. For the quantification, the signals of the protons H-5 (δ_H 6.89 ppm), H-9

(δ_H 4.21 ppm) and H-8a (δ_H 3.89 ppm) of APML as well as the *ortho*-aromatic protons of the tyrosine standard at 7,10 ppm were chosen. The concentration of the analyte was calculated by using the ERETIC 2 function of TopSpin 3.6.0 (Bruker BioSpin, Faellanden, Switzerland). The accuracy of the qNMR measurement was within an error limit of ± 2 %.

2.6 UV-Spectroscopy

Additionally, the absorption maxima as well as the respective extinction coefficients were determined using a UV/Vis spectrophotometer (Genesys, 10S, Thermo Fisher Scientific, Madison, Wisconsin, USA). The absorption maxima of APML were determined at a concentration of 15.0 µg/mL via full scan (200–450 nm, steps: 1 nm). The absorption spectrum is displayed in Figure S11. To determine the extinction coefficients, the absorptions of APML were recorded at 217.0, 258.0, 291.0, and 381.0 nm at different concentrations. Dilutions of 5.0 µg/mL, 10 µg/mL, and 15 µg/mL (each in triplicate) were prepared in acetonitrile and were measured against acetonitrile. The molar extinction coefficients ε were calculated for each dilution by using the equation $\varepsilon = (\text{absorption} \cdot 1000)/\text{concentration [mmol/L]}$. The absorption maxima and the respective extinction coefficients as well as the absorption minima of APML are listed in Table S4.

185 **Table S1** Optimized precursor and product ions, fragmentation parameters, and retentions times of
186 ATX I, ALTP, AOH, TEN, ATX II, STTX III, AME, and TA applying LC-MS/MS [12]

Analyte	Precursor ion *m/z*	Product ion *m/z*	Q1 Pre Bias voltage [V]	Collision energy (CE) [V]	Q3 Pre Bias voltage [V]	Retention time [min]
ATX I	351.20	297.20	25	28	19	9.2
		314.20	27	32	21	
ALTP	349.30	261.25	18	28	15	10.2
		303.00	18	19	18	
AOH	257.30	215.05	14	24	13	10.9
		213.00	15	23	12	
TEN	413.30	141.05	15	23	23	12.1
		271.30	15	17	17	
ATX II	349.40	313.25	16	25	12	13.2
		331.30	20	15	20	
STTX III	347.20	329.20	17	22	21	14.5
		301.10	17	35	18	
AME	271.30	256.20	20	23	25	17.9
		228.20	19	30	13	
TA	196.30	139.00	14	22	11	8.2
		112.05	22	26	20	

187

188 **Table S2** ^{1}H NMR (400 MHz) and ^{13}C NMR (101 MHz) data for the compound APML in
189 acetonitrile-d_3

Position	δ_C [ppm] (type)	δ_H [ppm] (J in Hz)
1	162.4 (C)	
2	120.2 (CH)	7.04 (d, 1H J = 8.9 Hz)
3	134.1 (CH)	8.04 (d, 1H, J = 8.9 Hz)
3a	126.2 (C)	
3b	125.4 (C)	
4	125.5 (CH)	7.58 (d, 1H, J = 8.7 Hz)
5	115.7 (CH)	6.87 (d, 1H, J = 8.7)
6	157.2 (C)	
6a	123.2 (C)	
6b	128.9 (C)	
7	63.0 (CH)	5.18 (bs, 1H)
7a	56.3 (CH)	3.53 (d, J = 3.7 Hz, 1H)
8a	50.5 (CH)	3.86 (d, J = 3.8 Hz, 1H)
8b	42.4 (CH)	3.74–3.78 (m, 1H)
8c	72.9 (C)	
9	49.1 (CH)	4.19 (t, J = 3.3 Hz, 1H)
10	40.7 (CH_2)	3.68 (dd, 1H, J = 17.3, 3.5 Hz), 3.00–3.18 (m, 1H)
11	204.6 (C)	
11a	114.9 (C)	
11b	137.8 (C)	
1’	174.1 (C)	
2’	72.1 (CH)	4.41 (dd, 1H, J = 6.6, 3.8 Hz)
3’	36.4 (CH_2)	3.00–3.18 (m, 1H), 3.09–3.17 (m, 1H)

