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Abstract

The main topic of this thesis was the development and application of GC-MS based analytical methods for the study of volatile as well as non-volatile, polar metabolites produced by the fungal pathogen *Fusarium graminearum* and its host plant wheat.
Fusarium Head Blight (FHB) is a severe plant disease of many grains caused by pathogenic fungi of the genus *Fusarium*. To better understand the pathogenicity of the fungus and the plant defense response, changes in metabolic profiles due to infection are of high interest. For this purpose, I developed different GC-MS methods which form a part of the metabolomics platform at the Center for Analytical Chemistry. For the analysis of volatile metabolites HS SPME coupled to GC-MS was employed in this thesis. A two-step derivatisation using methoxyamine (MOX) and N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) was performed before liquid injection to analyse non-volatile, polar metabolites using GC-MS. Both methods were adapted from literature to study living fungal cultures, as well as healthy as infected wheat ears.
First, two in-house libraries containing mass spectra and RIs, one library for volatile metabolites, one library for non-volatile, polar metabolites, were created to be used for compound annotation and identification in MetaboliteDetector. In total, the in-house library for volatiles contained 296 mass spectra and RI values: 139 mass spectra and measured RI values from authentic standards, 116 were NIST mass spectra (median RI values from NIST Chemistry WebBook), which were successfully used for metabolite annotation in fungal cultures and wheat ears, and 41 mass spectra and RI from unknown metabolites, which were detected in fungal cultures. For the in-house library for non-volatile polar metabolites, 147 standard substances were measured and their mass spectra and RIs were stored.
For the evaluation of the HS SPME GC-MS method, a qualitative, non-targeted characterization of *F. graminearum* PH-1 cultures was performed over one week. Based on these results, matrix effects on the SPME efficiency for target analytes were studied. The influence of the cultivation medium (PDA), of the sesquiterpenes and the fungal mycelium on target analytes and of selected volatiles on the sesquiterpenes were determined. As a result of this study, only the sesquiterpenes remained unaffected by the matrices tested. Quantification for commercially available sesquiterpenes was achieved for fungal cultures using this method. For minor constituents, such as the alcohols, esters, ketones and monoterpenes, the HS SPME GC-MS method was used for qualitative analysis only.
Knowing about the limitations of the HS SPME method regarding quantification, metabolic profiles of *F. graminearum* PH-1, strains of *F.graminearum* of the Northland population (newly discovered strains in Northern America) and the $\Delta tri5$ mutant, lacking the *tri5* gene encoding for trichodiene synthase, were generated. Morever, the relevance of flushing cultures with synthetic air prior to HS SPME and cultivation in the presence of light, was determined for cultures of PH-1. Although higher amounts of volatiles were expected in the non-flushed cultures because accumulated amounts of volatiles were measured in contrast to time-dependent synthesis of volatiles for flushed cultures, most sesquiterpenes were found in higher amounts in flushed cultures than in non-flushed cultures of PH-1. Cultivation in the light in comparison to the dark revealed for *F. graminearum* PH-1 cultures that the sesquiterpene biosynthesis was downregulated. Only few sesquiterpenes were synthesized in the presence of light and those were detected in much lower amounts (factor ≥ 50) compared to cultivation in the dark. There was hardly any effect of light

on the $\Delta tri5$ mutant, which synthesized only a few sesquiterpenes at all. Additionally, 1,2,4-trimethylbenzene was only annotated in cultures (of both genotypes) which were cultivated in the light.
Lately, a research group in North America described newly isolated strains of *F. graminearum*, the Northland population. The strains of the Northland population were shown to produce no known trichothecene, but great amounts of zearalenone. Since sesquiterpenes are important precursors for trichothecene biosynthesis, the sesquiterpene pattern of cultures of the Northland population was of high interest in comparison to cultures of *F. graminearum* PH-1 (a trichothecene producer). HS analysis of the volatile metabolites showed that many sesquiterpenes which were usually synthesized in significant amounts by fungal cultures of the reference strain PH-1 were produced by fungal cultures of the Northland population as well, including trichodiene, which indicated the presence of an active *tri5* gene. Several other sesquiterpenes, which could not be annotated, and longiborneol, the volatile precursor for culmorin synthesis, were synthesized by strains of the Northland population which were not found in PH-1 cultures. Based on these results, coworkers at our institute found that these *F. graminearum* strains are capable of the production of a newly described trichothecene.
In addition to the fungal volatiles, plant volatiles from healthy and infected wheat ears were studied using HS SPME. The volatile metabolic profiles of healthy wheat ears of different developmental stages and *Fusarium*-infected wheat ears were compared. A significant difference in the signal intensity of calamene (sesquiterpene) was found between wheat ears before and during flowering. α-Longifolene, β-gurjunene, β-bisabolene, trichodiene, longiborneol and five sesquiterpenes which could not be annotated using the set criteria, were detected in the infected wheat ears only.
The comparison of the volatile, metabolic profiles of wheat ears from cultivar 'CM' (resistent against FHB) and 'Remus' (susceptible to *Fusarium* infection) showed that especially sesquiterpenes were produced at 96 h after *Fusarium* infection and their number and signal intensity differed between the genotypes.
In the presented thesis, the two-step, online-derivatisation GC-MS method, developed for the metabolic profiling of non-volatile, polar (mainly primary) metabolites, was adapted for the targeted determination of a small range of plant and fungal metabolites such as ergosterol, sitosterol, stigmasterol, 1-aminocyclopropane-1-carboxylic acid (ACC) and 2-ketobutyric acid. In cooperation with the research group of Gerhard Adam (Department for Cell Biology and Applied Genetics) sterols from infected maize seedlings were extracted to be used as biomarkers for fungal and plant biomass. The correlation between absolute concentrations of ergosterol (fungal biomass), sitosterol and stigmasterol (plant biomass) was studied for different maize cultivars. The highest infection degree was determined for the cultivar 'Okato', followed by 'Gilberto', 'Gingko', 'Pandoso'. The lowest ergosterol/sitosterol ratio was determined for the cultivars 'Eduardo' and 'Fortress'.
A target method for ACC and its fungal degradation product 2-ketobutyric acid was optimized for the analysis of cell lysates from *E.coli*. *Fusarium* enzymes, ACC synthases and ACC deaminases, which had been cloned into bacterial cells were tested for their activity *in vitro*. None of the ACC synthases produced detectable amounts of ACC. Successfully, one of the two deaminases converted ACC to 2-aminobutyric acid which is the transamination product of 2-ketobutyric acid. The transamination was attributed to bacterial transaminases.

keywords: Fusarium Head Blight, metabolic profiling, HS SPME GC-MS, target analysis, MSTFA.

Kurzfassung

Das Hauptthema dieser Dissertation war die Entwicklung und Anwendung von GC-MS basierenden analytischen Methoden, um flüchtige und nicht-flüchtige, polare Metabolite des Pathogens *Fusarium graminearum* und seiner Wirtspflanze Weizen zu studieren.
Fusarium Head Blight (FHB) ist eine schwerwiegende Pflanzenkrankheit bei vielen Getreidesorten, verursacht von Pilzen der Gattung *Fusarium*. Um die Pathogenizität des Pilzes und die Pflanzenabwehrreaktionen besser zu verstehen, sind Veränderungen auf metabolischer Ebene nach einer Infektion von hohem Interesse. Daher habe ich verschiedene GC-MS Methoden, die einen Teil der Metabolomics Plattform am Analytikzentrum darstellen, entwickelt. Zur Analyse der flüchtigen Metabolite wurde HS SPME GC-MS in dieser Dissertation verwendet. Eine zweistufige, Online-Derivatisierung unter der Verwendung von Methoxyamin (MOX) und N-methyl-N-(trimethylsilyl)trifluoroacetamid (MSTFA) wurde vor der Flüssiginjektion zur Analyse von nicht-flüchtigen, polaren Metaboliten mit GC-MS durchgeführt. Beide Methoden wurden aus der Literatur angepasst, um lebende Pilzkulturen sowie gesunde und infizierte Getreideähren zu studieren.
Zuerst wurden zwei hausinterne Bibliotheken, die Massenspektren und Retentionsindices (RIs) enthalten, eine für flüchtige Metabolite, eine für nicht-flüchtige, polare Metabolite, zum Annotieren und Identifizieren in MetaboliteDetector erstellt. Im Gesamten, enhält die Bibliothek für die Flüchtigen, 296 Spektren und RIs: 139 Massenspektren und gemessene RIs von Standardsubstanzen, 116 NIST Spektren (und die Mediane von RI Werten aus dem NIST Chemistry WebBook), die erfolgreich für Annotierungen in Pilzkulturen und Getreideähren verwendet wurden, und 41 Massenspektren und RIs von unbekannten Metaboliten, die in Pilzkulturen gefunden wurden. In der hauseigenen Bibliothek für nicht-flüchtige, polare Metabolite befinden sich 147 Massenspektren und RIs von Standardsubstanzen.
Zur Evaluierung der HS SPME GC-MS Methode wurde eine qualitative, nicht-gerichtete Charakterisierung von *F. graminearum* PH-1 Kulturen über eine Woche durchgeführt. Basierend auf diesen Ergebnissen wurden Matrixeffekte auf die Extraktionseffizienz von Zielanalyten untersucht. Der Einfluss des Kultivierungsmediums (PDA), der Sesquiterpene und des Pilzmycels auf Zielsubstanzen und ausgewählten Flüchtigen auf die Sesquiterpene wurden bestimmt. Die Studie ergab, dass nur die Sesquiterpene nicht von den untersuchten Matrices beeinflusst wurden. Eine Quantifizierung der kommerziell erhältlichen Sesquiterpene wurde für Pilzkulturen mit dieser Methode erreicht. Kleinere flüchtige Metabolite, wie Alkohole, Ester, Ketone und Monoterpene, wurden mit HS SPME GC-MS nur qualtitative beschrieben.
Nachdem die Limitierungen der HS SPME Methode bezüglich Quantifizierung aufgezeigt wurden, erstellte ich Metabolitprofile von *F. graminearum* PH-1, *F.graminearum* Stämmen der Northland population (neu entdeckte Stämme in Nordamerika) und der Δ*tri5* Mutante, der das *tri5* Gen, das die Trichodiene Synthase kodiert, fehlt. Zusätzlich wurden die Relevanz, PH-1 Kulturen mit synthetischer Luft vor der Extraktion zu spülen oder in der Gegenwart von Licht zu kultivieren, bestimmt. Obwohl höhere Mengen von Flüchtigen in den nicht-gespülten Kulturen erwartet wurden, da eine akkumulierte Menge an Flüchtigen gemessen wurde, im Gegensatz zu einer zeitabhängiger Synthese von Flüchtigen in gespülten Kulturen, wurden die meisten Sesquiterpene in höheren Mengen in den gespülten Kulturen gefunden. Eine Kultivierung im Licht im Vergleich zu Kulturen im Dunkeln ergab, dass die Sesquiterpenebiosynthese runterreguliert war. Nur wenige Sesquiterpene wurden im Licht produziert und diese wurden in geringeren Mengen gemessen (Faktor $\geq$50) im Vergleich zu Kulturen im Dunkeln. Kaum einen Einfluss hatte das Licht auf die

Δ*tri5* Kulturen, die generell nur wenige Sesquiterpene synthetisierten. Zusätzlich wurde 1,2,4-Trimethylbenzen in Kulturen beider Genotypen entdeckt, das nur im Licht gebildet wurde.
Kürzlich hat eine Forschungsgruppe aus Nordamerika neu isolierte Stämme von *F. graminearum*, die Northland population, beschrieben. Diese Stämme produzieren keine bekannten Trichthecene, aber große Mengen an Zearalenone. Da Sesquiterpene wichtige Vorstufen für die Trichothecen Biosynthese sind, wurde ein Sesquiterpenprofil von Kulturen der Northland population im Vergleich zu PH-1 (der Trichothecene produziert) erstellt. Die Analyse der flüchtigen Metabolite zeigte, dass viele Sesquiterpene, die normalerweise von PH-1 in signifikanten Mengen synthetisiert werden, auch von den Kulturen der Northland population gebildet wurden, inklusive Trichodiene, ein Metabolit, dessen Biosynthese die Gegenwart eine aktiven *tri5* Gens voraussetzt. Viele andere Sesquiterpene, die nicht annotiert werden konnten, und Longiborneol, der flüchtige Vorläufer der Culmorinsynthese, wurden von den Northland population Kulturen, nicht aber von PH-1 gebildet. Basierend auf diesen Ergebnissen haben Mitarbeiter des Instituts herausgefunden, dass die Stämme ein bisher unbekanntes Trichothecen bilden.
Zusätzlich zu den Flüchtigen des Pilzes, wurden Flüchtige von gesunden und infizierten Weizenähren mit HS SPME untersucht. Die Metabolitprofile von gesunden Weizenähren aus verschiedenen Entwicklungsstadien wurden mit *Fusarium* infizierten verglichen. Ein signifikanter Unterschied in der Signalintensität von Calamenene (Sesquiterpen) wurde zwischen Getreideähren vor und während der Blüte beschrieben. α-Longifolen, β-Gurjunene, β-Bisabolene, Trichodiene, Longiborneol und fünf weitere Sesquiterpene, die nicht anhand der festgelegten Kriterien annotiert werden konnten, wurden nur in den infizierten Weizenähren gefunden.
Ein Vergleich der Metabolitprofile von Flüchtigen von Weizenähren der Gattung 'CM' (resistent gegen FHB) und 'Remus' (empfindlich gegen *Fusarium* Infektion) zeigte, dass vor allem Sesquiterpene 96 h nach der Infektion synthetisiert wurden und dass ihre Anzahl und Intensität zwischen den Genotypen unterschiedlich war.
In der vorliegenden Dissertation wurde die GC-MS Methode zur Erstellung von Metabolitprofilen von nicht-flüchtigen, polaren (hauptsächlich primären) Metaboliten, die eine zweistufige, Online-Derivatisierung beinhaltet, angepasst für die zielgerichtete Analyse eines kleinen Bereichs an Pflanzen- und Pilzmetaboliten, wie Ergosterol, Sitosterol, Stigmasterol, ACC und 2-Ketobutyrat. In Kooperation mit der Forschergruppe von Gerhard Adam (Department für Zellbiologie und Angewandte Genetik) wurden Sterole von infizierten Maispflänzchen extrahiert und als Biomarker für Pilz- und Pflanzenbiomasse verwendet. Die Korrelation zwischen absoluten Konzentrationen von Ergosterol (Pilzbiomasse), Sitosterol und Stigmasterol (Pflanzenbiomasse) wurde für verschiedene Maissorten studiert. Die höchste Infektionsrate wurde für die Sorte 'Okato' erzielt, gefolgt von 'Gilberto', 'Gingko', 'Pandoso'. Das geringste Verhältnis an Ergosterol/Sitosterol wurde für die Sorten 'Eduardo' und 'Fortress' bestimmt.
Eine zielgerichtete Methode für ACC und dessen pilzliches Abbauprodukt 2-Ketobutyrat wurde optimiert für die Analyse von Celllysaten von *E.coli*. Die *Fusarium* Enzyme, ACC Synthase und ACC Deaminase, wurden in Bakterienzellen kloniert und ihre Aktivität wurde *in vitro* bestimmt. Keine der ACC Synthasen produzierte detektierbare Mengen an ACC. Eine der beiden ACC Deaminasen konvertierte ACC erfolgreich zu 2-Aminobutyrat, was ein Transaminierungsprodukt von 2-Ketobutyrat ist. Die Transaminierung wurde bakteriellen Transaminasen zugesprochen.

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Chapter 1

Biological background and aims of the thesis

This thesis was carried out within the project SFB *Fusarium* F37, funded by the Austrian Science Fund FWF, in which *Fusarium* metabolites and detoxification reactions regarding Fusarium Head Blight (FHB) disease are studied.
FHB is a severe plant disease of many grains caused by pathogenic fungi of the genus *Fusarium*. FHB, also called scrab, causes hugh economic losses in Europe and Northern America. It affects grain quality and yield negatively due to plant dieback and mycotoxins which accumulate in the kernels. Mycotoxins, which play an important role as virulence factors of the FHB disease, cause toxicoses and other health threats. The Food and Agriculture Organisation (FAO) has compiled worldwide regulations and directives for allowed contents of several mycotoxins in food and feed (FAO, 2004). Mycotoxins might not be synthesized in the initial stage of infection by *Fusarium* as a first consequence to infection. *Tri5* gene expression, which is an indicator for DON biosynthesis, is not expressed in anthers, the initial targets of the pathogen. However, as the rachis is a formidable barrier to the spread of *Fusarium*, DON biosynthesis was found essential to overcome this major obstacle (Ilgen et al., 2009).
As a consequence there is an absolute need for early and readily applicable methods to detect *Fusarium*-infected grain and to distinguish between toxic and non-toxic strains. So far *Fusarium* or its metabolites have been detected and analyzed using various techniques including GC-MS, LC-UV or LC-MS, TLC, fluorescence immunoassays, NIRS, ELISA or PCR. *Fusarium* spp. have been shown to emanate a number of volatile compounds, specifically hydrocarbons, ketones, aldehydes, esters, terpenes and complex mixtures of alcohols Jelen et al. (1995). Production of volatiles might be a way of removing inhibitory intermediates from the metabolism under unfavorable conditions, have inhibitory effects on other fungi or act as self-regulator of growth and development (Linton and Wright, 1993). Several studies suggest that monitoring the appearance of fungal volatiles is a good and early indicator of infected grain and therefore quality loss and mycotoxin formation (Magan and Evans, 2000). Plant breeders all over the world are looking for resistant lines of crops and breeding techniques to enhance plant resistance against the fungi. Understanding resistance mechanisms in resistant plants helps transferring the knowledge in breading of susceptible wheat cultivars. Plant defense mechanisms and resistance genes are under current investigation of molecular geneticists and biochemists. A large number of quantitative trait loci (QTLs) have been identified (Buerstmayr et al., 2009), which can be studied in near isogenic lines (NILs) that differ by the presence of absence of a single QTL. Exchange of scientific knowledge between molecular biologists, analytical chemists

Figure 1.1: Project parts of the SFB *Fusarium* in collaboration with the analytical chemists (project part: 'Metabolomics of plant-*Fusarium* interactions').

and plant breeders is prerequisite to study the defense mechanisms of plants resistant against *Fusarium*.
The SFB *Fusarium* is an interdisciplinary project which allows the important collaboration between scientists of various scientific fields, including plant breeders, molecular biologists, analytical chemists and bioinformatic scientists. The overall aim is to study the interaction plant::fungus and to get an improved understanding of the role of (secondary) metabolites of the fungus in disease development. In addition the project aims at gaining more knowledge about resistance compounds in plant. The plants under investigation are model plants *Arabidopsis* and *Brachypodium* as well as the crop plants wheat and maize. *Fusarium graminearum* is mainly used for the studies, being the most frequent mycotoxin producer of DON. Some plants have evolved detoxification mechanisms against mycotoxins, for example by masking the mycotoxins (glycosylation). Still, the fungus can overcome many defense mechanisms of the plant by expression of a redundant set of metabolites. Using modern genomics, transcriptomics and metabolomics tools (Walter et al., 2010) plant breeders can assemble their breeding trials on molecular processes to receive *Fusarium*-resistant wheat cultivars with low or no mycotoxin content.
The working hypothesis underlying the SFB project is that (hemi-)necrotrophic fungi such as *Fusarium* synthesize low molecular weight metabolites which suppress defense response of the host, which could explain the wide range of host plants of this pathogen. Genome sequencing revealed the presence of genes for 15 polyketide synthases, 20 non-ribosomal peptide synthases and 17 terpenoid synthases, for most of these genes no corresponding metabolites are known (Adam, 2008). Of uttermost importance for the SFB *Fusarium* is the use of functional genomics and analytical methods for metabolite profiling to detect new suppressor metabolites and assess the relevance of fungal secondary metabolites for pathogenicity.

In this respect, the emerging discipline of systems biology is of great use. Systems biology

is a biology-based interdisciplinary approach to model, discover and describe properties of cells, tissues and organisms. Global studies of living systems improve knowledge about individual organisms and their interactions with other organisms in complex networks. Systems biology includes scientific disciplines such as genomics, transcriptomics, proteomics and metabolomics.
Project part 'Metabolomics of plant-*Fusarium* interactions', as the core analytical facility of the SFB *Fusarium*, provides various metabolomics tools and target methods for the collaboration partners, see figure 1.1. We aim at the development and establishment of a metabolomics platform using LC- and GC-MS instruments for stable isotope labeling (SIL)-assisted experiments, metabolite annotation and identification and statistical analysis. Metabolites from different chemical classes, size and properties are studied with the instruments available by generating metabolic profiles with single instruments. Metabolomics of fungi and plants include four different analytical approaches: metabolic profiling of secondary metabolites using HR LC-MS, metabolic profiling of polar metabolites using derivatisation GC-MS, metabolic profiling of volatiles using HS SPME GC-MS and target analysis of selected analytes. Metabolomics studies are accomplished to study resistance and defense mechanisms of plants against *Fusarium* and the infection process from the fungal side on a metabolic level. Therefore, the metabolomics research group at the Center for Analytical Chemistry provides the service to do metabolic profiles and target analyses of selected compounds for SFB internal collaboration partners. The projects of the collaboration partners deal with: the regulation of biosynthetic genes and epigenetics in *F. graminearum*, secondary metabolite production and virulence of *F. graminearum*, detoxification by plant UDP-glycosyltransferases, the role of zearalenone and other *Fusarium* compounds in *Arabidopsis* and the characterisation of bioactive compounds from FHB resistant wheat lines. Revealing metabolic processes contributes significantly to a better understanding of genomic and transcriptomic data. The information from genomic or transciptomics data can be linked to metabolic intermediates and end products. Hence, it helps to clarify pathways involved in interaction processes.
This thesis was carried out in the course of the establishment of a metabolomics research group at the Center for Analytical Chemistry. The aim of this thesis is to develop, establish and apply GC-MS based analytical methods for the study of volatile as well as non-volatile, polar metabolites produced by *Fusarium graminearum* and its host plant wheat. For the analysis of volatile metabolites HS SPME coupled to GC-MS was accustomed, whereas a two-step online-derivatisation method using methoxyamine (MOX) and N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) was required before liquid injection to analyse non-volatile, polar metabolites using GC-MS. Additionally, target methods for selected metabolites of interest are developed for project partners and applied to biological samples.