190

191 **Table S3** Optimized precursor and product ions as well as fragmentation parameters for APML
192 detection using LC-MS/MS

Precursor ion *m/z*	Product ion *m/z*	Q1 Pre Bias voltage [V]	collision energy (CE) [V]	Q3 Pre Bias voltage [V]
470.90	349.05	27	17	23
	285.10	35	39	23
	331.10	39	29	31
	313.10	47	32	45
	261.10	17	32	24
	452.90	33	21	29

193

194 **Table S4** UV absorption maxima and minima as well as extinction coefficients of APML determined
195 in 100 % acetonitrile

Absorption maximum λ_{max} [nm] (Log extinction coefficient ε [L/mol/cm])	Absorption minimum λ_{min} [nm]
217.0 (4.472)	272.0
258.0 (4.297)	239.0
291.0 (4.334)	328.0
381.0 (3.409)	

196

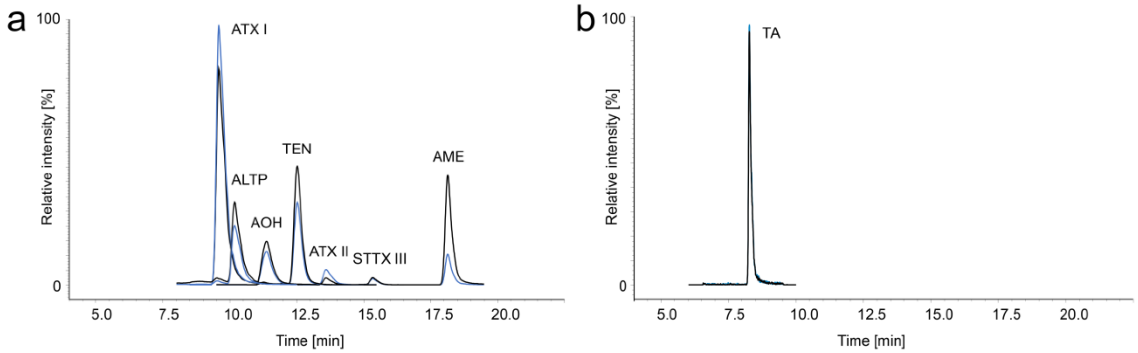


Fig. S1 Chromatogram a displays the *Alternaria* mycotoxins ATX I, ALTP, AOH, TEN, ATX II, STTX III, and AME. For each mycotoxin, two mass transitions are presented. The black and blue mass transitions represent the quantifier and qualifier of the analytes. Due to its higher polarity, TA was analyzed using a second chromatographic system. A chromatogram of TA is shown in b [12]

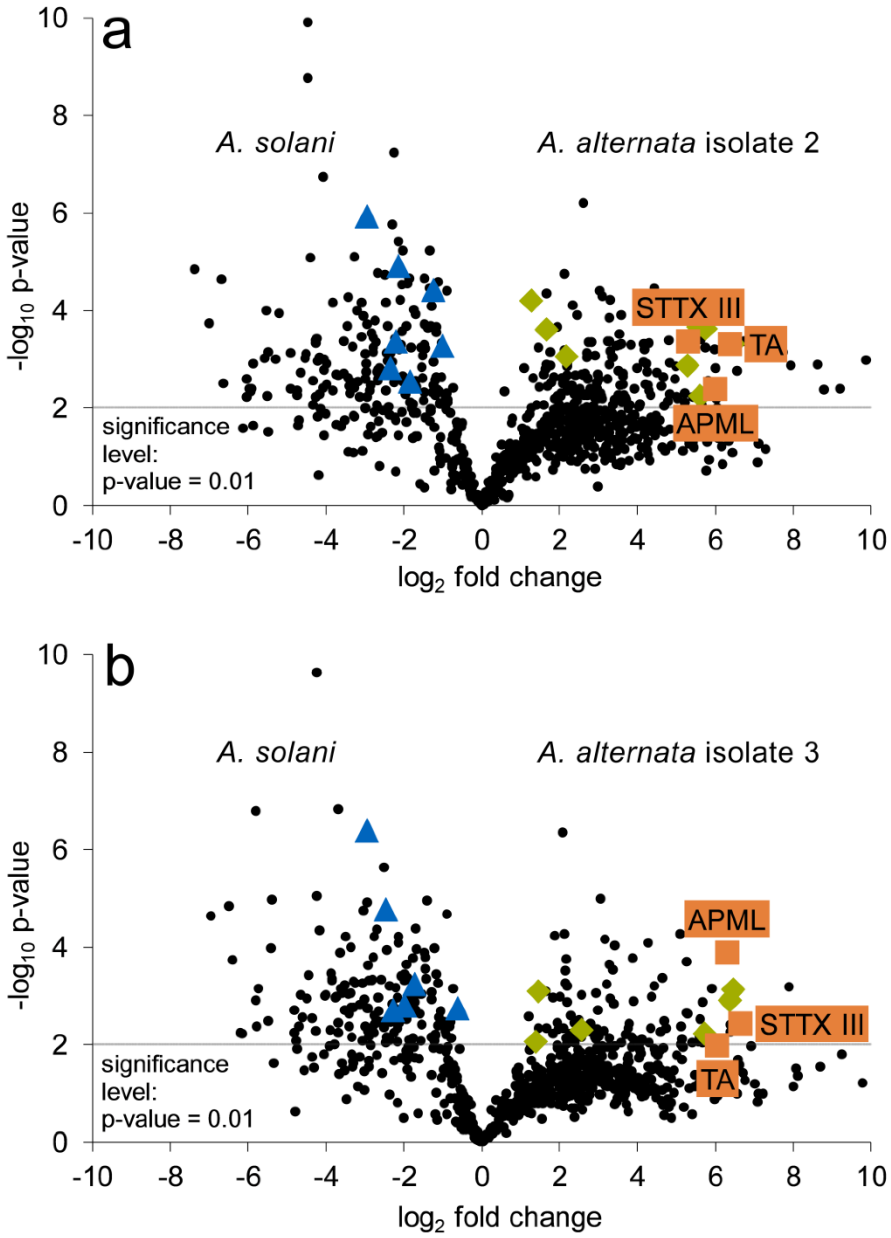


Fig. S2 Volcano plots a and b display the discriminant masses between *A. solani* and the *A. alternata* isolate 2 and between *A. solani* and the *A. alternata* isolate 3 at the 11th day of cultivation. The triangles and hashes represent molecular formulae that were assigned to metabolites by the Antibase database.[13] Due to the lack of reference compounds, the identification of these metabolites could only be performed for STTX III, APML, and TA (displayed as squares) using targeted LC-MS/MS analysis