The resulting technical and scientific questions for this thesis are:

1. Development and evaluation of HS SPME GC-MS method including

    - built-up of an in-house library containing spectra and RIs of authentic standards and annotated metabolites
    - characterization of the developed HS SPME GC-MS method regarding RI accurary and precision
    - qualitative evaluation of flushed and non-flushed *F. graminearum* PH-1 cultures and the determination of mycotoxin content in HS cultures

- assessment of matrix effects on SPME efficiency for quantification of target analytes

2. Application of the established HS SPME GC-MS method
   - for metabolic profiling of volatiles from fungal cultures
     - qualitative, non-targeted characterization of four *Fusarium graminearum* strains of the Northland population
     - comparison of the volatile metabolic profiles of *F. graminearum* PH-1 wild-type and Δ*tri5* mutant including a study of light influence
   - for metabolic profiling of volatiles from wheat ears
     - of different developmental stages and *F. graminearum*-infected ears
     - of two different wheat genotypes (one resistent, one susceptible cultivar) after infection with *F. graminearum*
3. Development and evaluation of an online-derivatisation GC-MS method including
   - built-up of an in-house library containing spectra and RIs of authentic standards
   - evaluation of different extraction conditions
4. Application of the established online-derivatisation GC-MS method
   - for metabolic profiling of non-volatile, polar metabolites from wheat ears
   - for target analysis of metabolites interesting to project partners
     - ergosterol, sitosterol, stigmasterol
     - 1-aminocyclopropane-1-carboxylic acid (ACC), 2-ketobutyric acid

Chapter 2

Introduction

2.1 Fusarium Head Blight

Fusarium Head Blight (FHB) is a destructive disease caused by ascomycete fungal pathogens, of the genus *Fusarium* spp. The most common species associated with FHB in Europe are: *Fusarium avenaceum*, *Fusarium culmorum*, *Fusarium nivale* and *Fusarium graminearum* (Gilchrist L, 2002). According to Kazan *et al.* the causative agent of FHB on wheat and barley and ear rot of maize is *Fusarium graminearum sensu stricto*. The elucidated life cycle of *Fusarium* comprises a saprophytic and a pathogenic phase. *Fusarium* spp. are hemibiotrophic fungi, they exhibit a biotrophic lifestyle during the initial stages of infection and switch rapidly (within a few days after inoculation) to necrotrophy in their pathogenic phase (Kazan et al., 2012). *Fusarium* spp. have a sexual form called *Giberella*. It is induced by light, air and humidity. The asexual form is induced if nutrients, light, air and humidity are deficient. The fungus is able to produce conidia (asexual form) or ascospores (sexual form), which are dormant bodies and can survive in the soil. The fungus overwinters on diseased kernels, plant debris or diseased kernels. These infected kernels provide primary inoculum which can be wide spread by wind or rain onto neighbouring plants, starting the infection cycle. The germination of conidia from primary inoculum is influenced by environmental conditions such as humidity, warmth and spore density. The first symptoms of FHB tend to occur in the middle of the heads, where flowering begins. Wheat heads are very resistant before flowering and most susceptible during anthesis. *Fusarium* spp. can entry plants passively through natural openings such as wounds or by direct penetration (Goswami and Kistler, 2004). Penetration of the host cuticule is facilitated by hydrolysis and cell wall-degrading enzymes secreted by the fungus. The fungus enters the softened cell wall and the appressorium (flattened hyphal turgor, the holdfast organ) is introduced. The appressorium develops enormous turgal pressure which leads to mechanical damage (lesions) of the plant cell wall. The fungus spreads from floret to floret over vascular bundles in the rachilla and from node to node in the rachis resulting in symptoms all over the wheat head. No kernels are produced in case of infections during anthesis. Older kernels are shriveled or wilted and contain mycotoxins (in case of *Giberella zeae* mostly deoxynivalenol (DON) is produced). In some cases the diseased kernels develop red or pink discoloration. Seed infection causes early infection of plant and dark brown lesions on the plant stem of seedlings. The disease cycle of *Fusarium* (*Giberella*) from plant infection to survival in the soil and plant debris is shown in figure 2.1.
Naturally infected wheat ears vary a lot in their symptoms and disease progress. There are significant differences regarding the infection degree per year. For research purposes, it is

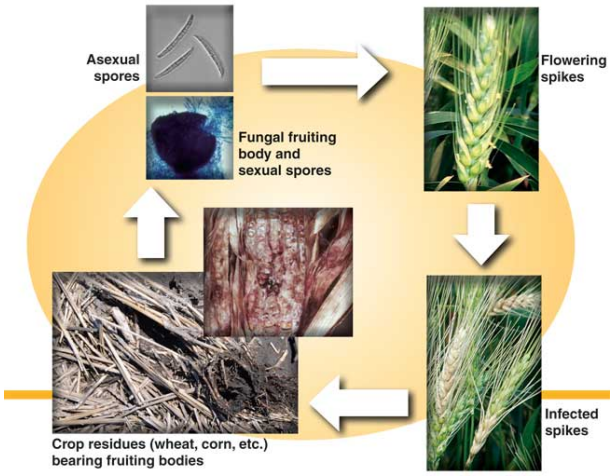


Figure 2.1: *Fusarium* (*Giberella*) disease cycle in the environment. Life cycle composition by Shaobin Zhong; photos by NDSU Department of Plant Pathology (http://www.ag.ndsu.edu/pubs/plantsci/smgrains/pp804w.htm)

necessary to have standardized, fixed and reproducible infection rates to be able to study the infection progress. Hence in most scientific studies investigating FHB, wheat ears are infected artificially during anthesis. The anatomy of a wheat plant is shown in figure 2.2. Researchers have different inoculation techniques, such as spraying the fungal inoculum or pipetting it directly into the spikelets. The pipetting allows targeted infection which is locally limited to see how fast and where the symptoms spread. The target for this technique is between palea and lemma of single spikelets (smaller units of a spike), where the ovary is located. The spikelets are therefore primary targets for sampling regarding metabolomics studies because here the initial reponse of the plant can be monitored. A single spikelet is not enough material for metabolomics studies, hence, a number of equally treated spikelets (prefereably from the same spike) are harvested as one biological sample. This was done in metabolic studies of wheat ears in this thesis. Collecting entire spikes including the rachis leads to metabolic studies of the initial and systemic response of the plant. Knowledge about the sampling material, the initial site of infection and the tissues involved in systemic response, is important for all studies in the field of systems biology to study resistance mechanisms *in planta*.

To describe FHB resistance in grains, different categories were defined: resistance to intial infection (type I), resistance to spread of symptoms in head from spikelet to spikelet (type II), resistance to effects of trichothecene toxins (type III) and resistance to kernel infection (type IV). A tolerant plant is resistant to yield loss in presence of disease (Schroeder HW, 1963).

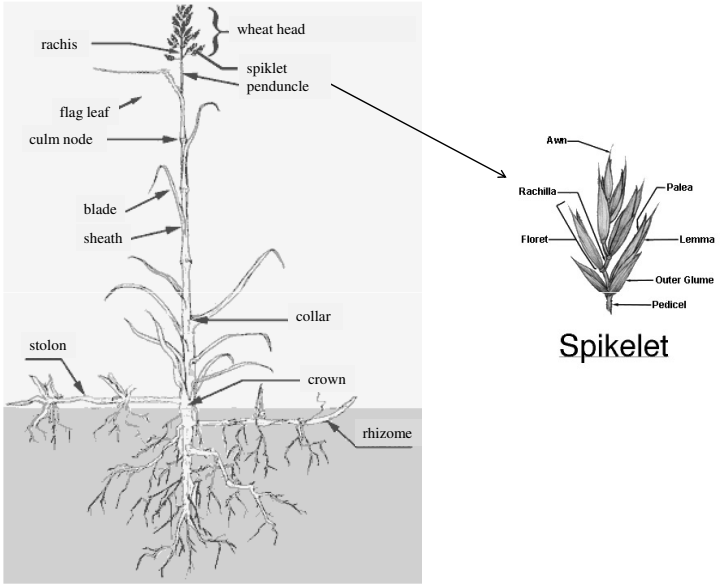


Figure 2.2: Wheat anatomy of the whole plant and spikelet in detail. (from Oregon State University, http://www.fsl.orst.edu/forages/projects/regrowth/print.cfm?PageID=11).

For determination of infection degree of a wheat plant, visual inspection and genetic markers are used. Infected wheat ears are usually classified visually in their infection degree by their area under diseased disease progression curve (AUDPC) (Zwart et al., 2008) or by counting the *Fusarium*-damaged kernels (FDK) (Miedaner et al., 2004) in comparison to mycotoxin contents. Quantitative trait loci (QTL) mapping allows marker-assisted plant breeding leading to more targeted resistance gene transfer (Buerstmayr et al., 2009). To study infected wheat ears on a metabolic level could help reveal the role of single QTLs and resistance mechanisms *in planta*. *Fusarium*-wheat interaction models show that the crosstalk between fungus and plant on a molecular level is very complex and that the response of the plant to infection can help restrict disease severity, but it can also be used by the pathogen to aid pathogenicity, see figure 2.3.
Metabolomics approaches are therefore used to study effects of fungal infection on resistance and susceptible plants on a metabolic and regulatory level for FHB (Gunnaiah et al., 2012; Kumaraswamy et al., 2011a,b; Bollina et al., 2011; Hamzehzarghani et al., 2005). In the following an overview of important pathways, which are involved in *Fusarium*-plant interaction, is given with emphasis on volatiles and small, polar metabolites which were studied in this thesis.

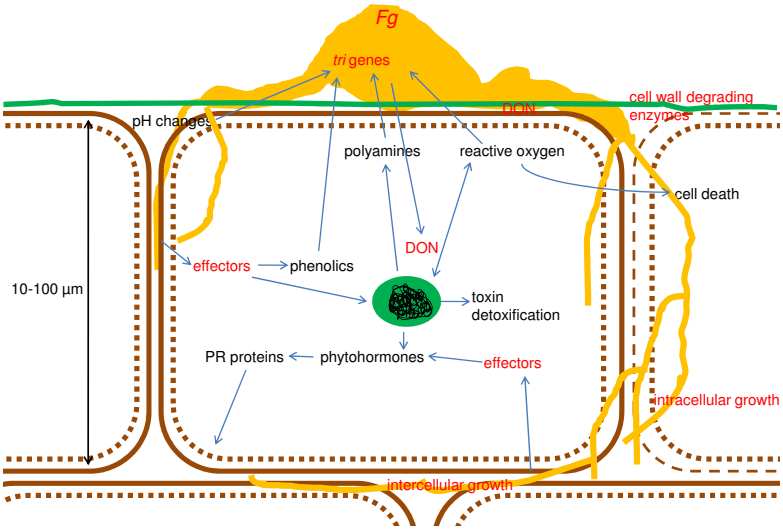


Figure 2.3: Scheme of *F. graminearum* (*Fg*)::wheat interaction. *Fusarium* produces cell wall degrading enzymes to penetrate the plant cell and effectors such as toxins (in red). *tri* gene expression is an important step during pathogenesis and production of mycotoxins, e.g. deoxynivalenol (DON). The plant responds to infection by producing pathogenesis-related (PR) proteins, reactive oxygen species, phenolics and phytohormones involved in cellular detoxification (according to (Kazan et al., 2012), modified).

2.2 Metabolic pathways involved in plant::pathogen interactions

Plants have evolved complex mechanisms to defend from pathogens, e.g. by the formation of pathogen-related (PR) proteins or oxidative burst. The majority of metabolites involved in defense response are organism specific secondary metabolites. Some of them are constitutively synthesized, others induced by biotic or abiotic stress. In case of toxic metabolites, they are frequently compartmentalised within vacuoles or other specialised cellular compartments to avoid self-toxicity. Enzymatic hydrolysis followed by vacuole disruption activates the defense-related metabolites (Du Fall and Solomon, 2011). In addition, phytoalexins, such as benzoxazanoids (e.g. DIMBOA, DIBOA), which are synthesized via shikimate pathway in the plastids, accumulate in the apoplasts.
For communication between plant and pathogen, volatiles play a crucial role. Volatiles are released by plants and can consist of inhibitory intermediates, self-regulators (hormones), attractants to herbivores or insects or important signals during pathogenesis. Either, they are external signaling molecules which contribute to the attractiveness of the flower to pollinators or seed dispersers. Healthy plants release these volatiles in the atmosphere, but abiotic stress, wounding, attacks by herbivores or infections by pathogens change expression pattern and target course. Or, plants emit volatiles from roots, leaves, fruits and flowers. These compounds are internal signals for defense and modulators of levels of systemic acquired resistance (SAR) to diseases and abiotic stress. Volatiles are mainly considered to be products of secondary metabolism and involve e.g. phytohormones like ethylene, methyl salicylate, methyl jasmonate and green leaf volatiles (GLVs, e.g. *cis*-3-hexenal, *cis*-3-hexen-1-ol, *cis*-3-hexenyl acetate) (Rowan, 2011). The production of VOCs is affected by abiotic conditions like nitrogen content of the soil, water availability and circadian rhythym (Schulze et al., 2006).

2.2.1 Signaling cascades during plant defense

Mechanical damage of the cell membrane, herbivore or pathogen attack induce a signaling cascade leading to the biosynthesis of phytohormones 12-oxophytodienoic acid (OPDA), jasmonic acid, salicylic acid and ethylene, see figure 2.4. N-acetylglutamines lead to in influx of Ca^{2+}-ions via receptor-mediated membrane depolarisation. This actives phospholipase A2 (PL), which releases linolenic acid from phospholipids. Lipoxygenase (LOX) uses linolenic acid as a substrate for 13-hydroperoxy-octadecatrienoic acid, which is cyclised via an instable allenoxide to OPDA. Linolenic acid itself was reported to inhibit mycelial growth of *F. graminearum* under in vitro conditions (Kumaraswamy et al., 2011a).
The biosynthesis of OPDA takes place in the chloroplasts, the following biosynthesis of *cis*-jasmonic acid is located in the peroxisomes. *cis*-Jasmonic acid is enzymatically transformed to the less bioactive *trans*-jasmonic acid. Salicylic acid, resulting from shikimate pathway via phenylalanine, see figure 2.5, is coordinated with the biosynthesis of jasmonic acid (Schulze et al., 2006). Salicylic acid and jasmonic acid show antagonistic activities whereas ethylene can induce or repress the activity of jasmonic acid. Salicylic acid primarily impacts in plant defense after pathogen attack which exhibit a biotrophic phase, whereas jasmonic acid is an important regulator of plant defense against necrotrophic pathogens. In *Arabidopsis* the nonexpressor of PR genes 1 (*NPR1*) is a key regulator in salicylic acid related signaling reponse (Makandar et al., 2010). NPR1 and salicylic acid per se might not have antimicrobial activity but in addition to basal defense they induce systemic acquired resistance (SAR) in uninfected plant parts after fungal infection. Although jasmonic acid and *NPR1* are coordinated during induced systemic resistance (ISR), where salicylic acid is not required, jasmonic acid can also antagonize signaling of *NPR1*.
Glucose oxidase induces the production of H_2O_2 to convert unsaturated fatty acids to fatty acid hydroperoxides, which are required for the biosynthesis of other oxylipins than OPDA or jasmonic acid (Schulze et al., 2006).
The largest group of secondary metabolites, which were shown to be related with defense responses or resistance mechanisms to biotic or abiotic stress, are synthesized via shikimate, chorismate and in the following by one of the amino acids tyrosin, phenylalanine or tryptophan (primary metabolism), see figure 2.5. Besides the three amino acids, which are used for the biosynthesis of a large variety of secondary metabolites with aromatic ring structures, *p*-amino- and *p*-hydroxybenzoate are synthesized in the shikimate pathway. The biosynthesis of aromatic compounds via the shikimate pathway is found in bacteria, fungi and plants. For monogastric animals, the essential amino acids phenylalanine and tryptophan are consumed in their diet and tyrosine is derived from phenylalanine (Herrmann, 1995). Phenylalanine is the precursor for cinnamic acid, which is in return a precursor for the biosynthesis of salicylic acid and phenylpropanoids. Phenylpropanoids originate from deamination of phenylalanine via phenylalanine ammonia lyase (Dixon and Paiva, 1995). It is thought that the molecular basis for the protective action of phenylpropanoids in plants are the antioxidant and free radical scavenging properties (Korkina, 2007), as well as their antimicrobial activity (Bollina et al., 2011). Phenylpropanoids are precursors for the cell wall biosynthesis (lignin biosynthesis), the synthesis of coumarins and flavonoids (Dixon and Paiva, 1995) and important for the cell wall fortification (Whetten and Sederoff, 1995). Figure 2.6 gives an overview of stress-induced phenylpropanoids dependent on the inducer. p-Coumaric acid and sinapic acid were reported to significantly

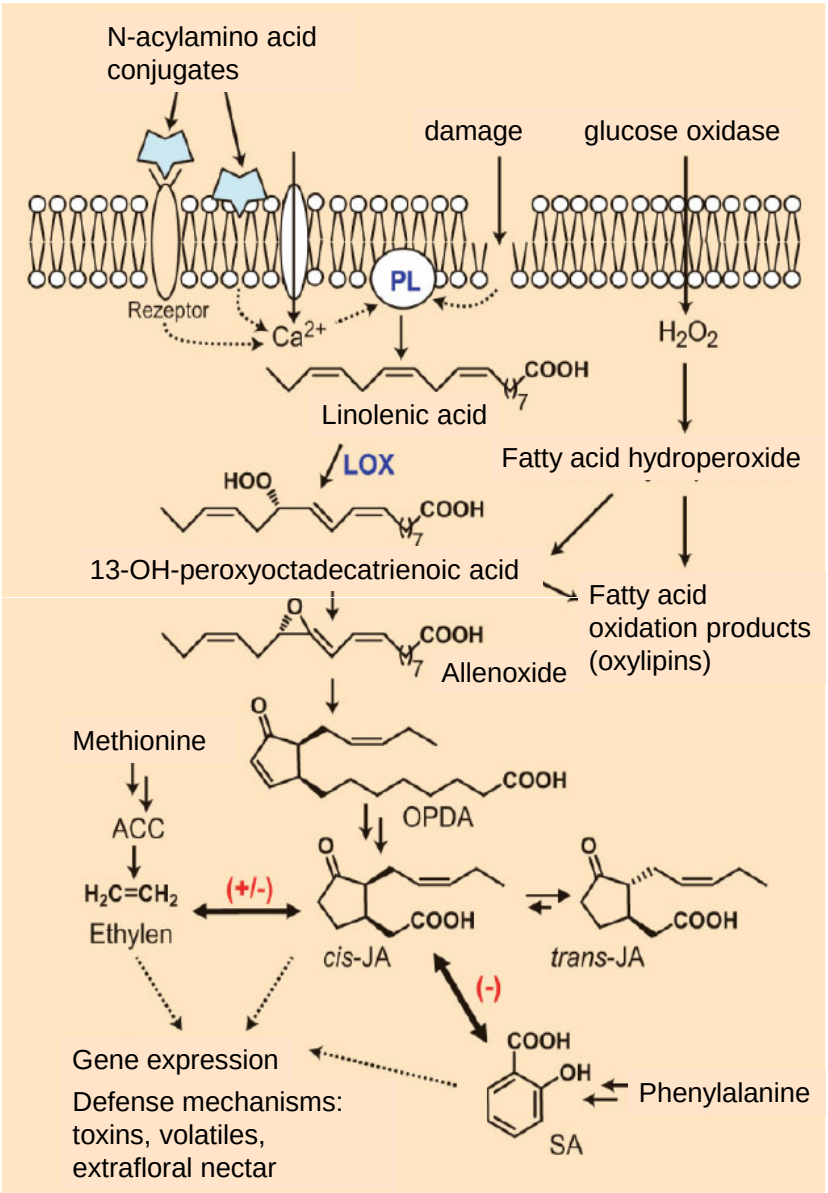


Figure 2.4: Signaling cascade during plant defense (Schulze et al., 2006). JA, jasmonic acid; LOX, lipoxygenase; OPDA, 12-oxophytodienoic acid; PL, phospholipase A2; SA, salicylic acid;

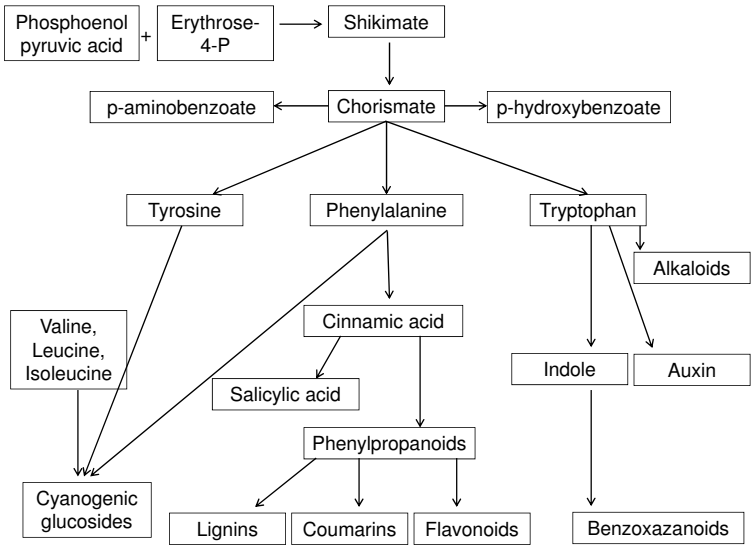


Figure 2.5: Biosynthesis of secondary metabolites, which play an important role plant::pathogen interactions, starting with the shikimate pathway (primary metabolism), based on Du Fall and Solomon (2011); Herrmann (1995).

reduce the biomass of *F. graminearum* under in vitro conditions (Kumaraswamy et al., 2011a; Bollina et al., 2010).
Tyrosine is used for the synthesis of cyanogenic glucosides, whereas tryptophan is required for the biosynthesis of alkaloids, auxin (indole-3-acetic acid) and indole. Phytoalexins, such as benzoxazinoids, are derived from indole.
The mechanisms of pathogenicity and host resistance in FHB disease are largely unclear Li and Yen (2008). Mycotoxins, such as DON, surely play an important role in disease development, but studies failed to establish DON as the causal agent of FHB Miedaner et al. (2004). Furthermore, there is no efficient chemical or biological treatment to date. There are few wheat lines resistant to FHB, such as Sumai 3 Kolb et al. (2001). The timing and strength of the defense response define the resistance level (Ding et al., 2011). So far, no gene or pathway is specified as a key player in FHB resistance. Related to FHB resistance were five wheat PR proteins, the NPR1 protein and several other enzymes, such as peroxidase. Jasmonic acid and ethylene signaling pathways were found to be involved in FHB resistance in Sumai 3, whereas salicylic acid, the PR genes of systemic acquired resistance (SAR) and active oxygen species (AOS) did not contribute to wheat defense response Li and Yen (2008). In contrast, Ding et al. (2011) reported that genes and proteins involved in salicylic acid, jasmonic acid, ethylene, calcium ions, phosphatidic acid, reactive oxygen species production, radical scavenging, antimicrobial compound synthesis, detoxification and cell wall fortification were significantly affected by *F. graminearum* infection. In this publication, it was demonstrated that resistance is a biphasic signaling event of coordinated defense related pathways. Genes related with salicylic acid and Ca^{2+} activation were expressed early after inoculation (3 hours), whereas genes involved in jasmonic acid and ethylene signaling were activated 12 hours after inoculation.
The complexity of pathways and networks in FHB disease make it hard to pin down key genes or metabolites. FHB resistance is a quantitative genetic trait involving multiple genes and pathways (Li and Yen, 2008). Therefore, the use of near isogenic lines (NILs) to study single quantitative trait loci (QTLs) is of great use. In addition, single gene

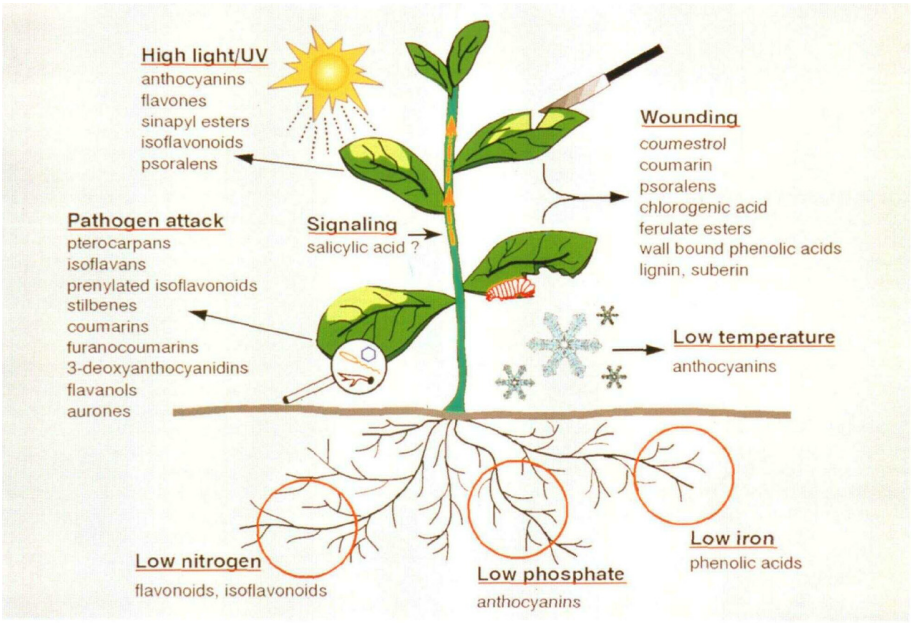


Figure 2.6: Stress-induced phenylpropanoids (Dixon and Paiva, 1995).

deletion in *Fusarium* helps to determine key genes and metabolites for pathogenesis. It was believed that *tri5* gene, encoding trichodiene synthase, is responsible for mycotoxin biosynthesis only, but Chen et al. (2011) revealed that *tri5* gene deletion in *F. graminearum* caused widespread changes in primary metabolism. A reduced amount of amino acids, choline and inositols was found in $\Delta tri5$ mutants. Interestingly, decreases in tyrosine, phenylalanine and tryptophan also indicate alterations in the shikimate pathway and therefore in the production of important secondary metabolites. Choline and inositols, which are important components of the cell membrane phospholipids, could alter cell integrity (Chen et al., 2011). In this thesis the effect of *tri5* gene deletion on volatile sesquiterpenes was studied.

2.2.2 Ethylene biosynthesis

Ethylene is produced by plants in large amounts during ripening as a growth regulator, as a stress hormone or during induced plant defense response against pathogens coordinated with jasmonate (Glick, 2005). Thus, it is one of the key volatiles which are of interest to researchers when studying plant::pathogen interactions. It is synthesized from methionine via S-adenosyl-methionine and 1-aminocylopropane-1-carboxylic acid (ACC) in the yang cycle, see figure 2.7.
In bacteria, ACC deaminases were described to be used for carbon and nitrogen generation (Thibodeaux and Liu, 2011). Saleem et al. (2007) reported the inhibitory effect of ethylene on plant root growth. Hence, microorganisms have evolved mechanisms to reduce the amount of volatile ethylene to adjust ethylene production and its inhibitory effect on plant growth. In plant growth promoting rhizobacteria (PGPR) bacterial ACC deaminases are found. They reduce the amount of ethylene by conversion of ethylene's precursor ACC to 2-ketobutyric acid and ammonia Glick (2005). Mainly enzymes which influence the concentration of S-adenosylmethionine or ACC, namely S-adenosylmethionine hydrolase, S-adenosylmethionine decarboxylase, ACC synthase, ACC deaminase and ACC oxidase, were shown to effectively reduce the concentration of ethylene without affecting the plant

physiology negatively.
In addition, Saleem et al. (2007) reported that PGPRs equipped with ACC deaminase showed antagonistic activities against *Fusarium* spp. Interestingly, also pathogenic bacteria were described to have ACC deaminases. Their role during pathogenesis is unclear, but Saleem et al. (2007) concluded that ACC deaminases could induce disease tolerance in plant by reducing the amount of ethylene during infection.
In *F. graminearum* three genes encoding for ACC synthases and two genes encoding for ACC deaminases were detected during genome sequencing (Cuomo et al., 2007). Their role during pathogenesis is unclear. Glick et al. (2007) reported an apparent paradox regarding ethylene level during environmental stress. Ethylene was suggested to both alleviate and repress some effects of pathogen infection. Induced by environmental stress, ethylene concentration was low in the beginning, which activated transcription of plant defense genes. Later a much higher ethylene level was detected which caused adverse reactions in the plant, such as senescence, chlorosis and abscission. This paradoxical effect could be used by *Fusarium* to enhance disease tolerance by the plant, followed by induced senescence and chlorosis. This effect could explain the presence of conflictive genes for ACC synthase and ACC deaminase in the *Fusarium* genome, the first synthesizing ACC from S-adenosylmethionine, the second degrading ACC to 2-ketobutyric acid and ammonium.