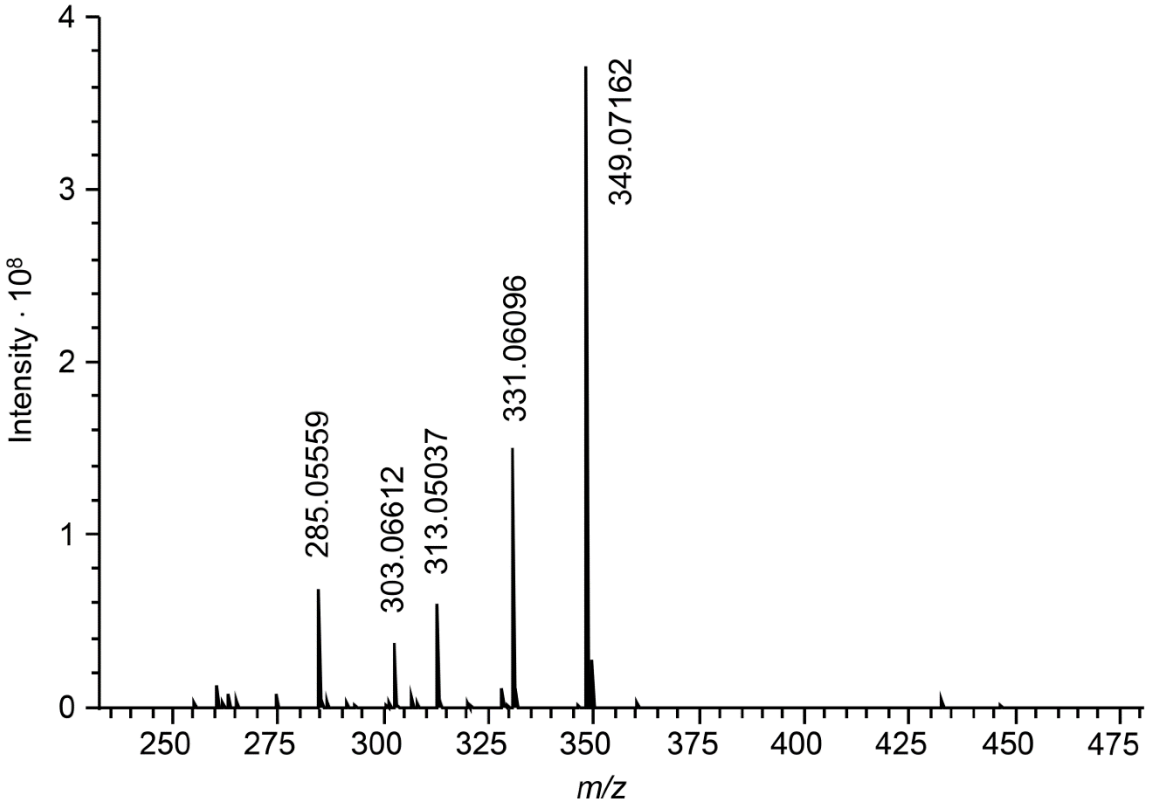


Fig. S3 MS/MS spectrum of APML obtained by FTICR-MS. 20 eV were used to fully fragment the $[M-H]^-$ ion of APML (*m/z* 471.07506). The five most intense fragment ions are displayed

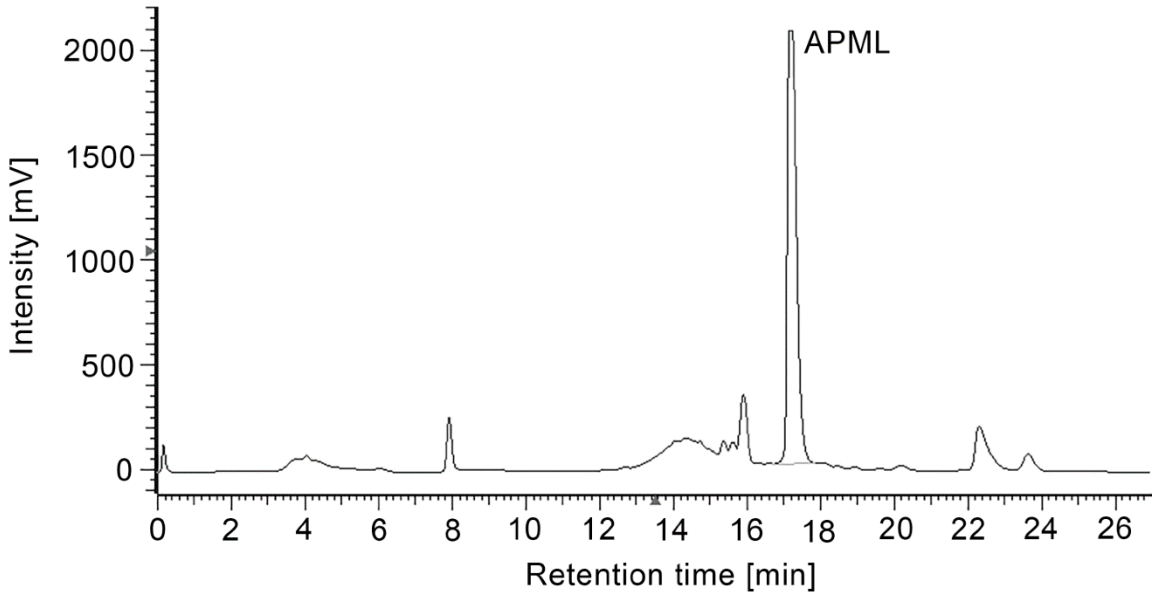
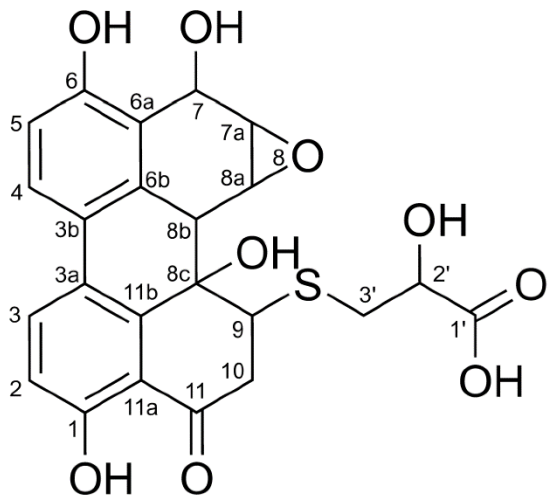