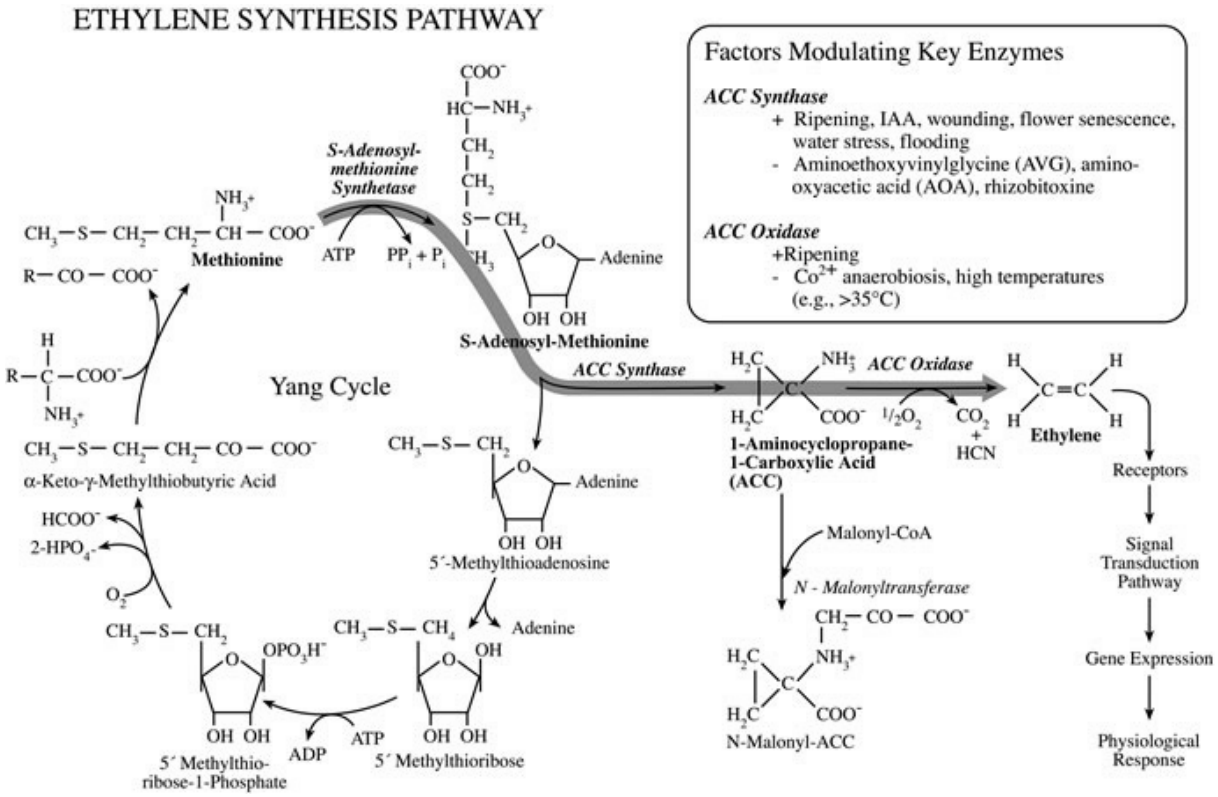


Figure 2.7: Ethylene biosynthesis via Yang cycle (from http://www.exonpress.com/EXONPRESS/Fig4_42.htm). FA, fatty acid; HOO, hydroperoxy

2.2.3 Fatty acid degradation for the biosynthesis of C6 GLVs (in plants) and C8-compounds (in fungi)

Fatty acids are a source of many volatile compounds for plants, mainly aldehydes, but also alcohols, acids and hydrocarbons (Combet et al., 2006). Alkanals, alkenals are most commonly produced from fatty acids, while heptanal, nonanal and decanal have the highest proportion of the oleic acid derivatives. Free acids can be the result of fungal lipase activity. The formation of methyl ketones is usually associated with fungal activity, which convert free fatty acid to corresponding alkan-2-ones and subsequently to alkan-2-ols (Jelen and Wasowicz, 1998). This is a possible source of 2-octanone, 2-nonanone. 3,5-octadien-2-one has been identified as a product of linolenic acid oxidation (Bogdan et al., 2012). The presence of styrene is also related to fungal metabolism and was detected before in *Penicillium*-infected barley samples (Wilkins and Scholl, 1989). Trimethylbenzene was identified as a metabolite of fungi of the genus *Fusarium* (Jelen and Wasowicz, 1998).
Apart from ethylene, jasmonate and C6 GLVs trigger jasmonate-dependent defense response. Kumar Choudhary et al. (2008) described the augmented defense expression upon stress treatment by C6 GLVs, but not their direct involvement in defense mechanisms of the plant. Wenda-Piesik (2011) reported the emission of 1.6 ng GLVs per 1 ng terpenoids in *Fusarium* infected wheat ears, suggesting the GLVs to play an important role in defense mechanisms to biotic stress. Piesik et al. (2011a) detected 6 GLVs (*cis*-3-hexenal, *trans*-2-hexenal, *cis*-3-hexen-1-ol, *trans*-2-hexen-1-ol, *cis*-3-hexen-1-ylacetate, 1-hexylacetate), five terpenes (β-pinene, β-myrcene, *cis*-ocimene, linalool, β-caryophyllene) and shikimic acid pathway derivatives (benzyl acetate, methyl salicylate, indole) in *Fusarium*-infected maize samples. The VOC emission of infected maize plants was higher for all of these metabolites than for the controls. Ton et al. (2007) and Baldwin et al. (2006) proposed that GLVs and terpenoids can stimulate plant response in uninjured neighbouring maize plants (priming), which was shown by Piesik et al. (2011a) for healthy plants even 3 m away from an infected plant.
Combet et al. (2006) reported that linoleic acid is the main substrate for the biosynthesis of C6 GLVs in plants and for C8-compounds in fungi, see figure 2.8. In plants, linoleic acid is used to synthesize hexanal, *cis*-3-hexenal and *cis*-3-nonenal via lipoxygenase and hydroperoxide lyases. Alcohol dehydrogenase, is subsequently responsible for the production of other C6 GLVs, including *cis*-3-hexenol, *trans*-2-hexenal and *cis*-3-hexenyl acetate. In fungi, C8-compounds, like 1-octen-3-ol, are synthesized from linoleic acid via haem dioxygenase and hydroperoxidase, which catalyse stereospecific oxidation of linoleic and linolenic acids to 10-monohydroperoxides. 10-Monohydroperoxide then undergoes decomposition to 1-octen-3-ol, 3-octanol, 1-octen-3-one and 3-octanone. Both pathways, seem to play an important role in plant::fungus interaction because they were found in *Fusarium*-infected wheat ears (Piesik et al., 2011b; Eifler et al., 2011).

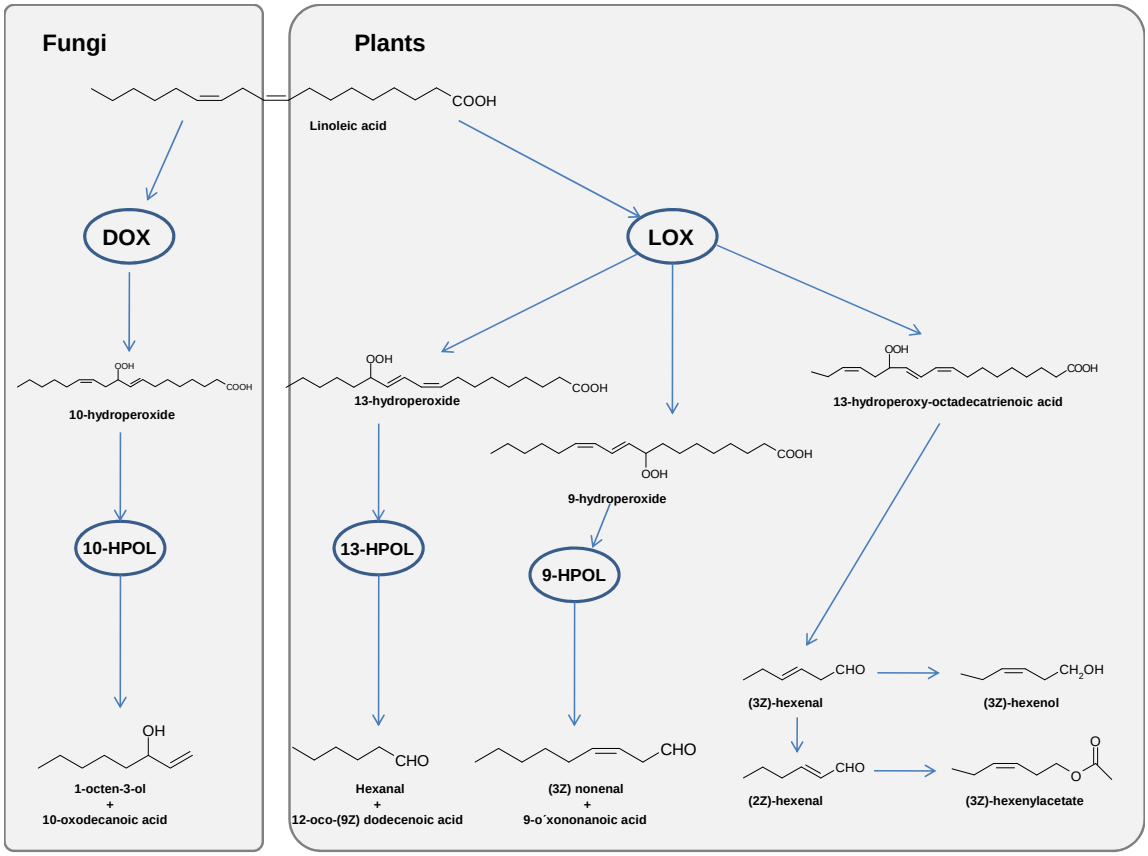


Figure 2.8: Biosynthesis of fungal C8-compound 1-octen-3-ol from linoleic acid in comparison to synthesis of hexanal and 3-(*cis*)-nonenal *in planta*, based on (Combet et al., 2006; Schulze et al., 2006). DOX, haem dioxygenase; LOX, lipoxygenase; HPOL, hydroperoxide lyase.

2.2.4 Terpenoids

The terpene biosynthesis is one of the key pathways concerning interactions with micoorganisms or plants in fungi (Kramer and Abraham, 2012). The majority of terpene compounds are hydrocarbons with hundreds of carbon skeletons. The terpenes and terpenoids (term used for oxygenated terpenes in this thesis, in general sometimes used as overall name for terpenes and their oxygenated products) are the largest and most diverse class of secondary metabolites.
The synthesis of mono-, sesqui- and diterpenes from plants is attributed to two main pathways (Dewick, 2002a): the mevalonate pathway, being used by fungi for the biosynthesis of terpenes, and the non-mevalonate pathway, 2-C-methyl-D-erythritol-4-phosphate (MEP) or 1-deoxy-D-xylulose-5-phosphate (DOXP). Plants use both pathways for biosynthesis of isopentenylpyrophosphate (IPP) and dimethylallylpyrophosphate (DMAPP), which are the precursors for geranylpyrophosphate (GPP) and farnesylpyrophosphate (FPP). IPP can be exchanged between cytosol and plastids. In the mevalonate pathway, three units acetyl CoA are needed for the synthesis of mevalonate. Mevalonate is then converted to IPP, which is in an equilibrium with DMAPP. In the non-mevalonate pathway, glycerinaldehyde-3-phosphate and pyruvate, both originating from glycolysis, are converted to 1-deoxy-D-xylulose-5-phosphate (DOXP). DOXP is subsequently rearranged to 2-C-methyl-erythritol-4-phosphate (MEP) which leads to IPP and DMAPP formation. The mevalonate pathway is located in the cytosol and is required for the biosynthesis of sesquiterpenes, whereas the non-mevalonate pathway, which takes place in the chloroplasts, is used to synthesize mono- and diterpenes, see figure 2.9. It is unknown whether FPP can also be exchanged between compartments.
The group of sesquiterpenes include several metabolites unique to fungi, e.g. trichodiene (Jelen et al., 1997). They are synthesized from five-carbon isoprene units (C_5H_8) in the mevalonate pathway only, starting with acetyl CoA (Kramer and Abraham, 2012). The precursors DMAPP and IPP are essential in fungi to synthesize all kinds of terpenes and terpenoids. IPP is consecutively added to the substrate DMAPP to elongate the terpene-skeleton. The monoterpenes (C_{10}) are the end products of GPP. The sesquiterpenes (C_{15}) are synthesized via FPP. The diterpenes (C_{20}) result from geranylgeranylpyrophosphate (GGPP).
Terpenes are the largest group of natural products (Dewick, 2002a). Their structural diversity is very high and therefore, terpene synthases are multi-product enzymes, in contrast to 'classical' enzymes which usually yield in one main product (Schulze et al., 2006). Terpene synthases are able to use one precursor, usually a carbocation, for the production of a high number of differing, but structurally related end products.
Sesquiterpene synthases catalyze the cyclization of the universal acyclic precursor FPP to form over 300 known monocyclic, bicyclic and tricyclic sesquiterpenes with a wide variety of structures and stereochemistry (Kramer and Abraham, 2012). In the following, the high diversity of mono- and sesquiterpenes synthesized from only few carbocations is shown *in planta*. However, the same carbocations and similar end products are produced by the fungus.

Starting with GPP, a hand full of different monoterpene skeletons are synthesized by cyclisation and isomerization. GPP is dephosphorylated in a first step before ionization of the skeleton to different carbocations, see figure 2.10. The carbocations can undergo a range of cyclizations, hydride shifts and rearrangements before reaction is terminated by deprotonation or water capture (Degenhardt et al., 2009). The formation of (A) cyclic monoterpenes requires a linalyl intermediate which is capable of cyclization. α-Terpinyl

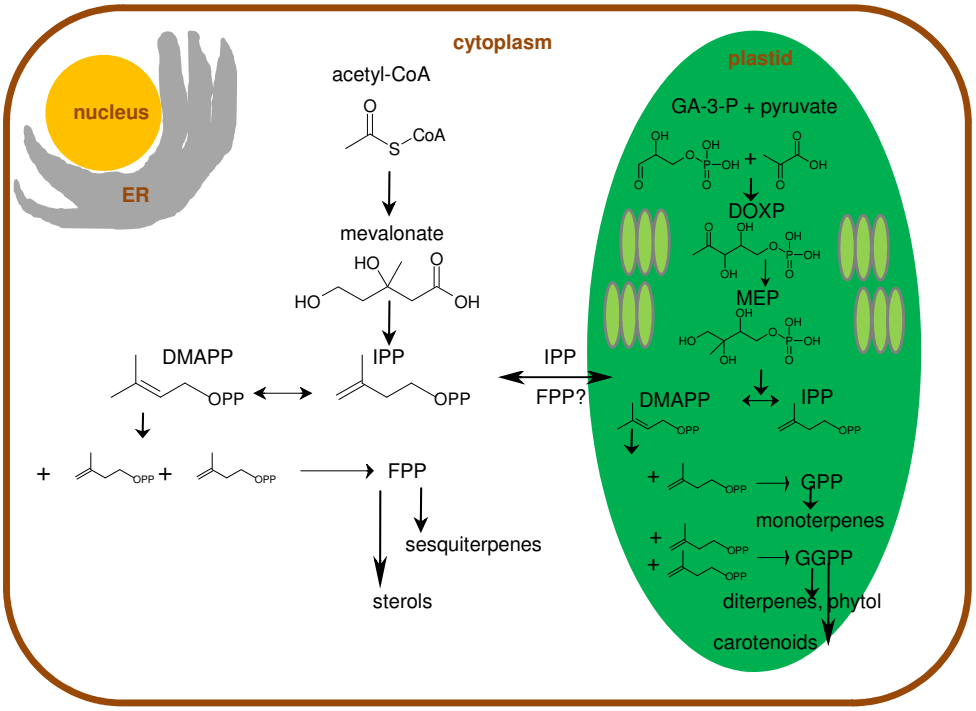


Figure 2.9: Terpene biosynthesis *in planta* by two pathways: mevalonate pathway in cytoplasm, non-mevalonate (MEP/DOXP) pathway in plastids, based on Lichtenthaler (1999). DMAPP, dimethylallylpyrophosphate; IPP, isopentenylpyrophosphate; GPP, geranylpyrophosphate; FPP, farnesylpyrophosphate; GGPP, geranylgeranylpyrophosphate; GA-3-P, glycerinaldehyde-3-phosphate; DOXP,1-deoxy-D-xylulose-5-phosphate; MEP, 2-C-methyl-erythritol-4-phosphate;

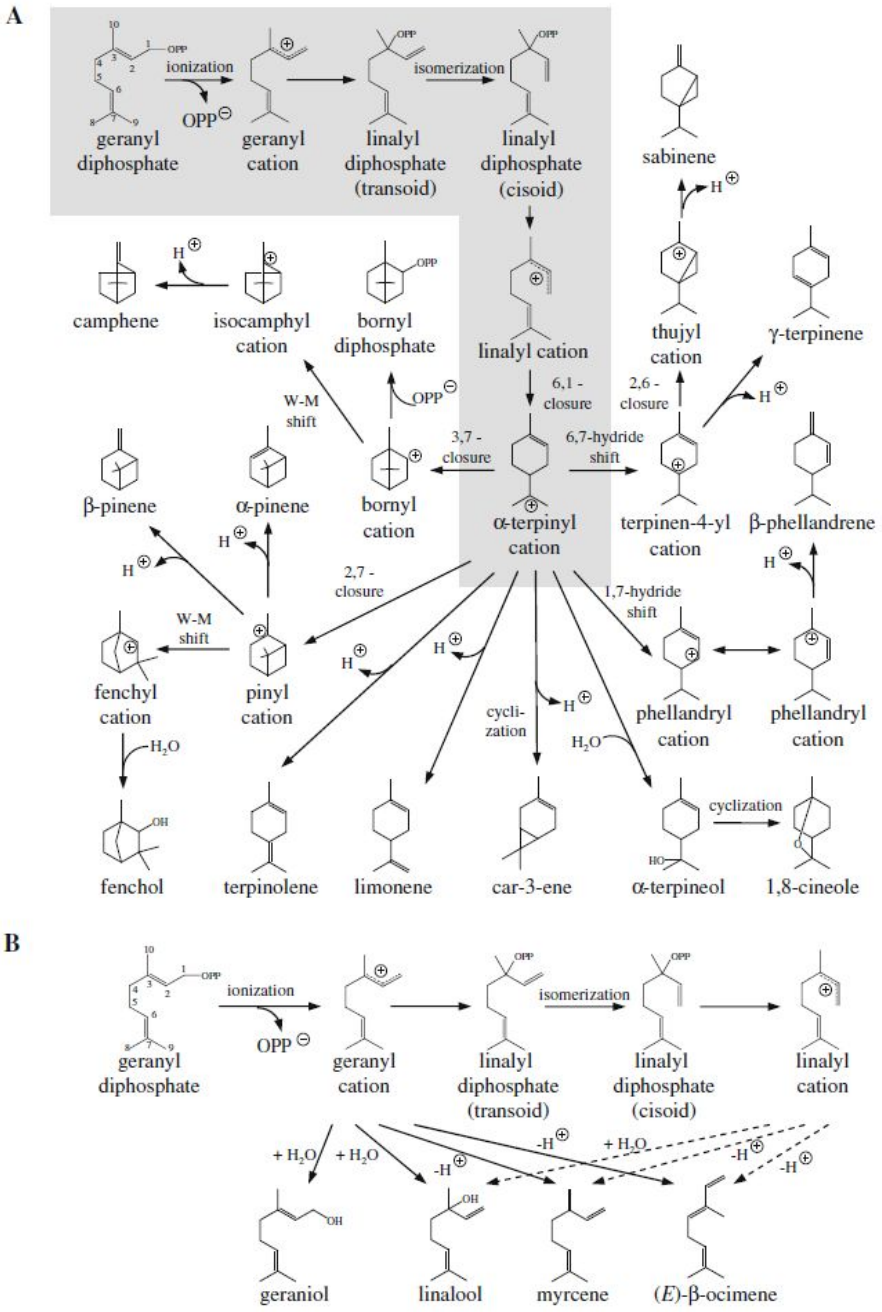


Figure 2.10: Biosynthesis of monoterpenes *in planta* (Degenhardt et al., 2009). The mechanisms of (A) cyclic and (B) acyclic monoterpene synthases are depicted separately. The route to the central α-terpinyl intermediate is highlighted in grey.

cation is the initial cyclisation product which is further used for secondary cyclizations. The formation of (B) acyclic monoterpenes, e.g. linalool, myrcene and (E)-β-ocimene might proceed either via the geranyl cation or via the linalyl cation, see figure 2.10

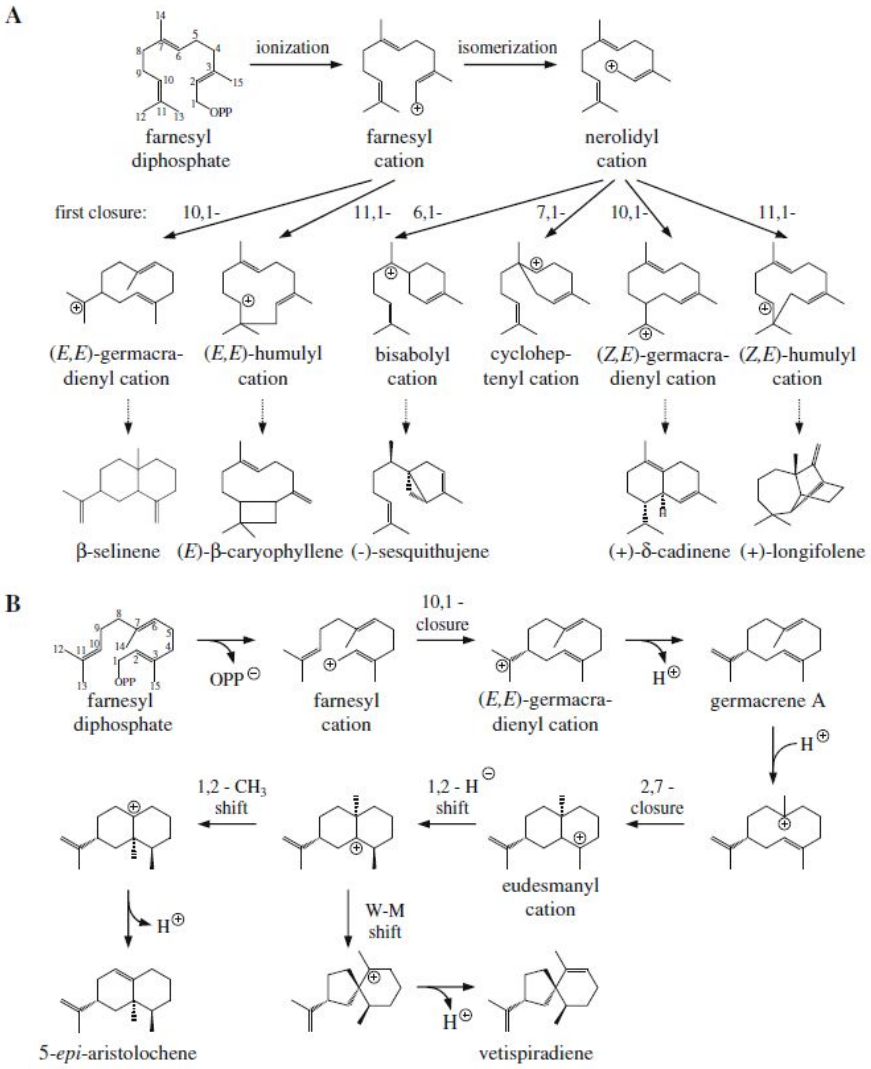


Figure 2.11: Biosynthesis of sesquiterpenes *in planta*, see (Degenhardt et al., 2009). (A) shows the variety of carbocations available from cyclisation of farnesyl and nerolidyl carbcationic intermediates. (B) shows the reactions from germacrene A intermediate to 5-epi-aristolochene by 5-epi-aristolochene synthase of *Nicotiana tabacum* in detail. W-M, Wagner-Meerwein rearrangement

FPP is used to built different sesquiterpene skeletons and intermediates by terpene cyclases. After removal of pyrophosphate, the farnesyl carbocation and its isomerization product nerolidyl intermediate (A) are available for the synthesis of a huge variety of sesquiterpenes, see figure 2.11. Steps following the initial cyclization of a carbocation, which include secondary cyclization, deprotonation to a neutral intermediate, hydride shifts, methyl shifts and Wagner–Meerwein rearrangements, are shown exemplary for (B) the 5-epi-aristolochene synthase. The reaction mechanism of this enzyme proceeds via a neutral intermediate, germacrene A, that is re-protonated to form the bicyclic reaction products.

Most of the terpene cyclases require magnesium as cofactor for proper folding (Vedula et al., 2007). The carbocation is channelled to other carbocation or sesquiterpene in-

termediates and often several products. The most important intermediate sesquiterpene skeleton and branching point for several other sesquiterpene types is farnesane (Kramer and Abraham, 2012). Interestingly, the enzymes are highly conserved between plant and fungi, however the pattern of terpenes and terpenoids produced is variable.