Fig. S4 HPLC-UV chromatogram of the semi-preparative purification of APML. The absorption was recorded at λ = 254 nm. APML eluted from the C18 material after 17.2 min



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216 **Fig. S5** Chemical structure of the discriminant metabolite APML detected in all *A. alternata* extracts

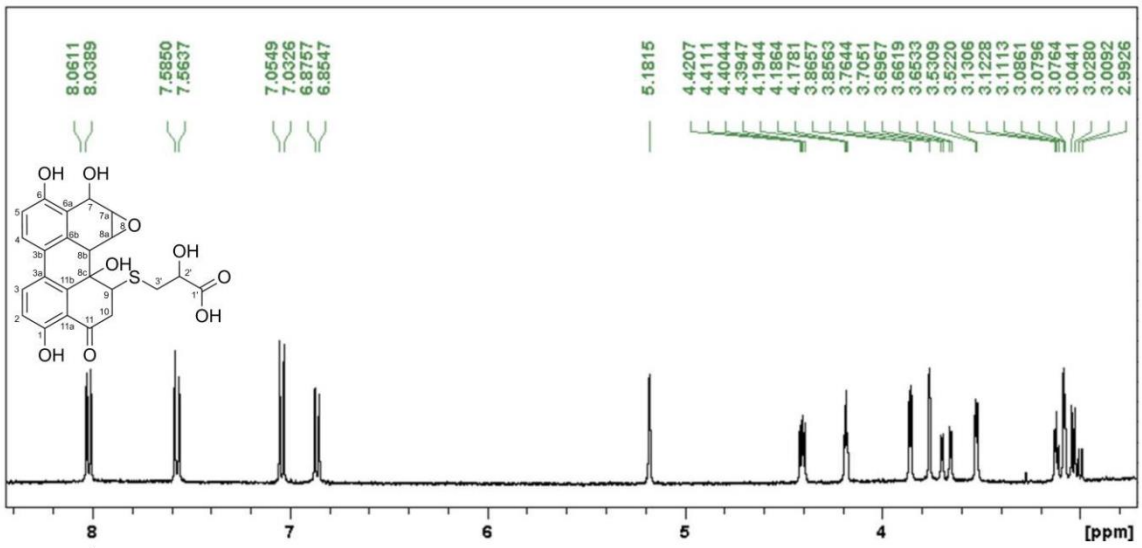


Fig. S6 ^{1}H NMR spectrum of APML

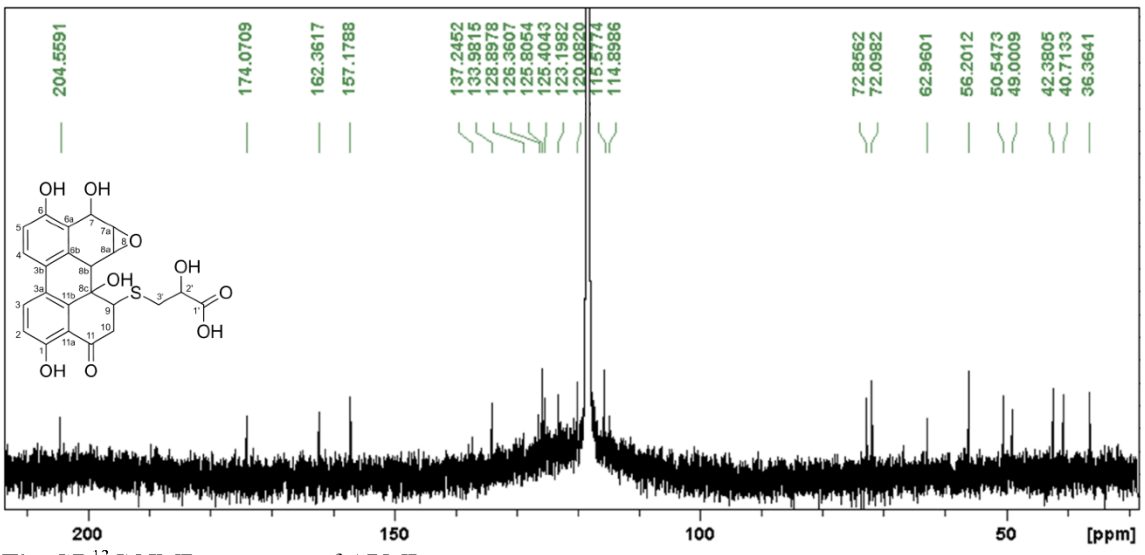


Fig. S7 ^{13}C NMR spectrum of APML

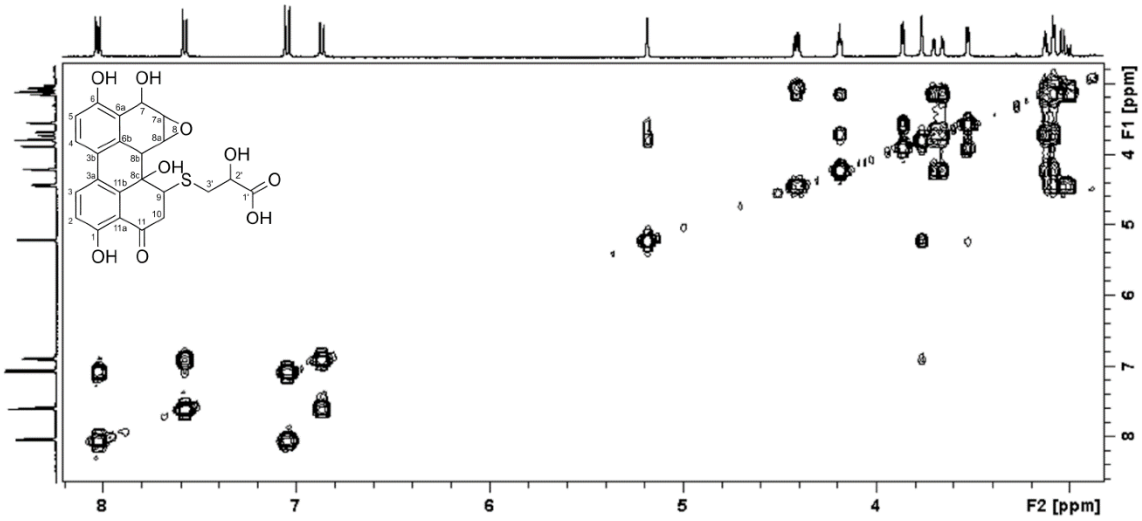