FPP is also used to synthesize triterpenes like squalene. Squalene is required for biosynthesis of sterols, like ergosterol in fungi, see figure 2.12. Sterols are the major component of membranes to regulate permeability and serve as precursors to steroid hormones involved in the sexual reproduction of some fungi (Combet et al., 2006). Ergosterol is the dominant membrane sterol in eumycota fungi and not found in bacteria, plants and animals. In Ascomycetes (e.g. *Fusarium*) 70-100% of the total sterol content is attributed to ergosterol (Ruzicka et al., 2000). Ergosterol is one of the principal components in fungal membranes and often serves as biomarker for the presence of fungal biomass (Ruzicka et al., 2000; Schnuerer et al., 1999).

Phytosterols, β-sitosterol or stigmasterol, see figure 2.13, are widely distributed over the plant kingdom. The two phytosterols are known to be involved in cell membrane stability, like the fungal sterols. Both phytosterols were reported to be synthesized via the mevalonate pathway in the cytosol or via the DOXP pathway in the chloroplasts (Lichtenthaler, 1999; Kongduang et al., 2008). According to Griebel and Zeier (2010) the β-sitosterol content in the host plant does not change after a pathogen attack. This makes sitosterol a suitable biomarker for the plant biomass. Thus, the ratio ergosterol/sitosterol was used as the ratio for fungal over plant biomass in this thesis to determine the infection degree in maize cultivars after *Fusarium* infection.

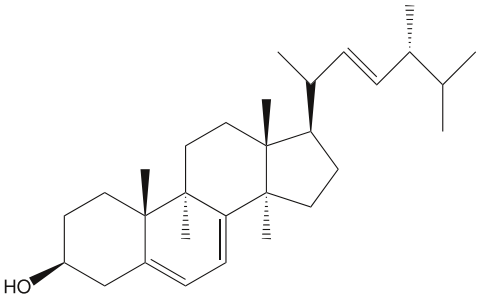


Figure 2.12: The structure of the fungal sterol ergosterol.

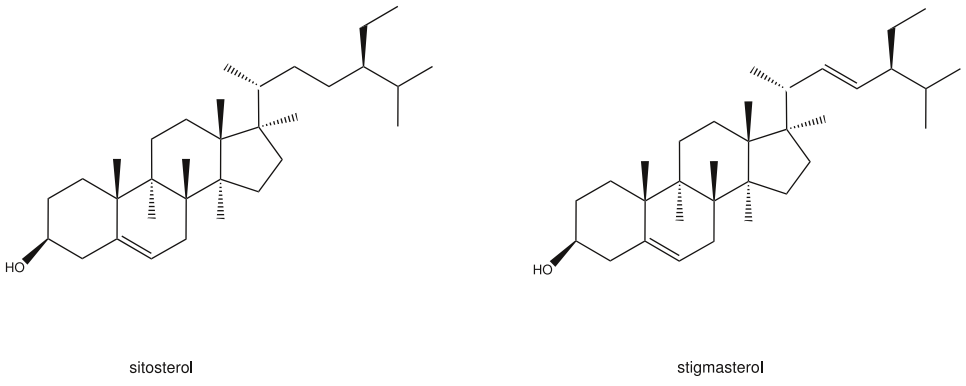


Figure 2.13: The structure of the phyotsterols β-sitosterol and stigmasterol.

Chapter 3

GC-MS in the field of metabolomics: state of the art

Numerous volatile and non-volatile, polar metabolites including those, which were shown to play important roles during plant defense against pathogens, can be measured with GC-MS systems. The general workflow comprises of quenching and grinding, sample extraction, enrichment and clean-up dependent on the substance class under investigation. For non-volatile metabolites, derivatisation is required prior to measurement. In this thesis, HS SPME was employed for the determination of volatiles, whereas a two-step derivatisation (methoxymation and silylation) was accustomed for the determination of non-volatile, polar metabolites. The separation and detection of the compounds was achieved using GC-MS instruments (equipped with a single quadrupole). For data processing the software tool MetaboliteDetector was used. Data evaluation was performed with R using uni- and multivariate statistics. In the following, the techniques and tools mentioned are critically reviewed with respect to metabolomics applications.

3.1 Metabolomics

Metabolomics is a term coined at the end of the 1990s (Oliver et al., 1998), followed-on from transcriptomics and proteomics. The concept entailed the analyses of metabolite composition of biological materials and aimed to be fully complementary with the other, potentially unbiased or non-targeted '-omics' approaches. Metabolomics - per definition - is specifically focused on the smaller metabolites, while the larger organic polymers ($>$ 1500 Da) were excluded for reasons of practicability related to extraction and detection limitations (Hall, 2006). Concentrating on these smaller molecules enabled the focus to be directed to global sets of compounds involved in both primary as well as secondary metabolism. Primary metabolites are commonly encountered in all organisms, whereas secondary metabolites are more specific to single organisms or groups of organisms. According to Du Fall and Solomon (2011), a wide range of plant's secondary metabolites are induced under certain conditions, such as pathogen attack. They function is often unknown but it is assumed that they play a role for the well-being of the producer (Dewick, 2002a).

Low molecular weight metabolites of primary and secondary metabolism are intermediates or the end products of a huge network of metabolic pathways and represent the activities of cell metabolic and regulatory processes (Goodacre, 2005). As such, they advertise the response of biological systems to a variety of genetical and environmental

responses. Detecting and monitoring such global sets of metabolites quickly enables us to assess changes in the distribution and concentration of a broad range of potentially biochemically-unrelated compounds and such strategies therefore permit the detection of pertubations at multiple levels of organization: from cell to whole organism (Goodacre, 2005).
The ultimate goal of a global metabolomics experiment is to measure the changes in the abundances of metabolites arising from natural fluctuations and external perturbations. As absolute quantification of all metabolites is not feasible, metabolite abundances are generally compared for biological samples of different conditions. Researchers try to gain a helicopter view of metabolism at a specific point in time, in a chosen tissue, obtained either under control or experimental conditions(Hall, 2006). Samples taken at appropiate time intervals, can then also introduce a degree of dynamics to the system. However, even by employing a number of extraction, separation and detection conditions the view gained will never be truly holistic as some element of bias will always be involved. This results from a failure to extract or detect certain compounds that perhaps are unstable or that have chemical or physico-chemical properties unsuited to the methodologies chosen. Nevertheless, optimization of data collection and mining strategies have greatly enhanced our capacity to expand our biochemical knowledge of biological organisms (Hall, 2006).

Table 3.1: Terms and definitions in the field of metabolomics (Fiehn, 2002; Hall, 2006; Dettmer et al., 2007)

Term	Definition
Metabolomics	An unbiased detection and quantification of all (low molecular weight) metabolites of a biological system, organism, tissue, or cell under well-defined conditions.
Metabolic profiling	Detection (and quantification) of a predefined set of metabolites, e.g. belonging to a substance class, to a particular pathway or measureable with a specific analytical technique.
Metabolic fingerprinting	A recognizable chemical pattern for individual samples where qualitative and quantitative assignment of metabolites is not necessary. Comparison of samples with respect to their metabolite pattern.
Target analysis	Identification and precise quantification of metabolites by using authentic standards, optimized extraction and dedicated separation/detection techniques.

The definitions for metabolomics and three important techniques, namely metabolic profiling, metabolic fingerprinting and target analysis are given in table 3.1. Metabolic fingerprinting is generally referred to the use machine output as a potentially recognizable chemical pattern, specific to an individual sample. Generally no attempt is made to annotate the metabolites present. These unique fingerprints are usually the starting point for comparative metabolomics where the researcher wishes to compare up to several hundred extracts in order to quickly assess the degree of variation and often selects the most divergent samples or genotypes for more detailed study (Fiehn, 2002).
Metabolic profiling can be defined empirically as the (semi-)quantitative analysis of a set of metabolites or derivative products (identified, annotated or unknown) of a sample. It

aims at the identification and quantification of a limited number of components, which are often chosen on the basis of multivariate analysis or on molecular relationships based upon molecular pathways (Hall, 2006).
Target analysis is performed for selected metabolites for which sample preparation, separation and detection were optimized by the use of authentic standards. The metabolites were identified and calibration was achieved with standards to allow proper qualitative and quantitative analysis. Target analysis cannot be performed for the whole metabolome because of lack of authentic standards. Furthermore, the metabolome shows high complexity and a high dynamic range (metabolite concentrations cover several ordners of magnitude typically $nmolL^{-1}$ to $mmolL^{-1}$). In general, single analytical instruments are used for metabolic profiling. The combination of different profiling methods typically derived from the use of more than one analytical instrument, give a more complete picture of the metabolome of the organism under investigation and thus is one step closer to 'true' metabolomics (Lei et al., 2011; Moco et al., 2007).

The strategies of the metabolomics research group at the Center for Analytical Chemistry for the SFB *Fusarium* project include metabolic profiling and target analysis. Aims of this thesis were the development and establishment of GC-MS based methods for metabolic profiling as well as their applications to biological samples. For the analysis of volatile primary and secondary metabolites, HS SPME GC-MS was employed. For the detection of non-volatile, polar (mainly primary) metabolites two-step derivatisation GC-MS was used, which is a key method in the field of metabolomics due to its robustness, reliability and low costs. The GC-MS methods will be reviewed in the following, starting with the sample preparation.

3.2 Sample preparation for metabolic profiling using GC-MS

Sample preparation includes quenching, grinding (homogenisation), extraction, enrichment and clean-up prior to measurement. In case of the non-volatile metabolites, derivatisation is necessary for GC-MS analysis. For target analysis, the analyte(s) of interest are separated from matrix compounds to increase the sensitivity of the method with this cleaning step. Sample preparation in metabolomics studies is supposed to lead to no (or necligible) changes in the metabolome. Therefore, samples are 'frozen' in a metabolic state and kept in this state. Influences which lead to sample degradation must be avoided. Rapid sampling and quenching are prerequisite to stop all enzymatic activities. Thawing must be prevented. Proteins, which could be used for proteomics studies, are removed during extraction. There is a lack of certified reference materials which would be of great help to monitor the efficiency of sample preparation and the integrity of samples. Compromises regarding extraction efficiency, accuracy and precision have to be taken and are accepted in the field of metabolomics in order to study as many metabolites possible with one analytical method (Fiehn, 2008). There is not one single technique which leads to extraction of the whole metabolome. Different methods need to be used complementary to get an overview of part of the metabolome by metabolic profiling of some chemical classes.

3.2.1 Quenching and grinding

Regarding metabolomics experiments, samples, independent from the following analytical method, must be rapidly harvested and enzymatic activity must be arrested, which is called quenching. Álvarez-Sánchez *et al.* (Álvarez Sánchez et al., 2010) listed the four most important requirements for quenching: (a) inactivation of the metabolism should be faster than metabolic changes occurring in the sample, (b) sample integrity must be carefully preserved,(c) the procedure should not induce significant variations in chemical and physical properties or in the concentration of metabolites, (d) the sample should be amenable to subsequent steps of the analytical process.
Rapid handling of samples is prerequisite for successful quenching. This is usually achieved by changing pH, either to high (e.g. by adding KOH, NaOH) or low pH (e.g. by adding perchloric, hydrochloric, trichloroacetic acid), or by instantly freezing the samples (at least -20 °C) (Álvarez Sánchez et al., 2010). Shock freezing can be performed with liquid nitrogen or prechilled organic solvents. Methanol is the preferred solvent in quenching solutions because it is miscible with water, possesses a low freezing point and the methanol:water mixture is not very viscous. Quenching with cold methanol is very common when the non-volatile metabolites are investigated. Liquid nitrogen can be used for quenching to study the volatile and non-volatile metabolome. It is a simple and fast method, feasible for lab and field samples and similar results are achieved to the cold-methanol quenching technique (Álvarez Sánchez et al., 2010). Further advantages of liquid nitrogen are its easy removal from the samples and its extremely low temperature (-196 °C). Thus, wound responses are suppressed immediately and the loss of compounds by adsorption to cell walls is avoided.
For homogenisation of the sample material mortar and pestle, ball mill, vibration mill, UltraTurrax, ultrasonic probe or thermomixer can be employed (Álvarez Sánchez et al., 2010). The homogenizer needs to be cooled (e.g. with liquid nitrogen) to avoid thawing of the sample.
In this thesis quenching of plant samples was performed with liquid nitrogen. The frozen samples were ground in prechilled beakers in a ball mill (Retsch mill). The resulting fine powder was weighed-in HS-vials or 1.5 mL tubes for sample preparation. All steps were performed using liquid nitrogen and short handling times to avoid thawing. The samples were stored at -80 °C until used for further sample preparation (HS SPME or liquid extraction and derivatisation).

3.2.2 Extraction of volatile metabolites

Volatiles were classified according to their boiling points in 1989 by the World Health Organisation (WHO, 1989), see table 3.2. There are different extraction techniques for these

Table 3.2: Classification of volatiles. Polar metabolites appear at the higher end of the boiling point range.

category	description	abbreviation	boiling-point range (°C)
1	very volatile (gaseous) organic compounds	VVOC	<0 to 50-100
2	volatile organic compounds	VOC	50-100 to 240-260
3	semivolatile organic compounds	SVOC	240-260 to 380-400
4	organic compounds associated with particulate (organic) matter	POM	>380

volatiles dependent on their classification and the degree of information which should be gathered. In general, there are two basic approaches to collect volatiles: direct sampling of volatiles from the headspace (HS) above a biological sample or solvent extraction followed by purification to remove non-volatile matrices which could interfere during instrumental analysis (Rowan, 2011). Static and dynamic HS sampling methods are commonly used to collect and concentrate volatiles from the HS of a biological sample (Tholl et al., 2006; Qualley and Dudareva, 2009). Therefore, a defined volume of gas sample can be removed with a gas-tight syringe or with a sorbent-coated fiber and introduced into the GC. Using a defined volume of gas sample, VVOCs and VOCs can be measured, but enrichment is not possible with this method, hence, sensitivity could be a limiting factor.
Direct-reading instruments for HS sampling (e.g. E-nose (Presicce et al., 2006)) are portable and make it possible to detect rapid changes of VOCs on the field. The output signal does not separate volatiles from each other. Therefore, no identification is achieved.
Sorbent materials are used for collection and enrichment of VOCs and SVOCs and further separation using GC instrumentation. Qualitative and quantitative information is received. Using MS coupled to a GC instrument, structural information is gained which helps to classify metabolites of which no reference standard is available. The sorbent materials cover a broad range of substances. Adsorption to charcoal or Tenax is most wide spread technique to collect VVOCs and VOCs. In the field of VVOCs, the purge-and-trap system is the commonly used method to extract volatiles from liquids.
SPME is currently the most widely used technique for static extraction of the HS above samples or for direct immersion of liquids (liquid sampling of volatiles) and shall be described in detail in the following.
Dynamic headspace sampling can be performed as 'push-and pull' technique or 'closed-loop stripping' method. Unlike SPME where the whole sample is desorbed in the GC instrument, dynamic methods collect larger quantities of volatiles because continuous streams of air allow the sorbent material to act as a trap. For 'push-and-pull' systems a unidirectional flow of air acts as mobile phase to carry volatiles to a sorbent (trapping) material like Tenax or charcoal. In 'closed-loop stripping' systems this airflow is recirculating between sample and trapping material, whereas for 'push-and-pull' systems a vacuum pump forces air to flow over the sample and trap as often as wished.

About Solid-Phase Microextraction

Solid-phase microextraction (SPME) methods are generally used to extract VOCs as well as several VVOCs and SVOCs. The volatility, the boiling point, the vapor pressure of volatiles and the interaction with the fiber coating determine if SPME is suitable for extraction. The higher the vapor pressure, the easier the substance evaporates from solid or liquid state (the more volatile). SPME is a passive technique for volatile collection which requires a certain amount of volatiles to successfully bind to the coating, but it also reduces background impurities. There are a number of coatings commercially available, which can be coated in different ways to the fiber material. To name some techniques, the coating can be on the outside of a fiber (classic SPME), in the fiber (in-tube sorptive extraction, called SPDE - solid phase dynamic extraction), on stir bars (SBSE - stir bar sorptive extraction, or the extension of this technique HSSE - headspace sorptive extraction, so called 'Twister' technique), on membranes (SMSE - silicone membrane sorptive extraction) (Rubiolo et al., 2010).
For high-throuput applications, an autosampler and heating stations of fiber and sample are required. The most widely used technique of sampling using classical SPME consists

of exposing an absorbent/adsorbent (coated on a fiber) to a sample for a determined amount of time (Pawliszyn, 2009a). Pawliszyn introduced the term and method SPME in the early 1990s. It has been more and more widely used in sample preparation, especially since the first fibers became commercially available from Supelco in 1993. SPME has been used routinely in combination with GC and GC-MS and successfully applied to a wide variety of compounds, especially for the extraction of volatile and semi-volatile organic compounds from environmental, biological and food samples. Lately, SPME was also introduced for direct coupling with HPLC in order to analyze weakly volatile or thermally labile compounds not amenable to GC. Still, SPME-LC has lagged behind SPME-GC. Some of the reasons are the small number of commercially available SPME sorbents for LC applications, the lack of commercially available interfacing options, long equilibration times and lack of automation (Pawliszyn, 2009a).
Classic SPME is based on the partitioning of analytes between a coated fiber, a sample and its environment. The coated fiber consists of a small fused-silica, stableflex or metal core coated with a thin layer of sorbent material (liquid or solid, or a combination). If the coating of the fiber is a liquid film, the analytes are absorbed by paritioning, depending on the thickness of the film. There is no competition for binding sites between analytes, whereas fibers with solid coating, which is a porous material, extract analytes by adsorption and have therefore limited active sites. During exposure to the vapour phase above a sample (headspace) or during direct immersion in a solution, a mass-transfer process begins, driven by the second law of thermodynamics, according to which the chemical potential of each compound should be equal throughout the system once equilibrium is reached. The amount of analyte sorbed to the SPME fiber is determined by the following equation (Pawliszyn, 2009b):

$$n = \frac{C_0 \cdot V_f \cdot V_s \cdot K}{K \cdot V_f + V_s} \tag{3.1}$$

C_0 is the initial concentration of the analyte in the matrix, V is the volume of fiber (f), headspace (h) or sample (s). n depends on the sample concentration and volume of the fiber coating and HS, and the partition coefficients K_{fh} and K_{hs}. The partition coefficient K is equal to $K_{fh} * K_{hs}$. It is controlled by the partition coefficient K_{fh}, between the fiber coating and the headspace, and the partition coefficient K_{hs}, between the HS and the sample. The partition coefficients can be determined reproducibly if the system is in equilibrium. Still, SPME is commonly used before equilibrium for all metabolites is reached. The duration for extraction is usually determined experimentally. It is often a compromise between the number of extracted metabolites, an acceptable intensity and the consumed time. However, SPME is sensitive to experimental conditions because quantitative recovery is not achieved.
After the coated fiber has been exposed to the sample for a given period, it is inserted into the injection port of a chromatograph in order to release the analytes. In GC, this is achieved by thermal desorption whereas in HPLC it is accomplished by dissolution in the elution solvent which is then injected into the HPLC system. Although a very small amount of analyte is transferred via the SPME fiber, it is sufficient to give rise to a reproducible analytical signal with modern detectors.
On the one hand, SPME has several advantages over traditional extraction methods. It is rapid, simple, solvent-free, sensitive, compatible with different analytical separation and detection techniques, and it is small in size and therefore convenient for designing portable field devices. It avoids losses that can occur during extraction, concentration and clean-up steps in traditional sample preparation. HS extraction is a very clean and

convenient way to extract volatiles from a living organism without major disturbances. It is very well suited to extract little amounts of volatiles from living organisms, e.g. fungal or plant cultures.
On the other hand, relatively low maximum operating temperatures (240-280 °C) are recommended to prolong the fiber's reliable usage. SPME fibers themselves are quite fragile and swell in organic solvent (which greatly restricts use in HPLC). In addition, low extraction efficiencies are found in certain cases, particularly for VVOCs or thermally labile analytes (Nerin et al., 2009). One of the main drawbacks of the technique is the limited number of active binding sites on solid fiber coatings commercially available. A list of commercially available fiber coatings is given in table 3.3. Absorption is described as the partitioning of analytes into a liquid film (fiber coating) (Shirey and Mindrup, 2000). The thickness of the coating and the size of the analyte define the retainment and release of individual analytes from the absorbent. In contrast, adsorption is the physical interaction of the fiber coating with a volatile compound (Shirey and Mindrup, 2000). Adsorbent materials are generally solid materials containing pores and high surface areas. Therefore, there is only a limited number of binding sites available for adsorbent materials. The DVB/CAR/PDMS coated fiber was designed to extend the molecular weight range of analytes, which are extracted with a single fiber, by placin a DVB layer suspended in PDMS over a layer of Carboxen suspended in PDMS, see figure 3.1. Each coating layer has less capacity compared to fibers with single coatings, but the total fiber capacity should be the same. CAR-PDMS is the strong adsorbent because it has less meso- and macropores than DVB-PDMS, but more micropores. The porosity of DVB-PDMS, the weaker adsorbent, is higher due to the presence of more meso- and macropores (Shirey, 2009). The adsorbents were layered in a way that the larger analytes (large in size) first contact the weaker adsorbent (DVB-PDMS), whereas the smaller analytes migrate through this layer to the strong adsorbent, CAR-PDMS. The smaller analytes travel much faster through the DVB-PDMS layer, whereas the larger molecules require more time. Hence, it is ensured that small and large molecules adsorb and desorb in comparable amounts over extraction time. This makes the DVB/CAR/PDMS coated fiber an interesting tool for metabolomics applications to analyze polar and nonpolar metabolites over a mass range of m/z 35-270.

Table 3.3: Commercially availabe SPME fiber coatings for GC-MS (Shirey, 2009)

type of coating	**extraction mechanisms**	**polarity**
7 μm polydimethylsiloxane (PDMS)	absorbent	nonpolar
30 μm PDMS	absorbent	nonpolar
100 μm PDMS	absorbent	nonpolar
85 μm polyacrylate (PA)	absorbent	polar
60 μm polyethylenglycol (PEG), Carbowax	absorbent	polar
65 μm Carbowax (CW)-divinylbenzene (DVB)	absorbent	polar
15 μm Carbopack Z-PDMS	adsorbent	bipolar
65 μm PDMS-DVB	adsorbent	bipolar
85 μm Carboxen (CAR)-PDMS	adsorbent	bipolar
55 μm/30 μm DBV/CAR/PDMS	adsorbent	bipolar

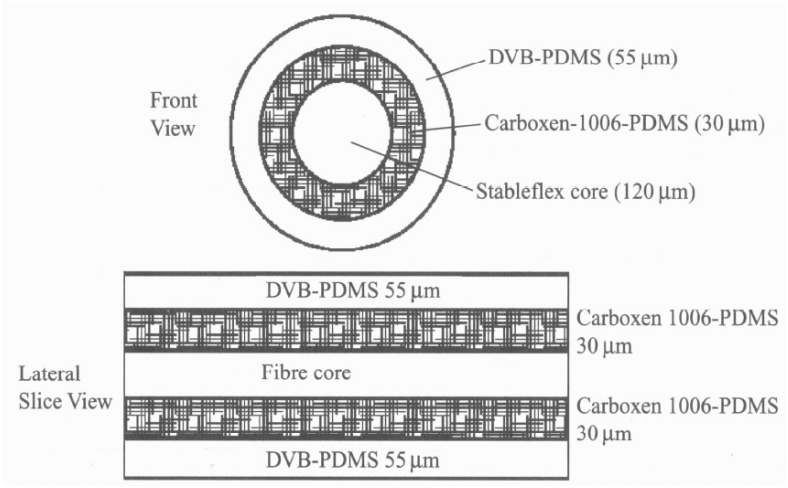


Figure 3.1: Front view and slice view from layers of DVB/CAR/PDMS coated fiber (from (Shirey, 2009)).

3.2.3 Extraction of non-volatile, polar (mainly primary) metabolites

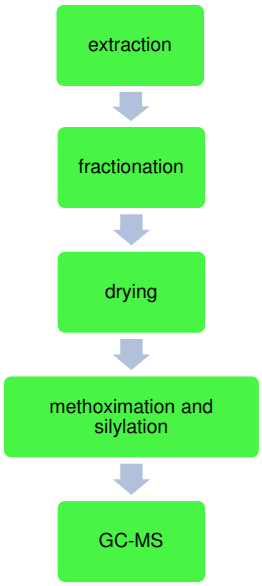


Figure 3.2: Sample preparation from extraction to GC-MS measurement for metabolic profiling of non-volatile, polar metabolites.

After having discussed the sample preparation steps for volatiles using HS SPME, I want to go on with the sample preparation of non-volatile, polar (mainly primary) metabolites revealing a different part of the metabolome. The sample preparation defines which part of the metabolome is studied. For the study of non-volatile, polar metabolites, sample preparation includes more steps than for volatiles using HS SPME, see figure 3.2. The ground plant powder is extracted with solvents (e.g. a one-phase solution of CH_3OH:H_2O:$CHCl_3$).

Usually, the addition of water leads to a two-phase solution. Subsequently, the polar phase (methanol-water phase) is separated from the lipophilic phase (chloroform). This fractionation is a separation step for polar and apolar metabolites. It reduces the number of metabolites which are studied. The dried residue of an aliquot e.g. from the polar phase is methoximated and silylated. Methoximation and silylation are necessary to get a defined number of derivatives for polar metabolites which are non-volatile and appear in different forms (e.g. linear and cyclic form of sugars in solution). Using online-derivatisation, the autosampler performs methoximation and silylation steps, which reduces degradation of derivatives and deviations between samples due to varying storage times. The individual steps of the sample preparation for non-volatile, polar metabolites will be discussed in the following.

Liquid extraction, fractionation and drying

Besides clean, solvent-free extraction methods like HS SPME, organic solvents are required for the extraction of analytes from complex matrices. Solvents of varying polarities, with varying pH-values and solvent mixtures are in use, e.g. perchloric acid, acetonitrile/water, methanol/water, and methanol/chloroform/water (Lin et al., 2007; Weckwerth, 2006). A number of publications are commonly adapted and applied to metabolomics studies using aqueous methanol for the extraction of polar metabolites from plant material, see table 3.4. The protocols published vary in the ratio of solvents, in the order of addition of single solvents and the extraction temperature. All of them use organic solvents to open and remove all cell debris and receive the soluble, intracellular metabolites. The residual cellular pellet can be further used for proteomics analysis or RNA analysis (Weckwerth et al., 2004). For there is no standardisation regarding the sample preparation for metabolomics studies, the researcher needs to test the appropiate protocols published for their suitability for his application.
Generally, a one-phase solution of aqueous methanol (with or without chloroform) is used for the first step of extraction, then water (and chloroform) is (are) added to achieve the formation of two separate liquid phases, the polar (aqueous methanol) and lipophilic (chloroform) phase. Extraction is performed at 60 °C (Gullberg et al., 2004), 70 °C (Lisec et al., 2006; Roessner et al., 2000) or on ice (Weckwerth et al., 2004; Morgenthal et al., 2006)). De Vos et al. (2007) used acidified, aqueous methanol and an ultrasonication bath at 22 °C for the extraction of polar metabolites only and subsequent HR LC-MS analysis. Methanol stops all enzymatic activity which should be independent from extraction temperature, but was reported to be enhanced at higher temperatures (Roessner et al., 2000). It is also in use as cold-methanol solution for quenching, see 3.2.1.
The addition of chloroform is necessary if polar metabolites should be separated from nonpolar metabolites. The fractionation can be used to analyse the polar and nonpolar phase individually or as a cleaning step for polar metabolites. The number of metabolites per analysis is reduced leading to less background signals, higher chromatographic resolution for metabolites of each polarity and more reproducible results. Besides these advantages for each substance class, only a smaller part of the metabolome is covered within a single measurement. Each phase needs to be measured individually resulting in a higher number of measurements. In this thesis, only metabolites present in the polar fraction were studied, therefore, only protocols for the analysis of polar metabolites were discussed here.
Subsequent to extraction, samples are dried for successful, reproducible methoximation

and silylation. Water residues could lead to degradation of samples if residual enzymes are reactivated and to unstable silyl-derivatives because ethers are sensitive to water. Drying can be achieved by applying a gentle stream of nitrogen, in a vacuum concentrator (Lisec et al., 2006; Gullberg et al., 2004) or by lyophylisation (Allwood et al., 2009).

Table 3.4: Selected extraction and derivatisation protocols published in the field of metabolomics. FAME, fatty acid methlester; IS, internal standard; speed vac, vacuum concentrator; US, ultrasonication bath;

	De Vos et al. (2007)	Lisec et al. (2006)	Allwood et al. (2009)	Gullberg et al. (2004)	Weckwerth et al. (2004)	Roessner et al. (2000)
sample matrix	plants	plants	melon varities	Arabidopsis	Arabidopsis	potato
sample amount [mg]	100	100	100	22	30-100	50-100
vol. extraction [ml]	0.4	3.55	2	1	2	1.4
extraction solvents (v/v)	MeOH:H_2O 3:1	MeOH:$CHCl_3$:H_2O 2:1:2	MeOH:$CHCl_3$:H_2O 2.5:1:1	MeOH:$CHCl_3$:H_2O 6:2:2	MeOH:$CHCl_3$:H_2O 2.5:1:1	MeOH:H_2O 1:1
extraction procedure, fractionation	0.4 mL MeOH:H_2O 3:1 + 0.1% HCOOH, US 15 min, centrifuged, filtered	1.4 mL MeOH, shake at 70°C 10 min, add 750 μL $CHCl_3$ + 1.4 mL H_2O	according to (Lisec et al., 2006), 3 extracts combined	200 μL $CHCl_3$, add 800 μL MeOH:H_2O 3:1 + IS 60°C 15 min, no phase separation	2 mL MeOH:$CHCl_3$:H_2O 2.5:1:1, centrifuge, extract pellet with 1 mL MEOH:$CHCl_3$, add to supernatant, add 500 μL H_2O	1400 μL MeOH, 15 min 70°C, add 1400 μL H_2O, centrifuge at 22000 g
steps to prepare for derivatisation		150 μL of polar phase drying in speed vac	1 mL polar phase lyophilised	180 μL dry in speed vac	drying in speed vac	250-1000 μL aliquot drying in speed vac 6-16 h
MOX		40 μL 20 mg/mL in pyridine 37°C 120 min	50 μL 20 or 30 mg/mL in pyridine 30°C/40°C 90 min	30 μL 20 mg/mL 60°C 1h 22°C 17h	50 μL 20 mg/mL in pyridine 30°C 90 min	80 μL 20 mg/mL in pyridine 30°C 90 min
MSTFA		70 μL 37°C 30 min	50 μL 40°C 90 min	30 μL 60°C 60 min	80 μL 37°C 30 min	80 μL 37°C 30 min
prep prior to GC-MS				wait 1 h at 22°C		transfer 80 μL to sample vials; precondition GC-MS with 100 μL MSTFA + 10 μL alkanemix
alkanes		20 μL/mL FAME together with MSTFA	20 μL 0.6 mg/mg pyridine alkanes		30 μL alkanes (C_{10}-C_{40} odds only) together with MSTFA	40 μL fatty acid mix (10 mg/mL each in tetrahydrofuran) together with MSTFA
IS used		60 μL 0.2 mg/mL ribitol in H_2O or ^{13}C-sorbitol during extraction	0.1 mL of 10 mg/mL succinic-d4-acid, glycine-d5, malonic-d2-acid per mL of extract	15 ng/μL methyl-octadecanoate and 11 stable isotope reference compounds added to $CHCl_3$ or MeOH:H_2O buffer before extraction	50 μL 10 mg/mL ribitol in H_2O added before drying aliquots of extraction	50 μL 2 mg/mL ribitol in H_2O added with MeOH

3.2.4 Two-step derivatisation

For GC-MS measurements, the analytes must be volatile and thermally stable. Non-volatile, small metabolites can be measured with GC-MS after chemical derivatisation to increase their volatility and thermal stability. Derivatisation is usually required to reduce the polarities of the functional groups, e.g. -OH, -NH, -SH, to ease their separation by GC and to influence their mass spectral properties. The facility of silylation of these functional groups follows the order: primary alcohol > secondary alcohol > tertiary alcohol > phenol > carboxylic acid > amine > amide (Villas-Boas et al., 2011).
The protocols listed in table 3.4 are selected from plant metabolomics studies and show that different temperatures and amounts of silylation reagents are in use. For target analysis, the derivatisation time and temperature are optimized for the selected analyte(s). For metabolomics studies, a compromise of the derivatisation conditions for the metabolites under investigation must be found.
Even after optimization of derivatisation parameters, silylation can lead to artefact formation, which results in multiple peaks of the same compound (Little, 1999). Especially mono- and disaccharides (furanoses and hexoses), such as glucose ($C_6H_{12}O_6$), which appear in the linear and cyclic forms in aqueous solution, need to be stabilized prior to silylation to reduce multiple products by enolisation (Halket et al., 2005). This is achieved by the conversion of the carbonylgroup to an oxime group. Commonly used are O-alkylhydroxylamines (Dunn and Ellis, 2005), e.g. methoxylamine (Lisec et al., 2006), which transfers the carbonylgroup to a methoxime. Using methoxylamine hydrochloride (MOX), cyclisation of glucose is inhibited and the linear form is methoximated. Two isomers in *syn* (Z) and *anti* (E) form are produced, see figure 3.3.

Silylation is the most widely used derivatisation technique, besides alkylation and acylation. Frequent silylation reagents are N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA), N,O-bis-(trimethylsilyl)trifluoroacetamide (BSTFA), N,O-bis-(trimethylsilyl)-acetamide (BSA) or N-(tert-butyldimethylsilyl)-N-methyltrifluoroacetamide (MTBSTFA) which result in trimethylsilyl (TMS) or tertbutyldimethylsilyl (TBDMS) side groups. Most TMS and t-butyldimethylsilyl derivatives offer excellent thermal stability and are suitable for a wide range of applications.
MSTFA is currently one of the key derivatisation reagents in the field of metabolic profiling, although alkylation using methyl chloroformate (MCF) is gaining interest. MCF derivatives were even described to have a wider dynamic and linear range compared to TMS derivatives (Villas-Boas et al., 2011). For target analytics of phytohormones, MTBSTFA was found the more suitable silylation reagent compared to MSTFA (Birkemeyer et al., 2003). Halket *et al.* (Halket et al., 2005) reported that its derivatives are less sensitive to the hydrolytic effects of moisture than the corresponding TMS derivatives. The disadvantage of MTBSTFA is the significant increase in molecular weight after silylation, especially if multiple functional groups are present. Furthermore, complete derivatisation might be sterically hindered.
The TMS groups are much smaller in size and molecular weight. Furthermore, MSTFA itself and its reaction by-product, N-methyltrifluoroacetamide, have a high volatility (boiling point of MSTFA 131 °C, boiling point of N-methyltrifluoroacetamide 157 °C) and therefore elute very early in a chromatographic run (in many methods during solvent delay). The attachment of TMS groups to functional groups via ester bond during silylation using MSTFA is shown in figure 3.4. Fiehn *et al.* (Fiehn et al., 2000) reported that the completeness of NH derivatization of amines and amino acids was higher using MSTFA

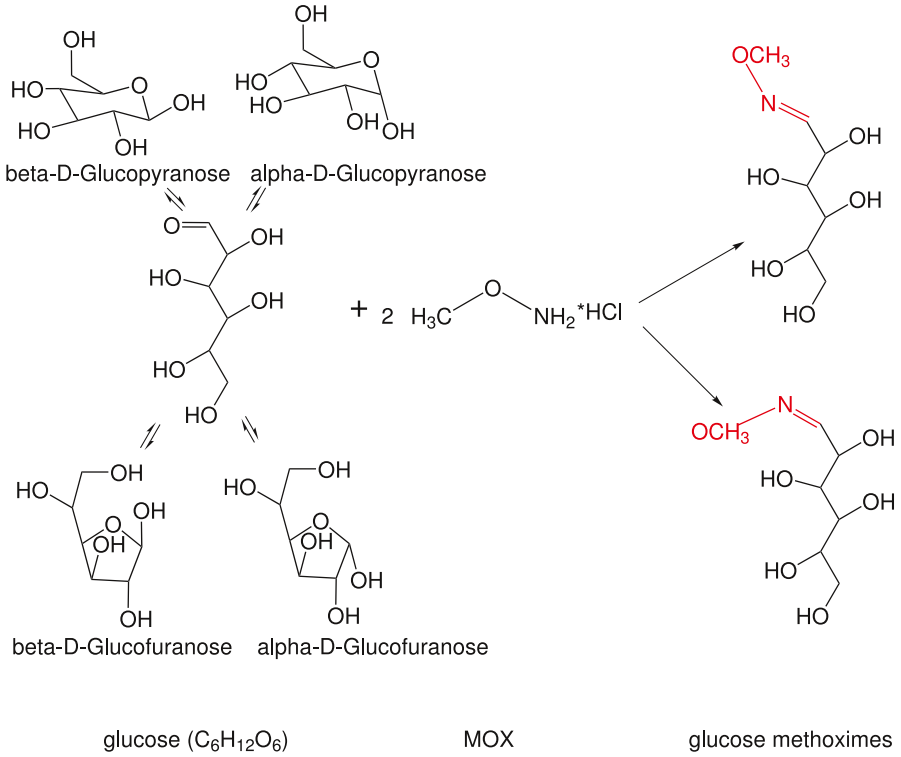


Figure 3.3: In aqueous solutions at 20 °C, D-glucose is present in form of a pyranose for more than 99 % (31-37 % α-D-glucopyranose, 64-68 % β-D-glucopyranose). The furanose form is limited to 1 % (0.5 α-D-glucofuranose, 64 % β-D-glucofuranose). The linear form is present in negligible amounts of 0.006-0.03 % (http://class.fst.ohio-state.edu/fst605/lectures/lect14.html). Transfer of the carbonylgroup to the methoxime during methoximation of the linear form D-glucose is achieved by adding methoxylamine hydrochloride in pyridine (MOX). The linear form is continuously removed from the equilibrium by methoximation and forces the cyclic forms to open to the linear form. Two isomers of glucose-methoxime in *syn* (Z) and *anti* (E) form are formed.

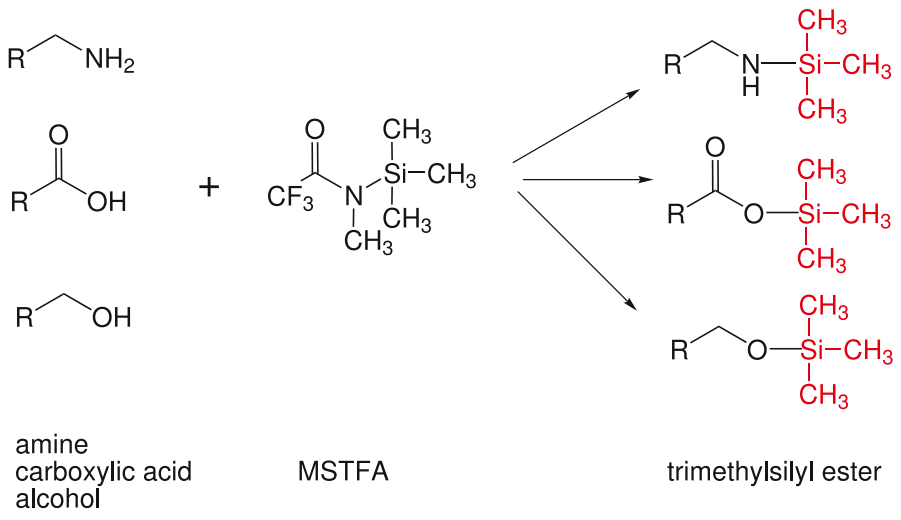


Figure 3.4: Attachment of trimethylsilyl (TMS) groups to functional groups exemplary shown for amines, carboxylic acids and alcohols during silylation using MSTFA.

in comparison to other silylation reagents. They also stated that unwanted side reactions of this derivatisation reagent were less. Still, silylation with MSTFA can result in artefact formation, which can lead to multiple peaks for the same compound or unexpected components (Little, 1999). Artefact formation can happen even under mild conditions. For example arginine loses a guanidine group during silylation with MSTFA and the resulting silylation product is ornithine-O,N,N,N-4TMS. In analogy, loss of the same guanidine group was found for agmatine, resulting in the silylation product putrescine-N,N,N,N-4TMS, see figure 3.5. TMS derivatives are hydrolytically unstable. According to Dunn

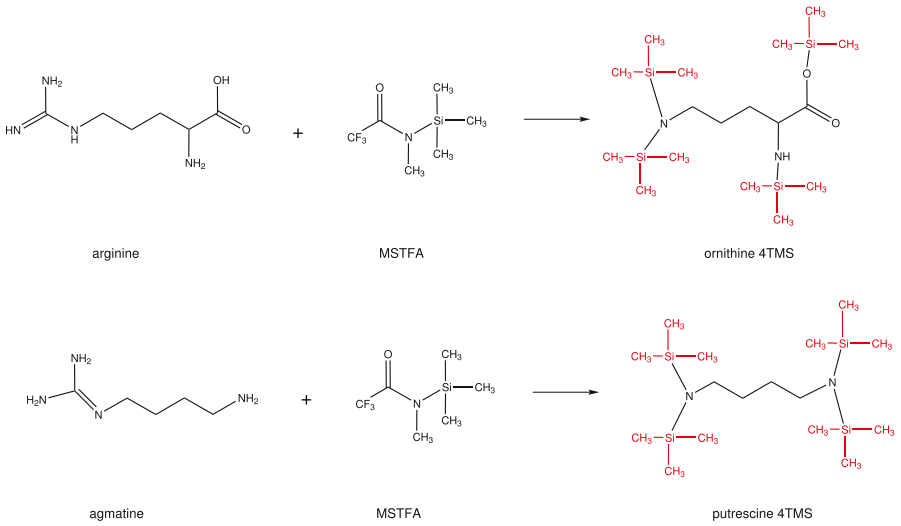


Figure 3.5: Silylation of arginine using MSTFA results in formation of ornithine 4TMS. The silylation product of agmatine is putrescine 4TMS. Both molecules lose a guanidine group during silylation.

and Ellis (2005), water can result in a breakdown of TMS esters because esterification is reversible process. Thus, the samples need to be protected from moisture. Extraction solvents must be evaporated completely before derivatization, either under a constant flow of nitrogen, in a vacuum concentrator or by lyophilisation. However, extensive sample drying can result in the loss of volatile metabolites as they can escape (Dunn and Ellis, 2005). Also the presence of excess silylation reagent helps to prevent from TMS bond breakages. The dried residues should not be kept at any temperature for longer time periods. Hence, the usage of an autosampler for online-derivatisation is a great advantage (Dunn and Ellis, 2005).

3.3 Gas chromatography-mass spectrometry (GC-MS)

3.3.1 Gas chromatography

GC-MS is considered a suitable and mature technique in the metabolomics field. In order to detect volatile metabolites originating from various living systems, GC-MS is a versatile, highly selective and sensitive detection system (Weckwerth and Morgenthal, 2005; Fiehn, 2008). It is the principal technique for separation and detection of volatile metabolites (e.g. alcohols, aldehydes, ketones, esters and terpenes). However, the technology is more broadly applicable to groups of nonvolatile, polar (mainly primary) metabolites, such as amino acids, sugars and organic acids, by converting these into volatile and thermostable compounds through chemical derivatization. These derivatized samples can then be analysed by GC-MS and detailed information on many of the primary metabolites can be

obtained in a single chromatographic run.
The instrumental setup of a GC-MS instrument with an autosampler for online-derivatisation or HS SPME is shown in figure 3.6. The autosampler consists of two injectors, which can be equipped with syringes or SPME devices. Dependent on the application, washing stations for the syringes, needle heaters for the SPME fiber, or agitators and sample trays are required. The inlet of the GC instrument consists of a heating unit which is flushed with gas (usually the mobile phase), where the glass liner is located and cleaned or replaced regularely. The inlet is closed with a septum on top, which is penetrated by the injector during injection and also needs regular replacement. The chromatographic column is introduced a few millimeters into the liner and its main part is kept on a column holder in the GC oven. The end of the column enters via heated interface the MSD. Controlling of the GC-MSD instrument can be done manually, which is usually done for maintenance, or via a personal computer (PC).
The mobile phase (carrier gas) is comprised of an inert gas e.g. helium, argon, nitro-

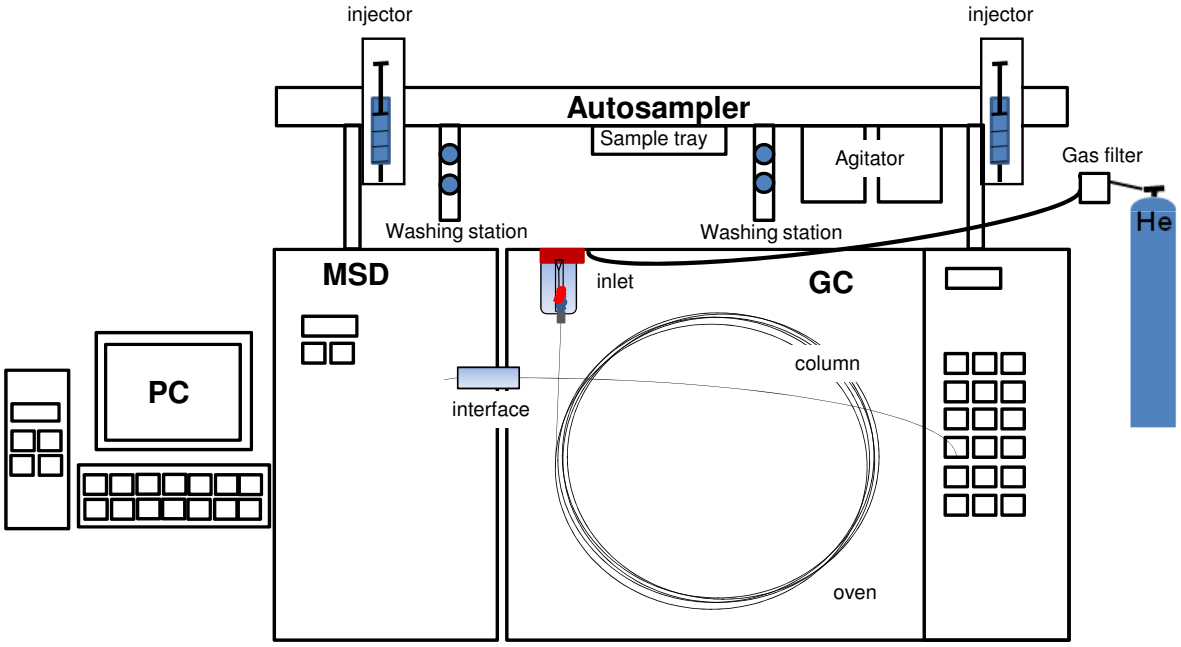


Figure 3.6: Instrumental setup of GC coupled to MSD. The autosampler contains two injectors, which can hold syringes for online-derivatisation or an SPME device for HS SPME.

gen, etc. The stationary phase can be solid or liquid. Solid stationary phases (packed columns, PLOT - porous layer open tubular) are mainly used for analyzing VVOCs. Liquid stationary phases are used to separate (volatile) organic compounds. There are two types of liquid stationary phases: liquid films (WCOT – wall-coated open tubular) and liquid stationary phase on solid carrier material (SCOT – support-coated open tubular). Most stationary phases, which are used for the separation in capillary GC consist of liquid films, which are highly viscous polymers such as polysiloxanes or polyethylenglycol. These columns are up to 100 m long (typically 25 - 60 m) and have an inner diameter from 0.15 – 1 mm.
Columns of different polarity, thickness of coating and length are commercially available by many manufacturers. Based on the application to be performed the coatings are chosen. Regarding metabolomics applications, 5 or 35% diphenyl-95% dimethyl polysiloxane column (length 30 m x inner diameter 0.25 mm x coating thickness 0.25 μm) is of general

use in metabolomics applications. The differences in the chemical and physical properties of injected analytes and their interactions with the stationary phase are the basis of the separation process. In the field of metabolomics, capillary columns (WCOTs) are routinely used. For their production, a protective coating is applied to the outer surface of fused silica capillary columns with polyimide being the most common coating material. The polyimide coating is responsible for the brownish color of fused silica capillary columns. Polyimide coated tubing often darkens after prolonged exposure to higher temperatures. The upper temperature limit of standard polyimide coated fused silica tubing is 360 °C. Stainless steel capillary columns are used for applications requiring very high column temperatures (up to 400 °C). The inner surface of fused silica tubing is chemically treated to minimize interactions of the sample with the tubing. The reagents and process used depend on the type of stationary phase being coated onto the tubing with silylation being the most widespread process. Polysiloxane columns, which are the most common stationary phases, are available in the greatest variety and are the most stable, robust and versatile columns for GC applications. Standard polysiloxanes are characterized by the repeating siloxane backbone. A 5% diphenyl-95% dimethyl polysiloxane contains 5% phenyl groups and 95% methyl groups (e.g. HP5 column). A low bleed or 'ms' version is available. These stationary phases incorporate phenyl or phenyl type groups into the backbone of the siloxane polymer and are commonly called arylenes (e.g. HP-5ms). A 5% diphenyl-95% dimethyl polysiloxane column is of general use in metabolomics applications and covers a wide range of metabolites of different polarity. However, columns with 35 % or 50% phenyl groups become more attractive.
Polyethylene glycols (PEG) are widely used as stationary phases to separate polar analytes. Polyethylene glycols stationary phases are not substituted, thus the polymer is 100% of the stated material. They are less stable, less robust and have lower maximum temperature limits than most polysiloxanes. Polyethylene glycol stationary phases are liquids under typical GC temperature conditions. There are also bonded and cross-linked stationary phases commercially available for special tasks in target analysis (e.g. chiral columns to separate D-, L-enantiomers).
There are various injection techniques available to introduce a certain amount of sample to the system: split/splitless injection, on-column injection, programmable temperature vaporizing (PTV) injection, cryo-focussing, gas-switching valves and purge-and-trap injection systems. In this thesis, split/splitless injection was used within all instruments. In splitless mode, the total amount of sample is transferred onto the column, whereas in split mode, only a defined ratio of sample is introduced to the column according to the split ratio. Split injections are very important for highly contaminated or dirty samples as well as highly concentrated samples. Injection is performed manually or as an automated process using an autosampler. The samples are introduced via syringe in liquid form (liquid injection) or in gas phase (headspace techniques) or bound to a fiber (solid phase microextraction). They are vapourized (and desorb from the fiber) due to high temperatures in the injector. The temperature in the GC oven, where the column is positioned is programmed to continuously rise temperature (temperature gradient) or keep at constant temperature (istothermal). The separation is an interaction process of the analyte with the stationary phase until it reaches the detector.
A mixture of analytes can be separated if the analytes reveal different interaction properties with the stationary phase. These interaction properties are influenced by the polarity of the stationary phase, the column length and diameter, the amount of material injected, the temperature in the GC oven, the carrier gas flow as well as the analyte's polarity, size, solubility and vapour pressure. The retention time of each analyte is dependent on its

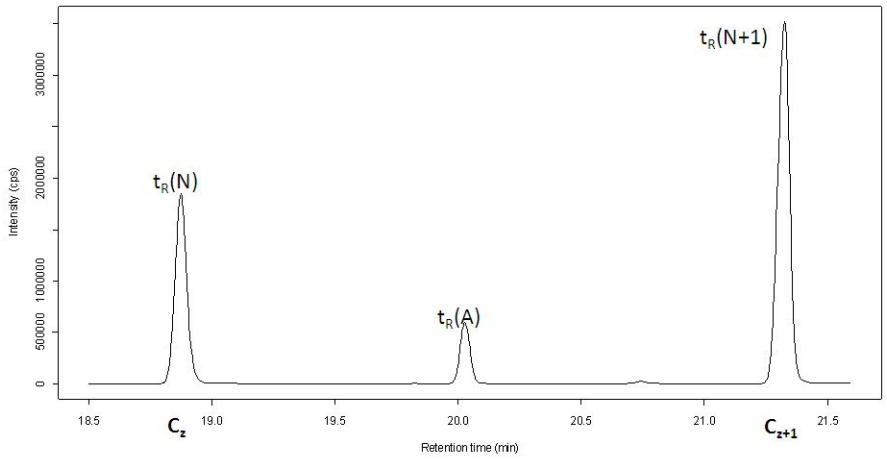


Figure 3.7: Calculation of Retention Index. c_z...number of carbon atoms of alkane eluting before analyte, c_{z+1}...number of carbon atom of alkane eluting after analyte, t_R...retention time of analyte (A), of alkane eluting before analyte (N), of alkane eluting after analyte (N+1)

interaction with the stationary phase.