Fig. S8 ^{1}H-^{1}H COSY NMR spectrum of APML

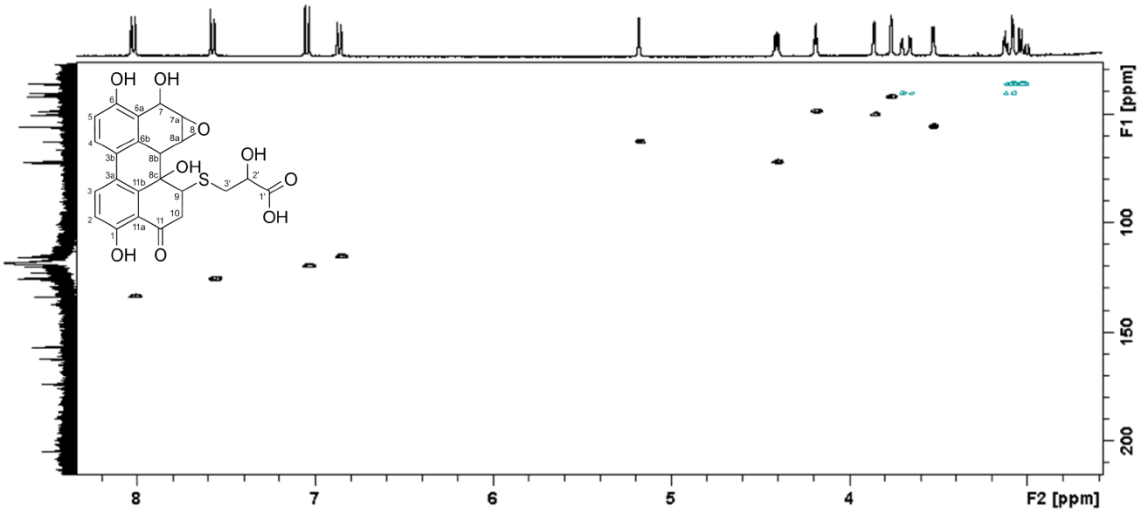


Fig. S9 HSQC 2D NMR spectrum of APML

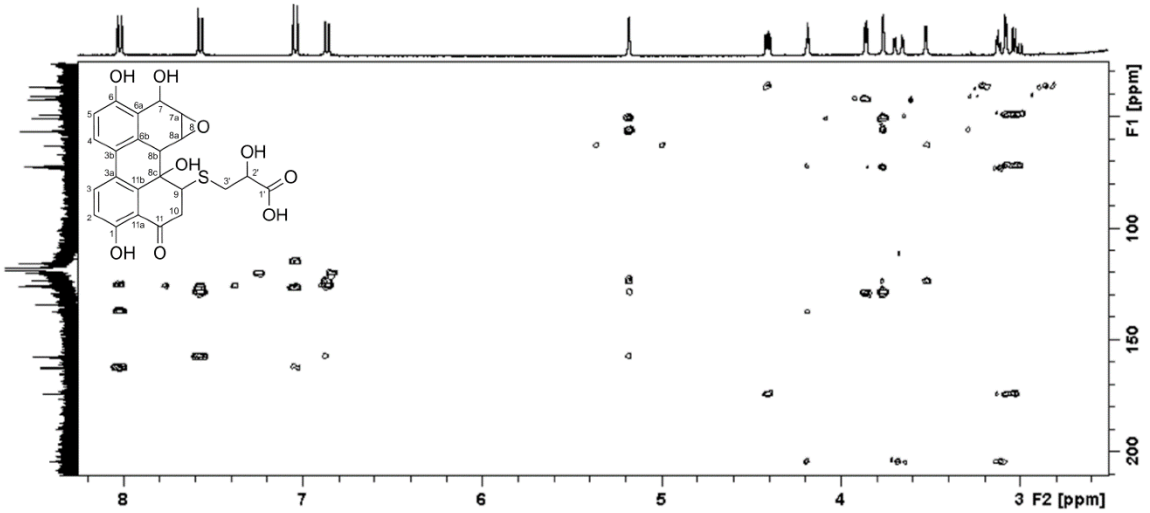
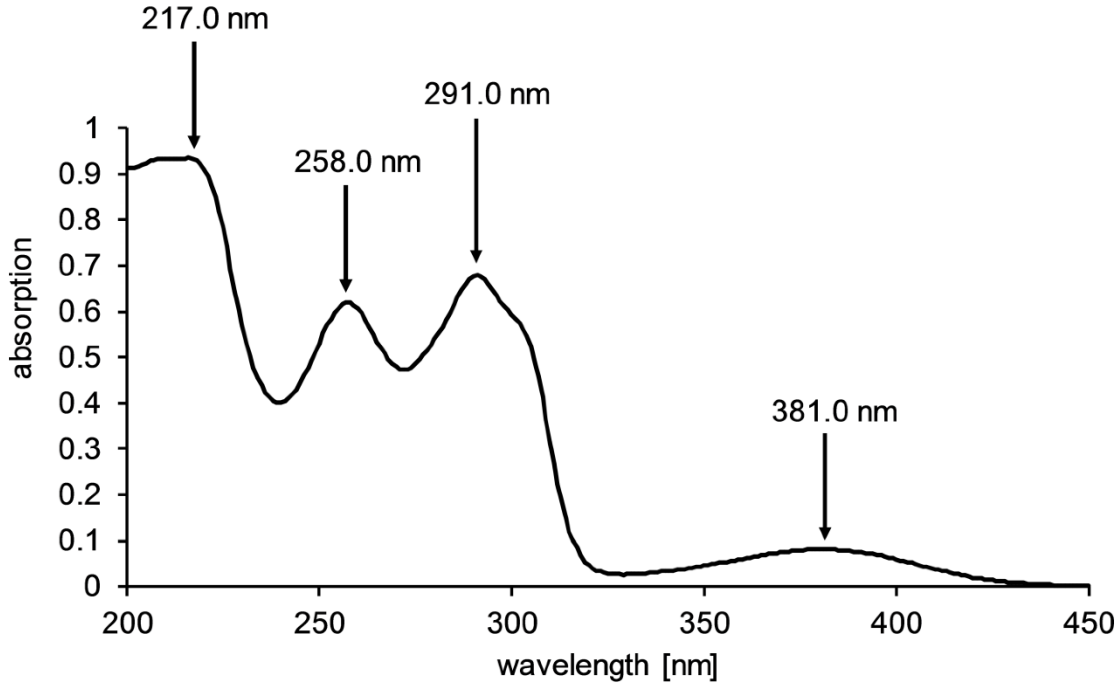


Fig. S10 HMBC 2D NMR spectrum of APML



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228 **Fig. S11** UV/Vis absorption spectrum of APML in acetonitrile at a concentration of 15.0 µg/mL

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Eidesstattliche Erklärung

Ich erkläre an Eides statt, dass ich die bei der promotionsführenden Einrichtung TUM School of Life Sciences der Technischen Universität München zur Promotionsprüfung vorgelegte Arbeit mit dem Titel:

Targeted and non-targeted studies on *Alternaria alternata* metabolites

am Lehrstuhl für Analytische Lebensmittelchemie unter der Anleitung und Betreuung durch Prof. Dr. Michael Rychlik ohne sonstige Hilfe erstellt und bei der Abfassung nur die gemäß § 6 Abs. 6 und 7 Satz 2 angegebenen Hilfsmittel benutzt habe.

(x) Ich habe keine Organisation eingeschaltet, die gegen Entgelt Betreuerinnen und Betreuer für die Anfertigung von Dissertationen sucht, oder die mir obliegenden Pflichten hinsichtlich der Prüfungsleistungen für mich ganz oder teilweise erledigt.
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Die öffentlich zugängliche Promotionsordnung der TUM ist mir bekannt, insbesondere habe ich die Bedeutung von § 28 (Nichtigkeit der Promotion) und § 29 (Entzug des Doktorgrades) zur Kenntnis genommen. Ich bin mir der Konsequenzen einer falschen eidesstattlichen Erklärung bewusst.

Biberach an der Riß, den 18.04.2020