Retention index

The retention time (t_R) is characteristic for each analyte and therefore one parameter for identification. The retention time itself varies slightly between consecutive measurements, but to a higher extent if different instrumental parameters are applied or a different instrument is used. To overcome these deviations and to make retention time information comparable between sequences and instruments, the retention time can be normalized to the retention index (RI). For metabolomics applications, RIs are generally calculated using alkane standards or fatty acid methylesters which are measured simultaneously with the samples. The main reason for this normalization is the comparability of retention indices independent from instrument, day of measurement or analyst. The values differ dependent on the polarity of stationary phase, but do not differ between manufacturers or coating thicknesses in theory. Kováts (1958) and Van den Dool and Kratz (1963) developed retention index systems which are valid and widely used today. Kováts' system (see equation 3.2) is based on isothermal GC separation, whereas Van den Dool and Kratz (see equation 3.3) developed a system for GC programs with temperature gradients. The only difference in the calculation of the KI and the LTPRI is that the logarithmic retention times $\log(t_R)$ of the analyte (A) and the alkanes eluting before (N) and after the analyte (N+1) are used for the calculation of the KI, but not for the LTPRI. In both equations, the number of carbon atoms of the alkane eluting before the analyte (c_z) is added to the ratio of the retention time of the analyte minus the retention time of the alkane eluting before the analyte divided by the difference in retention time between the alkanes eluting before and after the analyte and multiplied by 100.

$$KI = 100 \cdot (c_z + \frac{\log(t_R(A)) - \log(t_R(N))}{\log(t_R(N+1)) - \log(t_R(N))}) \tag{3.2}$$

$$LTPRI = 100 \cdot (c_z + \frac{t_R(A) - t_R(N)}{t_R(N+1) - t_R(N)}) \tag{3.3}$$

The reference substances for RI calculation in the field of metabolomics are generally alkanes (Lisec et al., 2006) or fatty acid methyl esters (FAME) (Allwood et al., 2009), see table 3.4. In this thesis, alkane standards were measured in parallel to samples for RI calculation according to Van den Dool and Kratz because temperature gradients were used for chromatographic separation. The calculated RIs can be compared with database entries of commercially available databases and other analysts.

Although capillary GC shows a very good chromatographic separation efficiency, which means a high number of theoretical plates and high peak capacity, coelution of analytes in biological samples cannot be avoided in metabolic profiling. Many metabolites show the same chromatographic behaviour and similar physical properties because they are structurally very closely related or even have the same sum formula, e.g. sugars ($C_6H_{12}O_6$) or sugar alcohols ($C_5H_{12}O_5$). GC-GC might help in this respect to separate these metabolites in a second dimension. Another challenge in metabolomics is to prevent or at least minimise the risk of producing artefacts. It is very important to test which substances originate from the GC inlet and column. The bleeding of substances from these devices cannot be completely avoided. They contain for example a high number of silicium and can therefore easily be excluded from the results list (e.g. m/z 207, 221, 281, 355). Furthermore, artefacts might be produced from sample constituents during injection into the hot GC liner. This can either be tested with authentic standards or by injection of aliquots of the same sample under varying inlet temperatures.

Detectors for GC applications

There are various detectors available coupled to GC instrument: flame ionization detector (FID), electron capture detector (ECD), thermal conductivity detector (TCD), nitrogen phosphorus detector (NPD) and photo ionization detector (PID). Besides this great variety of detectors, only mass spectrometers provide structural information and are therefore commonly used in the field of metabolomics. Many GC instruments are coupled with a mass selective detectors (MSD), see figure 3.8. The chromatographic information from GC and mass spectral information from MSD are required for reliable identification or annotation in the field of metabolomics, which was done in this thesis (Hübschmann, 1996).

3.3.2 Mass Selective Detector MSD

MS is a technique to determine the mass-to-charge-ratio (m/z) of a substance. It is the primary detection method in the field of metabolomics due to its sensitivity, selectivity, speed, broad application and structure information of measured metabolites. Still, fourier transform infrared (FT-IR) spectroscopy and nuclear magnetic resonance (NMR) spectroscopy are rapid, non-destructive techniques for high-throughput metabolite detection with minimal sample preparation. Especially FT-IR is routinely used for metabolic fingerprinting of biofluids (Dunn and Ellis, 2005). Using NMR, structure elucidation is achieved. Hence, NMR is often used for target analysis of selected metabolites to clarify their chemical structure. The majority of applications employ ^{1}H (proton) NMR for clinical studies and, as the majority of known metabolites contain hydrogen atoms, the system is nonbiased for detection of metabolites (Dunn and Ellis, 2005).

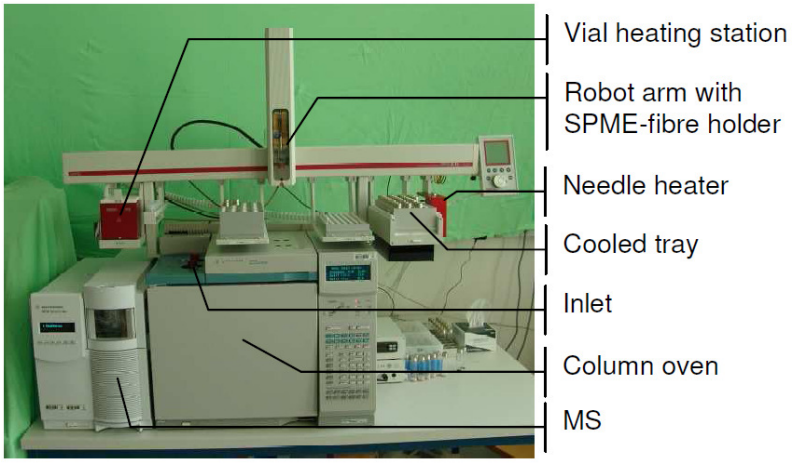


Figure 3.8: Agilent 7890 GC coupled to 5975B inert XL MSD equipped with Gerstel MPS XL autosampler at the Center for Analytical Chemistry

MS provides a fast, sensitive and selective platform for the qualitative and quantitative analysis of metabolites. It provides structural information and is currently the most widely spread technique in metabolomics research. Generally, quadrupoles or time-of-flight (TOF) instruments are accustomed for MS.
Regarding GC-MS, ionization for gas molecules is required. Both electron ionization (EI), formerly called electron impact, and chemical ionization (CI) are routinely used for metabolomics applications using MS. In this thesis EI was used exclusively. As shown in figure 3.9 the gaseous, uncharged molecules enter separately (due to chromatographic separation) the ion source (under vacuum) via heated interface from the GC column. They are hit by vertically emitted electrons which travel from cathode to anode. Due to the high ionisation energy (70 eV) most parent ions carry an excess of internal energy and consequently charged and uncharged fragments are generated, see equation 3.4. EI is a hard ionization technique. This means that not only one fragment per molecule is generated, but more fragments resulting in a fragmentation pattern which can be used for structure elucidation. The charged fragments, ions, are then pushed from repeller to the mass filter. The mass filter in case of this thesis was a single quadrupole, which will be explained in more detail now.

$$M + e^- \rightarrow M^{\bullet+} + 2e^- \tag{3.4}$$

Quadrupoles are mass filters, also called analyzers, which separate ions according to their mass-to-charge ratio, the m/z value. They are made up of four parallel rods, two negative, two positive rods, of hyperbolic or circular section (de Hoffmann and Stroobant, 2007). If a positive ion enters the space between the rods, it will be drawn to a negative rod. As soon as the potential changes sign, the ion will change direction. The only requirements are that the crossing time of the analyzer is short compared to the time of switching from mass to mass, and the ions must remain between the rods for a few oscillations of alternative potentials (de Hoffmann and Stroobant, 2007). The user of the instrument selects a scan range, using scan mode, or single ions for detection, single ion monitoring

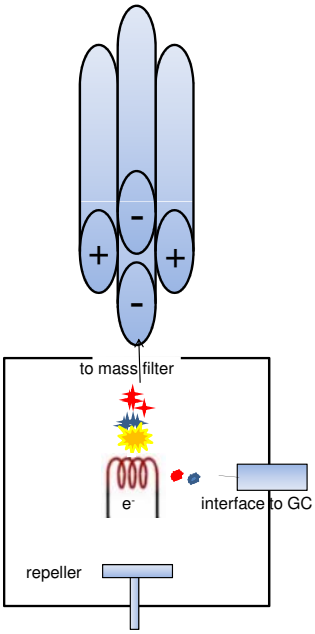


Figure 3.9: Electron ionization source

(SIM) mode. The selected ions are kept on a stable trajectory through the rods and finally reach the detector itself, whereas the residual ions hit the rods and leave this path through the mass filter. The detector is an electron multiplier (EM). Ions which made their way through the mass filter reach the conversion dynode, where their signal intensity is enhanced: One positive ion striking the conversion dynode causes the emission of several secondary negative ions and electrons. At the first dynode they are all converted to electrons. These electrons are amplified in cascades in the EM to produce an electrical current. This current is the actually measured signal by the instrument (de Hoffmann and Stroobant, 2007).

Besides quadrupoles, TOF instruments are commonly used in the field of metabolomics (Allwood et al., 2009). After an initial acceleration of ions by an electric field, TOF analyzers separate the ions according to their velocities in a free-field region (the flight tube). The detector is located at the other end of the tube (linear TOF) or next to the ion source (reflectron). M/z values are obtained by measuring the time which the ions require to travel through the free-field region. In principal, there is no limit in the upper mass range. Therefore, it is suitable for soft ionization techniques in LC as well as for EI in GC or MALDI.

In comparison to quadrupoles, TOF instruments have many advantages: First, their transmission efficiency is much higher, leading to higher sensitivity. Second, the scan speed of the analyzer is very fast leading to high number of scans per time interval over a wider mass range. This also improves spectra deconvolution positively. Third, the flight tubes are 1-2 m long resulting in high resolution, which can be even improved in delayed pulsed extraction or usage of a reflectron (de Hoffmann and Stroobant, 2007). Still, TOF instruments are much more expensive than quadrupoles. The data acquisition and handling is much faster and less space consuming for quadrupole data than TOF data, but the limited scan number is worse for deconvolution. In the field of metabolomics, both techniques are routinely found. In this thesis, Agilent 5975 MSD mass analysers (single quadrupoles) were used in full scan mode for metabolic profiling and SIM mode for target analysis.

3.3.3 Results from a GC-MS measurement

The raw total ion current (TIC), which is constructed from the sum of intensities of all ions measured using scan mode, is displayed in the chromatogram, see upper part of figure 3.10. The sum of intensities of all ions (y-axis) which reached the detector at a certain retention time (x-axis) are shown in one signal. Using selected ion current (XIC) chromatograms, the analyst can display the intensities of single ions (instead of the TIC) over time. XICs are calculated from TICs. In contrast, using SIM mode for data acquisition, only selected ions are monitored by the MSD and stored in the files.
The information which ions were detected at any retention time is stored in the mass spectrum (lower part of figure 3.10). For the ions in a mass spectrum are fragments from one compound (if no coelution occurs), structural information is gained from the mass spectrum. In theory, we no background ions are monitored, the highest m/z value shows the molecular mass of the compound. The isotopic pattern is very characteristic for the fragments, resulting from the natural occurrence of ^{13}C and ^{14}C in respect to ^{12}C.

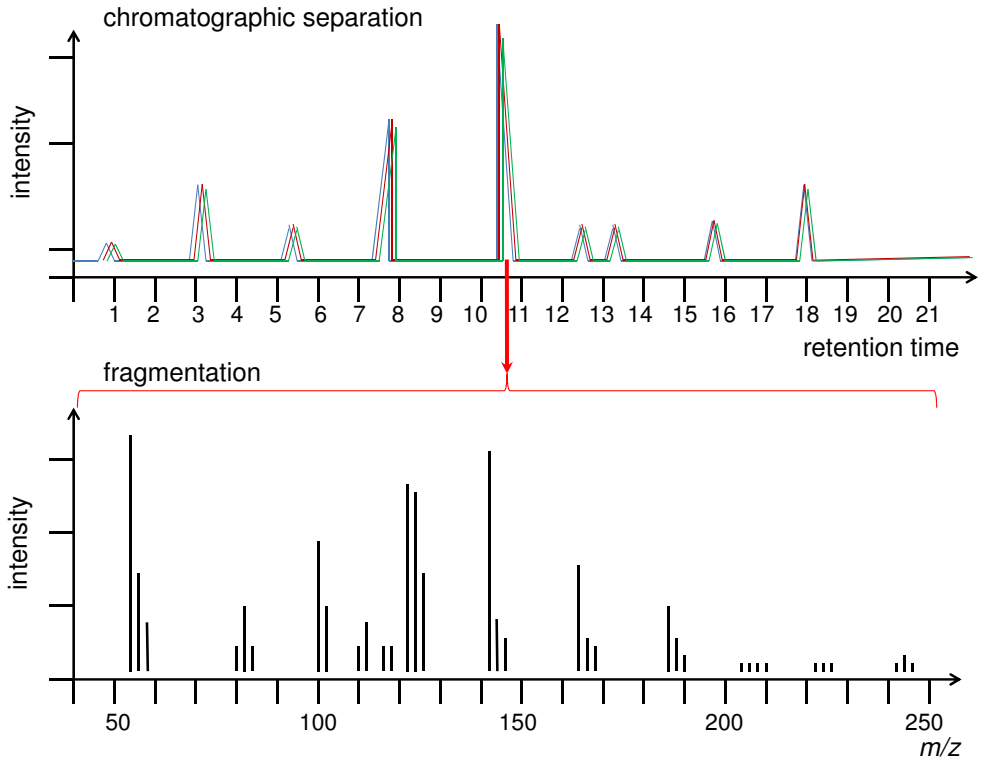


Figure 3.10: The TIC chromatograms of three samples (replicates) display the retention time on the x-axis and the intensity on the y-axis. The mass spectrum of the chromatographic peak at 10.5 minutes shows the m/z values on the x-axis and the intensity on the y-axis.

In the raw chromatogram retention time shifts can occur between samples. These shifts have to be corrected prior to comparison of the compounds in different samples. Chromatographic peak shapes can also be far from an ideal Gaussian peak shape. This can be a result of low chromatographic resolution for this analyte, background interferences or coeluting compounds. In all cases, data processing steps are necessary to smooth the peak shape mathematically, to eliminate background ions from the mass spectrum or to

separate and rearrange (deconvolve) the mass peaks from one mass spectrum to individiual mass spectra for the coeluting compounds.
Data processing of GC-MS data includes: data conversion in a suitable format for the data processing software, data smoothing and peak detection, deconvolution, baseline correction and RI calibration and compound annotation (or identification). These individual steps will be discussed in the following for the software tool MetaboliteDetector, which was used in this thesis.

3.4 GC-MS data processing and statistical evaluation

3.4.1 Data processing algorithms used by the software tool MetaboliteDetector

Metabolomics studies result in enormous amounts of data, while only a handful of it is biologically relevant for the individual study. Hence, the raw data in the chromatogram and the mass spectrum need adequate data processing prior to statistical evaluation. Huge data sets cannot be analysed manually or by vendor software. Several data processing and evaluation software tools have been developed for GC data. MetaboliteDectector (Hiller et al., 2009), Automated Mass Spectral Deconvolution and Identification System (AMDIS) (Stein, 1999) and SpectConnect (Styczynski et al., 2007), Tagfinder (Luedemann et al., 2008), MaltMS and ChromA (Hoffmann and Stoye, 2009) or XCMS (Smith et al., 2006) can be employed for data processing.
XCMS is mainly used for LC data processing, but the same algorithms can be employed for GC data processing. XCMS includes binning of data, peak detection and alignment resulting in a list of so called features. Each feature is characterized by a mass, retention time and intensity. Several features make up one mass spectrum of a substance. For GC data, it is much more suitable to work with deconvolution software tools because here the features belonging to one mass spectrum are not treated individually but together for one detected compound. They mass spectrum and RI comparison with reference libraries can only be made with these software tools.
AMDIS performs noise analysis, component perception, and spectral deconvolution, extracting pure component spectra and related information stored in different output files such as '.elu' and '.fin' file formats (Stein, 1999). Spectra can be searched against the NIST or any other user-defined database. Additionally, the generated files can be used by the software tool SpectConnect to add compounds, which cannot be annotated based on their mass spectral and RI information, to the list of detected components.
Although it is very useful for qualitative data processing including alignment and deconvolution of overlapping peaks, AMDIS cannot integrate user-selected XICs for detected compounds. MetaboliteDetector integrates user-selected ions for the detected metabolites of all samples in parallel and generates a batch report, including a statistical analysis. This is an important aspect in the field of metabolomics and one of the reasons why MetaboliteDetector was used in this thesis. In the following, I will focus on the individual steps of data processing for this software tool.

Data conversion

Data acquisition is done by the GC-MS instrument using vendor software. The file formats generated differ dependent on the supplier of the instrument. In this thesis Agilent instruments were used resulting in '.D' files for each measurement. Their size is approx.

10 MB per file. AMDIS supports the Agilent format, whereas open-source software tools like MetaboliteDetector require the files to be converted to '.CDF' (common data format) prior to data import. (Net)CDF is the oldest and currently most widely used data format in the field of metabolomics (Hoffmann and Stoye, 2012).
The Agilent software tools are able to convert the '.D' to '.CDF' files, which can be processed by MetaboliteDetector. Thus, '.CDF' files are generated as a first step of our data processing.

Data smooting and peak detection

Second step, to improve peak shape and the signal-to-noise (S/N) ratio of noisy data, a five-point cubic Savitzky-Golay filter has been applied to smooth both the spectral as well as the retention time dimension of the data, see equation 3.5.

$$f(x) = \frac{-3(x_{-2} + x_{+2}) + 12(x_{-1} + x_{+1}) + 17x}{35} \tag{3.5}$$

where f(x) is the filtered value for the intensity x of the mass peak and x_{-2}+x_{+2}, x_{-1}+x_{+1} are the neighbouring intensities of x.
A special form of the Savitzky-Golay filter is applied in MetaboliteDetector to determine the first derivative and the smoothing of the data within one computational step, see equation 3.6.

$$f'(x) = \frac{-2x_{-2} - x_{-1} + x_{+1} + 2x_{+2}}{10} \tag{3.6}$$

where f'(x) is the first derivative of f(x) in equation 3.5.
The beginning of a mass peak is set if f'(x) exceeds a predefined positive threshold. The mass peak ending is determined when two subsequent values of f'(x) pass a predefined negative threshold. Two criteria must be fulfilled to be nominated a peak: First, it must consist of more than three values of f'(x), second, the height above the baseline (in S/N units) of the mass peak maximum must exceed a predefined threshold, see figure 3.11. The detected peaks are now deconvolved, (base line corrected) and RI calibrated for compound detection.

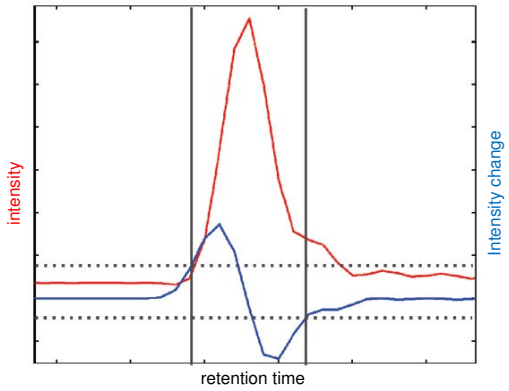


Figure 3.11: Peak detection in MetaboliteDetector. The red line represents an XIC chromatogram. The blue line displays the first derivative of the intensity. If the values of the first derivative cross the peak threshold (dotted lines), a mass peak begin or end is set. (from (Hiller et al., 2009))

Deconvolution

"The basic idea behind deconvolution is that the raw signal is the superposition of single-metabolite contributions from the metabolite responses in that signal." (Hendriks et al., 2011)

The mass spectral peaks of different compounds (or background) are often included in one compound and need to be separated before compound detection. Thus, deconvolution is a processing step to reduce background noise and to extract 'clean' or 'pure' spectra (Stein, 1999; Hiller et al., 2009). Different algorithms were developed for each software tool to detect the underlying MS spectra and chromatograms of the single-metabolite contributions. In figure 3.12 this process is explained in a simplified way for the deconvolution algorithm using MetaboliteDetector. The raw TIC chromatogram (in red, see (A)) results in the deconvolved TIC chromatogram (in blue, see (B)). The deconvolved TIC chromatogram shows at least four separated chromatographic peaks, which were not observed in the raw TIC chromatogram. This is achieved by classifying the scans to subintervals (bins) and applying the second derivative Gaussian filter for the intensities of the XICs for each bin. The XICs which belong to one of the coeluting compounds were found under the peaks resulting from the Gaussian filter (dotted red line, see (B)). These XICs were combined to individual mass spectra for each detected compound. This step is necessary for accurate and reproducible compound detection.

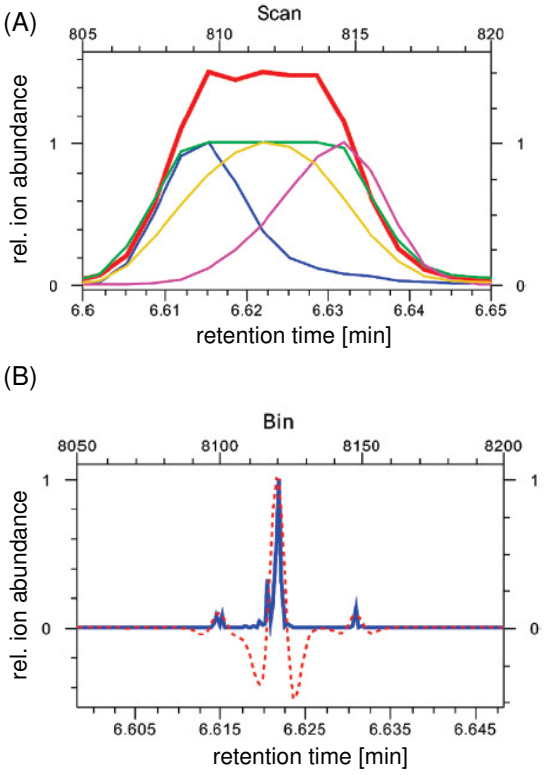


Figure 3.12: Deconvolution algorithm using MetaboliteDetector. (A) shows the TIC chromatogram, normalized to 1.5, and XIC chromatograms of coeluting compounds (m/z 158, m/z 205, m/z 299, m/z 314), normalized to 1. (B) The scans are assigned to subintervals (10 bins per scan). The deconvolved TIC clearly shows the separation of the coeluting compounds. Finally the compounds are detected by the application of a second derivative Gaussian filter (dotted red line). All XICs assigned to bins that are lying under peaks of the Gaussian filter are used to form the mass spectra of the detected compounds (from (Hiller et al., 2009))

Baseline correction and advanced RI calibration

MetaboliteDetector also offers the possibility for baseline correction. This task is especially important if column bleed in chromatography, non-linearities in detectors or accumulation of less abundant compounds (background signals) appear (Liland, 2011). Baseline elevations can lead to a number of false-positive metabolite detections because software tools cannot automatically distinguish between baseline and signal. This also affects quantification results. However, during this thesis baseline correction was never a necessary task.
The next step in data processing, is the RI calibration, which is essential to calculate the corresponding RI values for the compounds detected. The RI calibration for samples in MetaboliteDetector includes the compound detection step. There is the possibility to do a compound detection without RI calibration. For these samples, compounds are only compared for their spectral similarities with library entries. Since, annotations in the field of metabolomics are generally based on the comparison of mass spectra and RIs between a detected compound and a libary entry, the RI calibration is necessary for each analysis. Moreover, the retention time correction using advanced RI calibration is an extension of the RI calibration. The retention time of a compound, e.g. an internal standard (IS), is locked and the time shift in each sample is corrected for this retention time. If the alkane standards for RI calibration are measured in parallel to the samples, e.g. every tenth sample, the use of the advanced RI calibration is that the RI correction is made based on a compound, which is measured in every sample.

Compound annotation

The last step of data processing is the compound annotation. It is one of the major bottlenecks in metabolomics. In case of GC-MS data, a detected compound is characterized by the signal intensity, its mass spectrum and its retention time (or its retention index). Annotations are resolved by libraries, public or private, using appropiate matching criteria for the mass spectrum and the RI. The identification of a compound is achieved if the annotation is confirmed by the measurement of an authentic standard.
MetaboliteDetector calculates similarity scores for the mass spectral and RI match between detected compound and library entry separately. The overall score similarity is based on equation 3.7. Since it can be expected that the spectrum of a compound contains more information than the RI, it is weighted stronger.

$$S_{total} = \sqrt[3]{S_{spec}^2 \cdot S_{RI}} \tag{3.7}$$

Since the ionisation energy in GC-MS is standardised (70 eV), mass spectra with nearly identical fragment patterns are obtained for substances independent of the instrumentation. It is state of the art in GC-MS analytics to use mass spectral and retention time information for annotation or identification (Schauer et al., 2005; Dettmer et al., 2007). For identifications, the mass spectrum and RI need to be confirmed with an authentic standard.
Besides the metabolites which can be annotated, there are very often metabolites monitored which remain unknown despite data processing and the application of annotation algorithms as either mass spectrum and/or RI do not match with library reference values according to the set criteria. For metabolomics questions, non-targeted approaches are of uttermost importance to include unknowns in statistical evaluation as well.

Mass spectral libraries are commercially available, e.g. Wiley 8^{th} and NIST/EPA/NIH Mass Spectral Library - Version 2008, or open-source (Hummel et al., 2007). RIs are published in literature, as additions to spectral libraries (NIST Chemistry WebBook - NCWB) or in combined databases (Schauer et al., 2005; Hummel et al., 2007). NIST database provides many GC-MS mass spectra and RI values. However, the user has to take care with the spectra available. They were not checked with existing entries or reference standards. Some entries contain spectra which were collected in SIM mode or with instruments which revealed different fragmentation patterns than today's instruments. Most spectra are not deconvolved or cleaned from noise (e.g. column bleed). The RIs which are stored with the entries give no hint about the column and GC parameters and need to be checked, e.g. with entries in the NCWB. NIST database is of great help to get a first impression of the origin of the metabolite. The NIST database is much too big to be used with MetaboliteDetector, which deals well with less than 1000 entries. Only subsets of interesting metabolites can be imported, checked and used.
The two main advantages of MetaboliteDetector over AMDIS regarding the compound annotation are: Quantification of user defined ions is possible and the compound list includes metabolites, which could not be annotated, in the results table. Therefore, even though no annotation of a compound was possible and it was found significant in a number of samples, it is included in the list for statistical evaluation. The integrated areas of user-selected ions of detected compounds are listed in the results table. By changing the settings for deconvolution, the integration if single metabolites can also change. The user can inspect automatically integrated XIC peaks manually, which is important for quantification purposes.

Quantification in the field of metabolomics

Metabolomics aims at the qualitative and quantitative information from all metabolites in the metabolome (Hall, 2006). However, for absolute quantification, reference standards are required for every compound. Since only a limited number of analytes is commercially available or can be synthesized, this level of quantification can hardly be reached for most of the compounds. Thus, absolute quantification is only feasible for target analysis.
Metabolomics applications aim therefore towards relative quantification, which can imply the comparison of a group of samples based on their signal intensities (Lei et al., 2011). The precision of the signal intensities and therefore the reliability of data comparison within a sequence can be enhanced by the use of internal standards (IS). The signal intensities of the sample are divided by the signal intensity of an IS to correct drifts in the signal intensity resulting from the detector or the sample preparation.
The number of IS and which IS to use, is up to the researcher. Dependent on the type of correction (for detector drifts or sample preparation deviations) the IS must be added prior to extraction, prior to derivatisation or prior to measurement. Ideally, stable isotopically labeled internal standards are used, but their availability is limited and the costs are high in particular for high-throughput applications.
The introduction of stable isotope tags to metabolites can be achieved by chemical labeling. If this labeling is applied to standards of known concentrations as well, absolute quantification can be considered (if the metabolite shows the same chemical and physical characteristics as the standard) (Lei et al., 2011).
In this thesis, the integrated areas of user-selected ions of the detected compounds, which are corrected with the exact sample weight, are used to build up the data matrix for statistical evaluation, which is described in the following.

3.4.2 Statistical evaluation

Signal intensity (area/height)

variable (i)/ object (j)	Met 1	Met 2	Met 3	Met 4	...
C1	3000	10	250	12	...
C2	2500	20	290	15	...
C3	3200	15	220	56	...
T1	1000	100	3	45	...
T2	780	120	4	30	...
T3	890	150	3	10	...
...	...	...	...	...	...

Figure 3.13: Data matrix X containing signal intensities (area/height). The columns contain the variables i (metabolites), the rows contain the objects j (samples). C1-C3... control samples, T1-T3...samples after treatment, Met1-4...metabolites 1-4;

The peak areas obtained from integration of XIC chromatograms of the detected compounds, including annotated metabolites and metabolites, which cannot be annotated, need to be arranged in a data matrix X, see figure 3.13. This data matrix contains the objects (samples) in rows, the variables (metabolites) in columns. It is the starting point for univariate or multivariate statistical tools for statistical evaluation.
For statistical evaluation the commercially available tools Matlab or Unscrambler are employed as well as R (R Development Core Team, 2010), which is an open-source tools for statistics.
Univariate statistical methods can be considered for selected metabolites of interest. The comparison between samples is based on one variable (metabolite) only. Students t-test or the Wilcoxon-Mann-Whitney-test (Mann-Whitney-U-test or Wilcoxon rank-sum test), which is more robust to outliers, can be employed to test the null hypothesis that there is no significant difference between two samples with a certain confidence limit. The result of a t-test is a probability value (p-value) which is above or below a critical threshold, usually 0.05 for the 95 % confidence interval is taken. This p-value is used to decide whether the null hypothesis must be refused or accepted.
To reveal differences between the samples for more than one metabolite at once, multivariate statistics is necessary. The data matrix needs to be reduced in its dimension to display significant differences in new coordinate systems of 2-3 levels. For univariate statistics, no data pretreatment is required. However, the first critical step of statistical evalution using multivariate statistics is the pretreatment method. Signal intensities can require rather profound pretreatment(s) to overcome deviations from the sample preparation or measurement drifts.

Pretreatment methods

Procedures which can be used for pretreatment of data are centering, scaling and transformation. A list of pretreatment methods currently used in the field of metabolomics was presented by (van den Berg et al., 2006) and is shown in figure 3.5.
There are some characteristics of metabolomics data which need to be considered for appropriate centering, scaling or transformation (van den Berg et al., 2006): First, the abundances of metabolites detected differ in orders of magnitude. Very abundant metabolites

are not necessarily of more biological relevance than low abundant metabolites. Second, the biological variance is often very high for secondary metabolites and might not be attributed to a biological induction. Third, large fluctuations in abundances can occur under identical experimental conditions (uninduced biological variation). Fourth, technical variation from sample preparation or the analytical instrument can influence the abundances detected. Fifth, heteroscedasticity introduces an additional structure in the data.
The total variation in the data is a result of induced (resulting from changes in the metabolome) and uninduced variations. Data pretreatment methods emphasize different aspects in the data to reveal biologically relevant information for statistics. Centering is achieved by subtracting the mean of each metabolite (for each column) from all samples (rows) from all signal intensities in the data matrix X, resulting in values around zero which show the high fluctuations but not similarities between samples. Centering is prerequisite for all scaling methods described. Scaling methods divide each (mean-)centered variable by a factor, e.g. the standard deviation (autoscaling), the highest minus the lowest abundance (range scaling), the variance (pareto scaling), the standard deviation times the inverse coefficient of variation (vast scaling) or the mean (level scaling). Two types of factors can be used for the scaling methods: data dispersion (e.g. standard deviation) and size measure (e.g. the mean).
Transformations are non-linear conversions of the data, e.g. the log or the power transformation, which are required to correct for heteroscedasticity or multiplicative relations between variables. They have a pseudo-scaling effect because differences between large data are relatively more reduced than those of small data. Transformation like scaling intends to achieve normal distribution for abundance values of individual variables. Still, it can be useful to apply a scaling method after transformation (van den Berg et al., 2006). Generally, one has to consider that pretreatment methods might overfit the data, which means that random errors or noise might get more or equal weight as the underlying relationship between samples, and therefore give a significance to biologically not relevant data. The same holds true for the statistical methods described in the following. Therefore, it is always important to go back to the raw data for the metabolites of interest and check the conclusions made with multivariate statistical methods.

Statistical methods for data analysis

Statistical methods can be sorted in two groups, supervised (predictive) and unsupervised (descriptive) methods, to discriminate, with a certain level of certainty, between treated and untreated (control) samples (Broadhurst and Kell, 2006). The biological question in a metabolomics experiment frequently refers to the difference in the metabolome of a control (untreated) and a treated sample group. For this purpose, unsupervised techniques are the simplest and most widely used (Hall, 2006). Unsupervised methods are used for dimension reduction, e.g. principal component analysis (PCA), independent component analysis (ICA), or cluster analysis, e.g. hierarchical cluster analysis (HCA). Supervised methods classify unknown data sets if classes or values of the responses are known using predictive models, e.g. discriminant analysis (DA), partial-least square analysis (PLS), support vector machines (SVM), neuronal networks (NN) (Goodacre et al., 2007).
PCA is an unsupervised method commonly used in metabolomics (Hall, 2006) to enable the illustration of the n-dimensional data by reduction to two or three dimensions. PCA, an exploratory analysis, is a linear data transformation with a new coordinate system, where the uncorrelated principal components (PC) serve as coordinates. The first PC rep-

Table 3.5: Overview of the pretreatment methods used in this study. In the Unit column, the unit of the data after the data pretreatment is stated. O represents the original Unit, and (-) presents dimensionless data, i and j label the variables and objects, respectively, (see figure 3.13) and J stands for the number of the objects. The mean ($\bar{x}_i$) is estimated as:

$$\bar{x}_i = \frac{1}{J}\sum_j x_{ij} \tag{3.8}$$

and the standard deviation (s_i) is estimated as:

$$s_i = \sqrt{\frac{\sum_j (x_{ij} - \bar{x}_i)^2}{J-1}} \tag{3.9}$$

$\tilde{x}$ and $\hat{x}$ represent the data after different pretreatment steps. (from (van den Berg et al., 2006))

Class	Method	Formula	Unit	Goal	Advantages	Disadvantages
I	Centering	$\tilde{x}_{ij} = x_{ij} - \bar{x}_i$	*O*	Focus on the differences and not the similarities in the data	Remove the offset from the data	When data is heteroscedastic, the effect of this pretreatment method is not always sufficient
II	Autoscaling	$\tilde{x}_{ij} = \frac{x_{ij}-\bar{x}_i}{s_i}$	(-)	Compare metabolites based on correlations	All metabolites become equally important	Inflation of the measurement errors
	Range scaling	$\tilde{x}_{ij} = \frac{x_{ij}-\bar{x}_i}{(x_{i_{max}}-x_{i_{min}})}$	(-)	Compare metabolites relative to the biological response range	All metabolites become equally important Scaling is related to biology	Inflation of the measurement errors and sensitive to outliers
	Pareto scaling	$\tilde{x}_{ij} = \frac{x_{ij}-\bar{x}_i}{\sqrt{s_i}}$	*O*	Reduce the relative importance of large values, but keep data structure partially intact	Stays closer to the original measurement than autoscaling	Sensitive to large fold changes
	Vast scaling	$\tilde{x}_{ij} = \frac{x_{ij}-\bar{x}_i}{s_i} * \frac{\bar{x}_i}{s_i}$	(-)	Focus on the metabolites that show small fluctuations	Aims for robustness can use prior group knowledge	Not suited for large induced variation without group structure
	Level scaling	$\tilde{x}_{ij} = \frac{x_{ij}-\bar{x}_i}{\bar{x}_i}$	(-)	Focus on the response	Suited for identification of e.g. biomarkers	Inflation of the measurement errors
III	Log transformation	$\tilde{x}_{ij} = log^{10}(x_{ij})$ $\hat{x}_{ij} = \tilde{x}_{ij} - \bar{\tilde{x}}_i$	$log(O)$	Correct for heteroscedasticity, pseudo scaling. Make multiplicative models additive	Reduce heteroscedasticity, multiplicative effects become additive	Difficulties with values with large relative standard deviation and zeros
	Power transformation	$\tilde{x}_{ij} = \sqrt{x_{ij}}$ $\hat{x}_{ij} = \tilde{x}_{ij} - \bar{\tilde{x}}_i$	$\sqrt{O}$	Correct for heteroscedasticity, pseudo scaling	Reduce heteroscedasticity, no problems with small values	Choice for square root is arbitrary

resents the direction of the maximum variance in the data. The second PC is displayed orthogonal to the first PC and represents the direction of the variance which describes the highest amount of the total variance after PC1. Typically, the result of a PCA is presented in two graphs: the score plot and the loadings plot. The score plot shows the clustering of objects (samples) in the new coordinate system of PC1 versus PC2. The loadings plot displays the variables (metabolites) which contribute in which direction to the clustering of the objects. PCA results show only where the greatest variance of data exists, but a great variance does not necessarily mean that this is a significant biological difference between samples.
Another unsupervised method for classification is hierarchical clustering analysis (HCA). Clustering itself can be distinguished in hierarchical clustering and partitioning (k-means). HCA is the most widely used clustering method in the field of metabolomics which is well suited for discriminatory analysis (Liland, 2011). It is applied to explore relationships and similarities within groups. The distance (or similarity) measures are usually Minkowski distance, with Manhattan, Hamming and euclidean distances as special measures, or Mahalanobis distance (Varmuza and Filzmoser, 2009; Wehrens, 2011). Methods used to cluster objects in groups are: complete linkage (the maximum of object distances), single linkage (the minimum of object distances), average linkage (the mean of object distances) or ward method (Ward, 1963). Furthermore, hierarchical clustering can be agglomerative (starting with single elements and aggregating them into clusters) or divisive (starting with the complete data set and dividing it into partitions). The result of clustering is displayed as a dendrogram or a heatmap. Clustering can be performed on objects (samples) or variables (metabolites). A dendrogram is a tree diagram. A heatmap displays the dendrogram of samples on one axis, the dendrogram of metabolites on an orthogonal axis. In between there is a colour-coded map showing different expression pattern in samples sorted according to their similarities found by HCA.

Finally, effective visualisation of pathways is important to display the biological output from the metabolomics experiment. There are a number of pathway databases freely available, e.g. KEGG (http://www.genome.jp/kegg/pathway.html), PlantCyc (http://pmn.plantcyc.org/PLANT/class-tree?object=Pathways), MetNet (http://metnetonline.org) or MetaCyc(http://metacyc.org). Software tools like Pathos (Leader et al., 2011) enable visualisation of metabolites, which were detected in metabolomics studies, and changes in their abundances in the context of the metabolic pathways in which they occur. The list of metabolites can be a list of m/z values with corresponding intensities or metabolites with their corresponding KEGG ID and intensity. Tools like Pathos help to put results from MS experiments into biological context.

In this literature review about GC-MS in the field of metabolomics, I discussed the important aspects and individual steps which have to be considered in a metabolomics experiment using GC-MS analysis. The sample preparation of volatile metabolites using HS SPME and of non-volatile, polar metabolites using solvent extraction and a two-step derivatisation using MOX and MSTFA followed by GC-MS measurements were reviewed under the aspects of a metabolomics-based approach to study as many metabolites possibly and reliably detected. Moreover, I described the individual steps of data analysis, including data processing with MetaboliteDetector and statistical evaluation. In the following, the experiments which were carried out to develop and establish GC-MS methods for metabolic profiling of volatiles and non-volatile, polar metabolites and their applications for fungal cultures, healthy and infected wheat ears are described.

Chapter 4

Materials and Methods

4.1 Chemicals, materials and reagents

4.1.1 Chemicals, materials and reagents for HS SPME GC-MS

Acetonitrile was obtained from VWR (Vienna, Austria). Benzaldehyde, 2-pentylfuran and phytol, 3-methyl-1-butanol, β-caryophyllene, 2-ethylfuran, 1-tridecanol were purchased from SAFC Supply Solutions (St. Louis, USA). 2-Methyl-1-butanol acetate, acetic acid nonylester, 5-methylfurfural, nonanal, methylsalicylate, pentadecane, ethyl acetate, 2-methyl-1-propanol, 2-methyl-1-butanol, isobutylacetate, p, m-, o-xylene, 1,3,5-cycloheptatriene, 2-(*trans*)-hexenal, 2-heptanone, 1-octen-3-ol, 3-octanone, 3-octanol, β-myrcene, S-(-)-limonene, p-, m-, o-cymol, α-pinene, terpinolene, (+)-α-longipinene, (-)-α-cedrene, (-)-thujopsene, (+)-aromadendrene, farnesene and the alkane standards C_8-C_{20} and C_{21}-C_{40} were bought from Sigma Aldrich (Vienna, Austria). The following reference substances were sent from Georg Weingart: butylpropionate, 2-pentanone, 5-hexen-1-ol, methylhexanoate, (E,E)-2,4-hexadienol, hexanoic acid ethylester, 2-propyl-1-pentanol, (E)-2-hexenylacetate, 6-methyl-5-heptene-2-ol, 3-ethoxy-1-propanol, R(-)-2-methyl-1,4-pentanediol, (E)-2-methyl-2-butenal, (E)-2-pentenal, 3-pentanol, 1,3-propanediol, isopropylacetate, 3-pentanone, 2-hexanone, propylpropionate, 2-methylbutylacetate, 2-methyl-1-pentanol, 2-methyl-2-pentanol, 5-methylfurfural, 4-hexen-3-one, 4-methylvaleric acid, 2-methylbutyric acid ethylester, allylpropionate, anisole, ethylvalerate, propylbutyrate, heptan-3-one, heptylformiate, butylbutyrate, 2-phenoxyethanol, 2-octanone, (Z)-5-octen-1-ol, γ-octalactone, 1-butanol-2-methyl acetate, acetic acid nonylester, 2-octanone, isolongifolene, 3-carene, 2-heptanol, (E)-β-farnesene, (-)epiglobulol, R(+)-limonene, α-, β-γ-humulene and three mixtures G09 (geranyl acetate, linalyl acetate, acetic acid n-butyl ester, i-pentyl acetate, benzyl acetate, neryl acetate, citronellyl acetate, pentyl butyrate, amyl propionate), G10 (ethyl phenylacetate, ethyl butyrate, ethyl heptanoate, ethyl octanoate, ethyl dodecanoate, ethyl isovalerate, ethyl decanoate, ethyl salicylate, ethyl nonanoate, ethyl hexanoate, n-pentyl acetate, ethyl sorbate) and G12(1-pentanol, benzyl alcohol, benzaldehyde, 1-hexanol, 1-octanol, 1-decanol, 3-methyl-1-butanol, (E)-3-hexen-1-ol). 10 mL and 20 mL HS-vials with screw caps and PTFE/Silicone septa (1.3 mm, 45° shore A) were obtained from Gerstel (Mühlheim an der Ruhr, Germany). SPME inlet glass liners, 1.5 mm i.d (Supelco, Bellefonte, USA) were required for fast desorption and consistent peak shape of analytes. 2 mL dark glass vials and natural rubber/butyl septa (1 mm, 45° shore A) were purchased from Wagner Munz (Munich, Germany). $ZnSO_4$*$6H_2O$, $Fe(NH_4)_2(SO_4)_2$*$6H_2O$, H_3BO_4, $MnSO_4$, Na_2MoO_4*$2H_2O$, $CuSO_4$*5 H_2O were bought from Sigma Aldrich (Vienna, Austria). KH_2PO_4, $MgSO_4$*$7H_2O$, KCl, $NaNO_3$

and potato dextrose agar (PDA) were bought from Carl Roth (Karlsruhe, Germany). Agar agar and citric acid were purchased from Serva (Heidelberg, Germany), sucrose and mung beans from local supermarket. 50 mL tubes and baffled flasks were obtained from Greiner AG Holding (Kremsmünster, Austria).
A Retsch mill (ball mill) with 50 mL stainless steel beakers was available at the Institute of Biotechnology in Plant Production. The Institute of Biotechnology in Animal Production provided liquid nitrogen for quenching.

Preparation of standard solutions

All standard solutions were handled with Hamilton syringes and kept at 4 °C. Individual stock solutions (6 g L^{-1}) of all standard substances were prepared using pure acetonitrile (precooled at 4 °C) and mixed at 4 °C over night. Stock solutions of target analytes for the evaluation of matrix effects (3-methyl-1-butanol, limonene, 3-octanone and thujopsene) were diluted using 50 % aqueous acetonitrile (v/v) in 10 mL HS-vials or 2 mL glass vials. The stock solutions of residual standards were diluted in 50 % aqueous acetonitrile (v/v) in defined mixtures: Mixture 1 consisted of sesquiterpenes only, 40 mg L^{-1} each: (+)-alpha-longipinene, (-)-alpha-cedrene, (+)-aromadendrene and farnesene. Mixture 2 contained 6.7 g L^{-1} ethyl acetate, 0.8 mg L^{-1} p-xylene, 0.8 mg L^{-1} S-(-)-limonene, 0.8 mg L^{-1} terpinolene, 40 mg L^{-1} 2-methyl-1-propanol, 40 mg L^{-1} 3-methyl-1-butanol, 40 mg L^{-1} 3-octanone, 40 mg L^{-1} (+)-alpha-longipinene, 40 mg L^{-1} (-)-alpha-cedrene, 40 mg L^{-1} (+)-aromadendrene and 40 mg L^{-1} farnesene. Mixture 3 corresponded to Mixture 2 without 3-octanone.

4.1.2 Chemicals, materials and reagents for online-derivatisation

All reference substances and internal standards (IS) were bought from Sigma Aldrich (Vienna, Austria).
Stock solutions of pure standards were prepared in 50 % aqueous methanol at 1-10 gL^{-1}, dependent on the solubility of the substance. They were diluted with 50 % aqueous methanol to 10-200 mgL^{-1} solutions for measurement.
Standard mixtures from Nils Hoffmann (Bielefeld, Germany) were obtained as a gift as 1 mM solutions in water and used directly for measurement. The following substances were included: Mix 1 (pyruvic acid, alanine, valine, fumaric acid, serine, threonine, citric acid, glucose, adenosine, sucrose), Mix 2 (leucine, glycine, succinic acid, aspartic acid, α-ketoglutaric acid, fructose, fructose-6-phosphate, maltose, malic acid, α-ketocaproic acid), Mix 3 (isoleucine, homoserine, cysteine, ornithine, dihydroxyacetone phosphate, arginine, galactose, tyrosine, trehalose), Mix 4 (erythrose-4-phosphate, norleucine, citrulline, phenylalanine, mannitol, lactose, ribose, ornithine, isocitric acid, phosphoenolpyruvic acid, adenine), Mix 5 (urea, maleic acid, glutamine, methionine, xylose, glyceric acid-2-phosphate, histidine, cellobiose), Mix 6 (thymine, β-alanine, α-hydroxyglutaric acid, glutamic acid, rhamnose, shikimic acid), Mix 7 (uracil, pantothenic acid, myo-inositol, melibiose), Mix 8 (homocysteine, arabinose, gluconic acid, tryptophane, spermidine), Mix 9 (glyceric acid, S-methylcysteine, 2-aminoadipic acid, pinitol, 2-methylcitric acid, glucose-6-phosphate, myo-inositol-phosphate), Mix 10 (glutamine, 4-aminobutyric acid, 2-isopropylmalic acid, arginine, histidine, gluconic acid-6-phosphate), Mix 11 (α-ketocaproic acid, malic acid, cytosine, phosphoenolpyruvic acid, asparagine, α-glyco-phosphate, glycerol-3-phosphate, glyceric acid-3-phosphate, gluconolactone, erythrose-

4-phosphate, mannitol, glucuronic acid, ribose-5-phosphate, ribulose-5-phosphate).
Alkane standards C_8-C_{20} and C_{21}-C_{40} were diluted with isooctan to a final concentration of 5 mgL^{-1} of each alkane.

4.2 Instrumentation

4.2.1 Instrumentation for metabolic profiling of volatiles

Two GC-MS systems were used for the measurement of volatile compounds using HS SPME: Agilent GC 6890N coupled to 5975B inert XL MSD and Gerstel MPS2XL autosampler, which was used with Agilent MSD Chemstation G1701EA E.02.00.493 and Gerstel Maestro 1.3.20.41 (Mühlheim, Germany), and Agilent 7890N GC and 5975C triple axis detector MSD with CTC Dual Pal autosampler and Chronos 3.1 software from Axel Semrau (Sprockhövel, Germany).
For HS SPME, 50/30 μm DVB/CAR/PDMS, 23 gauge, 2 cm stable flex fibers were purchased from Gerstel (Mülheim an der Ruhr, Germany).
Polar and apolar columns from Agilent Technologies (Waldbronn, Germany) were used for the chromatographic separation of the analytes: DB-Wax (30 m x 0.25 mm x 0.25 μm), OPTIMA Wax (30 m x 0.25 mm x 0.25 μm), DB5-ms (30 m x 0.25 mm x 0.25 μm) and two HP5-ms (30 m x 0.25 mm x 0.25 μm) columns. After the column, a guard column (0.5 m x 0.1 mm) from Agilent Technologies was used as a linker of the GC with the MSD interface to allow fast change of columns. This guard column needed replacement during studies and was finally completely removed.

4.2.2 Instrumentation for metabolic profiling of non-volatile, polar-metabolites

The Agilent 7890N GC and 5975C triple axis detector MSD with CTC Dual Pal autosampler and Chronos 3.1 software from Axel Semrau (Sprockhövel, Germany) was employed for online-derivatisation and GC-MS analysis of non-volatile, polar metabolites. An HP5-ms (30 m x 0.25 mm x 0.25 μm) column was used for the chromatographic separation. It was replaced by a new column of the same type and from the same supplier after appr. 3000 injections.
Conditions for derivatisation using MOX and MSTFA were optimized for selected target analytes by Katharina Köstlbauer in her diploma thesis at the Center for Analytical Chemistry using a different autosampler (from Agilent) but the same GC-MSD instrument. The autosampler was removed because it was not suitable for our purposes. It was replaced with CTC Dual PAL autosampler which held two agitators, the left one was kept at 37 or 60 °C, the right one at 60 °C, and two autosampler arms, the left one equipped with a 10 μL syringe, the right one with a 100 μL syringe.
The autosampler software Chronos 3.5 from Axel Semrau (Sprockhövel, Germany) and Agilent MSD Chemstation were used for online-derivatisation, data acquisition and spectra collection.

4.3 GC-MS based methods

4.3.1 HS SPME GC-MS method for the determination of fungal volatiles

For GC-MS measurements, authentic standard solutions or fungal cultures were equilibrated at 30 °C for 10 minutes and extracted for 45 minutes at 30 °C. The extraction of 10 μL alkane standard C_8-C_{20} was performed at 30 °C for 45 minutes, whereas the extraction of 1 μL alkane mixture C_5-C_{10} (ratio pentane:hexane:heptane:octane:nonane:decane 17:6:3:2:1:0.5 (v/v)) was performed for six seconds at room temperature only, due to their high volatility and high interaction with the fiber coating material.
The fiber was introduced 67 mm into the GC injector for 2 minutes for desorption and then cleaned in a gentle stream of nitrogen for 10 minutes at 250 °C. 1 mL min^{-1} helium flow was applied, the oven was kept at 40 °C for 2 minutes (during desorption), then heated with 6 °C min^{-1} to 280 °C (250 °C for polar column). The transfer line was kept at 270 °C, the ion source at 230 °C and the MS quadrupole at 150 °C. No solvent delay was applied, the scan range was set at m/z 45-400 . The MSD was autotuned before every sequence and after replacement of spare parts like the liner (every 300 samples).

4.3.2 HS SPME GC-MS method for the determination of plant volatiles

Volatiles from wheat samples and reference substances for plant volatiles were extracted at 90 °C for 60 min with 30 min equilibration prior extraction. The extraction of alkane standard C_8-C_{20} and C_5-C_{10} was performed as described in 4.3.1. Additionally, 20 μL of alkane standard C_{21}-C_{40} were pipetted in an empty HS-vial, equilibrated at 120 °C for 30 min and extracted at 120 °C for one hour. The oven was kept at 35 °C for 2 minutes (during desorption), then heated with 5 °C min^{-1} to 280 °C (250 °C for polar column), which was kept for 10 minutes.

4.3.3 Online-derivatisation and GC-MS method for the determination of non-volatile, polar metabolites

200 μL of the upper polar phase were dried under a constant flow of nitrogen in microinserts in GC vials. Dried samples were stored at 4 °C on the autosampler. 40 μL MOX (20 $mgmL^{-1}$) were added from the right autosampler arm and the samples were shaken at 60 °C for 90 min in the right agitator. Samples were transported back to cooled tray before 50 μL MSTFA and 10 μL IS (370 mgL^{-1} nonadecanoic acid methylester) were added from the right autosampler arm and samples were shaken for 60 min at 60 °C in the left agitator. Samples were kept for 10 min at 4 °C to allow sedimentation of particles. Then 1 μL of liquid sample was injected using the left autosampler arm in the injector at 250 °C , holding a split/splitless glass liner in splitless mode and using a split of 1:10. A constant flow of 1 $mLmin^{-1}$ helium) was used. An HP5-ms column (30 m x 0.25 mm x 0.25 μm) from Agilent Technologies (Waldbronn, Germany) was used for chromatographic separation. The oven was kept at 50 °C for 2 min, heated with 10 °Cmin^{-1} to 310 °C which was kept for 15 minutes. The MSD interface was kept at 310 °C throughout the run. After 7 minutes of solvent delay, the detector was used in scan mode m/z 50-1000. MSD ion source was kept at 230 °C, MSD quad at 150 °C. The MSD was autotuned at least once

a week or after exchange of spare parts, e.g. the liner (every 100 samples).

4.3.4 Data processing and statistical evaluation of volatile metabolites

MetaboliteDetector 2.07 (Hiller et al., 2009) was used for data processing, including deconvolution, RI calculation, component detection, comparison with in-house library entries and quantification of selected ions. GC-MS spectra were collected in centroid mode, therefore the standard settings for centroidation in MetaboliteDetector were used: peak threshold begin 10, peak threshold end 5, maximum baseline distance 30, FHWM 0.5. The settings for deconvolution of mass spectra were optimized for standard substances which resulted in the following settings: peak threshold 10, minimum peak height (noise units) 7, no baseline adjustment, 10 bins/scan, deconvolution width (scans) 5, required intensity (% base peak) 5, required number of peaks 3. Alkanes standards C_5-C_{20} were used for RI calculation of compounds detected in samples. For annotation of metabolites an in-house library for volatiles was build and used, see chapter 4.4.1, a spectral match of the compound of at least 80 % compared to the library entry and a RI deviation of maximum 20, peak purity 0.5, cutoff score 0.8 and a mass filter of m/z 0-45 were set in MetaboliteDetector. MetaboliteDetector was used for data processing of blank samples as well as biological samples. The metabolites detected in blank samples were eliminated from the list of metabolites. Only metabolites present in at least 2 of 3 biological replicates were considered for qualitative data evaluation.
Quantification ions for standard substances were determined by the software automatically and corrected manually in the library editor. For quantification, the minimal distance between subsequent ions was set 0.5, minimal required quality index 1, excluded ions for annotation and quantification were m/z 0-45, 207, 221, 281, 355.
R 2.13.0 (R Development Core Team, 2010) was accustomed for plotting signal intensities of detected compounds from MetaboliteDetector and statistical evaluation. ValiData (Koeck et al., 2012) is a Microsoft Excel macro used for method validation.

4.3.5 Data processing and statistical evaluation of non-volatile, polar metabolites

MetaboliteDetector was used for data processing of non-volatile, polar metabolites as well as for the volatile metabolites. GC-MS spectra were collected in centroid mode, therefore the standard settings for centroidation in MetaboliteDetector were used here as well: peak threshold begin 10, peak threshold end 5, maximum baseline distance 30, FHWM 0.5. The settings for deconvolution of mass spectra were optimized for standard substances which resulted in the following settings: peak threshold 5, minimum peak height (noise units) 5, no baseline adjustment, 10 bins/scan, deconvolution width (scans) 7, required intensity (% base peak) 0, required number of peaks 30. Alkanes standards C_8-C_{20} and C_{21}-C_{40} were used for RI calculation of compounds detected in samples. An in-house library for non-volatile, polar metabolites was build and used, which will be explained in chapter 4.6.1. For annotation of metabolites, a spectral match of the compound of at least 80 % compared to the library entry and a RI deviation of maximum 20, peak purity 0.5, cutoff score 0.8 and a mass filter of m/z 0-50 were set in MetaboliteDetector.
Quantification ions for standard substances were determined by the software automatically

and corrected manually in the library editor. For quantification, the minimal distance between subsequent ions was set 0.5, minimal required quality index 1, excluded ions for annotation were m/z 0-50, 207, 221, 281, 355 and for quantification m/z 0-50, 73, 147, 207, 221, 281, 355.

4.4 Development and evaluation of HS SPME GC-MS methods

4.4.1 Built-up of an in-house library for volatile metabolites containing mass spectra and RIs of authentic standards and annotated metabolites

To develop and establish a GC-MS based method for metabolic profiling of volatiles in *Fusarium* and wheat, a library containing mass spectra and RIs from authentic standards and annotated metabolites, is prerequisite. Throughout the thesis, mass spectra and RIs were measured and collected in an in-house library for volatiles.
For authentic standard, standard solutions containing 0.1-62.5 μgL^{-1} of each standard were prepared by dilution with 50 % aqueous acetonitrile (v/v) from stock solutions. The extraction of authentic standards, which were purchased for the confirmation of annotations of fungal metabolites, was performed as described in 4.3.1, whereas reference substances, which were used to confirm annotations of plant metabolites, were extracted as described in 4.3.2.
Moreover, annotated metabolites using Automated Mass Spectral Deconvolution and Identification System (AMDIS) (Stein, 1999) and Wiley 8th and NIST/EPA/NIH Mass Spectral Library - Version 2008, which were detected in metabolomics experiments of fungal cultures or wheat samples, were manually inspected using MSD Chemstation. Mass spectra and RIs from authentic standards and annotated metabolites were added to a user-defined library in MSD Chemstation. This library was converted from '.D' to '.msl' format using Lib2NIST converter, which is a tool from NIST Mass Spectral Database purchased together with MSD Chemstation. RI values from measurements of authentic standards and median RIs from NIST Chemistry WebBook (NCWB) were added to the mass spectra manually. The '.msl' file was imported to MetaboliteDetector where it was used to create an in-house library for volatile metabolites. Furthermore, mass spectra and RIs from metabolites, which were detected repetitive in metabolomics experiments of fungal cultures or wheat samples, but could not be annotated, were manually inspected and added to the in-house library. In the final '.lbr', the in-house library file for volatiles in MetaboliteDetector, ions for quantification were selected for each reference substance.

4.4.2 RI accuracy and reproducibility

Since the RI information (besides the mass spectral information) is required for compound annotation in the field of metabolomics using GC-MS, the accuracy, precision and reproducibility of experimentally determined RI values was studied before the average values were added to the in-house library for volatile metabolites.
The RI accuracy and precision of 29 authentic standards was monitored over half a year on three columns with an apolar stationary phase (DB-5ms, HP5-ms) and one column with a polar stationary phase (DB-Wax) on both GC-MS instruments available using HS SPME. Standard solutions were measured individually and in mixtures (up to 10

standard substances per mixture), in concentrations of 6.25-625 mgL^{-1} in 50% aqueous acetonitrile. The measured retention time values were used to calculate the KI values according to equation 3.2 and the LTPRI values according to 3.3 (see page 37). Median values of the KI and LTPRI values listed in NCWB were used for comparison with the arithmetic mean (average) of the experimentally determined KI and LTPRI values and calcuation of the accuracy. RI median values from NCWB were used because the range of LTPRIs listed was often very big and the values representing the most often measured values were assumed to be more reliable than the arithmetic mean or any of the single values. R was used to build boxplots showing the median, the 25th (lower quartile) and 75th (upper quartile) percentiles and the whiskers, which correspond to 1.5 times the interquartile range.
The following influences on the RI values were studied regarding reproducibility of the experimental LTPRI values: replacement of the guard column (independent from the chromatographic column), column ageing, heating rate, standard concentration measured (regarding quantitiy loaded on the column) and potato dextrose agar (PDA), the cultivation medium for fungal cultures. Different heating rates in the oven were: 3 °C min^{-1}, 6 °C min^{-1} and 10 °C min^{-1} from 40 to 280 °C (250 °C for polar column) and 100 °C isothermal.
The absolute standard deviation σ was used as a measure of the precision and the reproducibility for the experimentally determined RI values.

4.4.3 Spore production and subsequent cultivation of *F. graminearum*

Cultivation was essentially performed as described in Stoppacher *et al.* (Stoppacher et al., 2010) and Kluger *et al.* (Kluger et al., 2012). In brief, *F. graminearum* PH-1 was grown on an FMM (1 g KH_2PO_4, 0.5 g $MgSO_4$*$7H_2O$, 0.5 g KCl, 2 g $NaNO_3$, 30 g sucrose, 20 g agar agar, 10 mg citric acid, 10 mg $ZnSO_4$*$6H_2O$, 2 mg $Fe(NH_4)2(SO_4)2$*$6H_2O$, 1 mg H_3BO_4, 0.1 mg $MnSO_4$, 0.1 mg Na_2MoO_4*2 H_2O, 0.5 mg $CuSO_4$*5 H_2 in 1 L deionized water) plate at 20 °C in the dark until the mycelium reached the edge of the Petri dish. Small pieces of the mycelium were suspended in 50 mL of mung bean substrate (MBS) medium and shaken in baffled flasks at 120 rpm at 20 °C until spores were formed (typically 2-4 days). The spores were separated from the mycelium by filtration through glass wool filters, concentrated and resupended in sterile water. The spore count was determined using a hemocytometer (Fuchs-Rosenthal chamber).

4.4.4 Influences from the cultivation medium and flushing with synthetic air before HS SPME on the volatile metabolite pattern of *F. graminearum* PH-1 cultures

The experimental conditions to study living fungal cultures, can influence the biosynthesis of volatiles and therefore, the results of a metabolomics experiment. The following three different cultivation media were used for the cultivation of *F. graminearum* PH-1 cultures: potato dextrose agar (PDA), Czapek Dox and *Fusarium* minimal medium (FMM). 2 mL of each PDA and FMM (with agar) were placed in separate, horizontally laid HS-vials. This bottom medium was covered with 1 mL 0.8 % agarose (top agar, kept at 35 °C to

stay liquid) containing 10^3 fungal spores (spores were added shortly before placing the top agar on the bottom agar). In parallel, 2 mL of each FMM without agar and Czapek Dox were pipetted in vertically placed (free standing) HS-vials. The liquid media were directly inoculated with the same amount of spores. The fungal cultures were grown for five days at 22 °C in the dark. Each day, two cultures were flushed with synthetic air and measured six hours later to determine the number of volatile metabolites produced on each medium.

Moreover, the influence from flushing the fungal cultures prior to HS SPME with synthetic air was determined in this experiment. Fungal cultures were cultivated at 22 °C in the dark up to 172 hours. Three cultures per sampling point (28, 35, 52, 59, 76, 83, 100, 107, 124, 131, 148 and 172 hours) were consecutively flushed with synthetic air six hours before measurement (flushed cultures). In parallel, three cultures per sampling point were consecutively measured without prior flushing (non-flushed cultures). This resulted in three independent, biological replicates per treatment per sampling point. This experiment was performed twice.

Extraction using the DVB/CAR/PDMS coated fiber was carried out essentially as described in chapter 4.3.1, but at 22 °C (from sample tray) for 52 minutes instead of 30 °C. The analytes were separated on an DB5-ms column and introduced to the MS interface via a non-coated guard column. The oven was kept at 40 °C for 2 minutes, then heated with 5 °C min^{-1} up to 280 °C, and with 70 °C min^{-1} up to 350 °C for cleaning. The scan range was set at m/z 45-270.
Data processing was carried out using MetaboliteDetector. The growth medium was measured as blank sample. The metabolites detected in the blank samples were not considered biologically relevant. Only metabolites which were detected in at least two of three biological replicates from one experiment were considered for data evaluation.

4.4.5 Qualitative, non-targeted characterization of the volatile profile of *F. graminearum* PH-1

Regarding FHB disease, two interaction partners are involved: *Fusarium* and wheat. The individual study of volatiles produced from fungal cultures and wheat ears is important prior to interaction studies between fungus and wheat. Starting with the metabolic profiles of fungal cultures, the HS SPME GC-MS method was evaluated for the qualitative, non-targeted characterization of *F. graminearum* PH-1.
For this purpose, 2 mL PDA were put into a 20 mL HS-vial in a laminar flow workbench. After cooling of PDA, 8000 spores (preparation of spores and inoculation see 4.4.3) were pipetted on top of PDA and the HS-vials were closed immediately with screw caps containing 1.3 mm gas-tight silicone/teflon septa. All fungal cultures were cultivated at 22 °C in the dark up for defined time periods to one week. They were flushed six hours before measurement with synthetic air.

The DB-Wax and an HP5-ms column were used for chromatographic separation (the guard column was not installed). The oven was kept at 40 °C for 2 minutes, then heated with 6 °C min^{-1} up to 280 °C, and with 70 °C min^{-1} up to 350 °C for cleaning (250 °C for polar column). The scan range was set at m/z 45-270. MetaboliteDetector was used for data processing, see 4.3.4.

4.4.6 Determination of mycotoxins in *F. graminearum* PH-1 cultures on PDA using LC-MS/MS

Correlating with the production of volatile precursors, like trichodiene, the presence of mycotoxins, like DON, is expected. To get a greater picture of the mycotoxin pattern that is produced by *F. graminearum* PH-1 under the experimental conditions for HS SPME GC-MS, the multimethod for mycotoxins using LC-MS/MS was applied to these fungal cultures.
After HS profiles were determined according to 4.3.1, liquid extraction and the determination of mycotoxins was performed by Michael Sulyok from the Center for Analytical Chemistry using LC-MS/MS. 10 mL of (acetonitrile/water/acetic acid 79:20:1, v/v/v) were added for extraction, which was carried out at 22 °C shaking for 90 minutes. Subsequently, samples were centrifuged for 2 min at 3,000 rpm (15 cm radius). Aliquots of 100 μL of raw extract were transferred into glass vials equipped with glass microinserts. Samples were diluted with the same volume of dilution solvent (acetonitrile/water/acetic acid 20:79:1, v/v/v), vortexed and 5 μL were injected without further pretreatment.
Instrumental parameters for LC-MS/MS instrumentation see (Vishwanath et al., 2009).

4.4.7 Quantification of fungal volatiles using HS SPME

The purpose of the evaluation of matrix effects was to study the factors affecting extraction efficiency of the DVB/CAR/PDMS coated fiber regarding the different chemical classes of metabolites synthesized by *F. graminearum* PH-1. Therefore, the SPME extraction efficiency of a single component in the HS of a 20 mL HS-vial was analyzed in the presence of different matrix components: in presence of nutrition medium PDA, in presence of other HS constituents of fungal cultures (the effect of other volatiles on sesquiterpenes and the effect of sesquiterpenes on others) and in presence of fungal mycelium. These experiments were carried out in four spiking experiments.

Influence of the nutrition medium PDA on the SPME efficiency of target analytes

This spiking experiment was carried out for each target analyte (representing different chemical classes) individually. 10 μL of calibration solution of target analyte (0.1-10 mg L^{-1} in 50 % aqueous acetonitrile) were pipetted on top of 2 mL solidified PDA in HS-vials (corresponding to fungal cultures). As a reference, 10 μL were pipetted in empty HS-vials. The HS-vials were kept on the sample tray of the instrument at 22 °C at least an hour before measurement.

Influence of other volatiles on the extraction efficiency of the sesquiterpene thujopsene

For thujopsene calibration, solutions of 0.1-10 mg L-1 thujopsene in 50 % aqueous acetonitrile were used. 10 μL were pipetted onto PDA and into empty HS-vials. The influence of other volatiles on the extraction efficiency of thujopsene was tested by the addition of three volatile mixtures, in dilutions of 1:4, to different concentrations of thujopsene in HS-vials.

Influence of the sesquiterpenes on the signal intensity of other target analytes

To determine the influence of the sesquiterpenes on the extraction efficiency of other volatiles, (+)-α-longipinene, (-)-α-cedrene, (-)-thujopsene, (+)-aromadendrene and farnesene (final concentration of 10 mg L-1 each in 50 % aqueous acetonitrile (v/v)) were added to different concentrations of 3-methyl-1-butanol, 3-octanone and S-(-)-limonene (0.1-5 mg L-1 in 50 % aqueous acetonitrile). 10 μL were pipetted on top of 2 mL PDA and in empty HS-vials.

Influence of the fungal mycelium on the extraction efficiency of volatiles

F. graminearum PH-1 was cultivated on 2 mL PDA. The fungal cultures were flushed with synthetic air directly before the first measurement. After the measurement, the same fungal culture was flushed again with synthetic air and agar which was not covered with mycelium was spiked with 10 μL of a mixture of different metabolites (no direct contact with mycelium) to test the influence of the mycelium on their extraction efficiencies. A mixture of the following substances at the given concentration was tested: 1.6 g L^{-1} ethyl acetate, 100 mg L^{-1} 2-methyl-1-propanol, 100 mg L^{-1} 3-methyl-1-butanol, 1 mg L^{-1} p-xylene, 0.2 mg L^{-1} (+)-alpha-pinene, 0.2 mg L^{-1} beta-myrcene, 0.2 mg L^{-1} p-cymol, 0.2 mg L^{-1} S(-)-limonene, 0.2 mg L^{-1} terpinolene, 10 mg L^{-1} isobutyl acetate, 10 mg L^{-1} 1-octen-3-ol, 10 mg L^{-1} 3-octanone, 10 mg L^{-1} 2-heptanone, 10 mg L^{-1} (+)-alpha-longipinene, 10 mg L^{-1} (-)-alpha-cedrene, 10 mg L^{-1} (-)-thujopsene and 10 mg L^{-1} (+)-aromadendrene. This volatile mixture corresponded to previously estimated amounts of volatiles produced by the fungal cultures. The experiment was repeated with three independent fungal cultures. As a reference, 10 μL of 50 % aqueous acetonitrile were spiked on PDA and in empty HS-vials. The recovery rate was determined by comparison of signal intensities for each concentration and each analyte individually: $c_{fungal+added}/(c_{fungal}+c_{added})*100=\%$ recovery, with c being the concentration of analyte produced by the fungus (fungal) and spiked (added).

An HP5-ms column was used for chromatographic separation (the guard column was not installed). The oven was kept at 40 °C for 2 minutes, then heated with 6 °C min^{-1} up to 280 °C, and with 70 °C min^{-1} up to 350 °C for cleaning (250 °C for polar column). The scan range was set at m/z 45-270. MetaboliteDetector was employed for data processing and quantification of selected ions. The slope of the recovery function (obtained from linear regression of concentration added versus concentration measured) was taken as a measure of matrix effect. The coefficient of variation (CV_{method}) was calculated using ValiData, which is a makro for method validation in Microsoft Excel, and used as a measure of method precision.

Quantification of the sesquiterpenes

Although there are matrix effects observed that cannot be overcome by matrix calibration on PDA for minor constituents, the sesquiterpenes, the dominating group of volatiles produced by *F. graminearum* PH-1, were relatively quantified using commercially available reference substances for the calibration. Regarding FHB disease progress, it is very interesting to determine the time-dependent production of volatiles, especially precursors for mycotoxins, which are sesquiterpenes in case of trichodiene or longiborneol.
For time series experiment, fungal cultures of *F. graminearum* PH-1 were cultivated for a maximum of 131 hours in HS-vials. Three independent fungal cultures for each of ten

time points (28, 35, 52, 59, 76, 83, 100, 107, 124 and 131 h after inoculation) were flushed with synthetic air and extracted to determine sesquiterpene production over cultivation time (corresponding to time after inoculation). The signal intensities of three independent fungal cultures which were sampled at the same time point were averaged. Limit of detection (LOD) and quantification (LOQ) were calculated from signal to noise ratios (S/N) of the lowest calibration concentration of each sesquiterpene on PDA from MSD Chemstation and MetaboliteDetector, which could be reliably annotated and quantified.

4.5 Application of HS SPME GC-MS methods

The established HS SPME GC-MS method for the metabolic profiling of fungal volatiles was applied for the following experiments: the comparison of the volatile metabolic profile of *F. graminearum* PH-1 with four strains of *F. graminearum* of the Northland population and the Δ*tri5* mutant cultivated in the dark and in the presence of light. The results should provide information about influences on the volatile biosynthesis due to genetic and environmental changes, which could help reveal metabolic pathways involved in the fungal pathogenesis.

4.5.1 Qualitative, targeted and non-targeted characterization of four *Fusarium graminearum* strains of the Northland population

Strains of *F. graminearum* of the Northland population (Gale et al., 2010), were shown to produce no known trichothecene, but a lot of zearalenone and a novel mycotoxin. Regarding metabolic pathways for fungal virulence, it is interesting to know whether trichodiene is not produced and/or if other sesquiterpenes are synthesized indicating other metabolic pathways besides *tri5* gene induced DON production. *Fusarium*, which is commonly used used by many researchers since its complete genome sequence was determined (Cuomo et al., 2007), is used as a reference strain for the sesquiterpene production in general, and as a reference for the HS SPME method for the successful biosynthesis of trichodiene because no reference substance was available.
In total, fungal cultures of five different *Fusarium graminearum* strains were studied in this experiment: *F. graminearum* N2, N3, N4, N5 (Northern population) and PH-1 (reference). Spore suspensions of each *Fusarium* strain were provided by Gerlinde Wiesenberger from the Departement of Applied Genetics and Cell Biology. All fungal cultures in HS-vials were cultivated at 22 °C in the dark for 60, 70, 90, 100, 120 and 140 hours. They were inoculated at different time points to allow consecutive measurements of all fungal cultures on one day. The HS SPME was performed at 22 °C for 52 minutes. The analytes were separated on an DB5-ms column and introduced to the MS interface via a non-coated guard column. This experiment was carried out once. The abundances of the detected sesquiterpenes using MetaboliteDetector were compared over the cultivation time for five different *Fusarium*strains. The 2D plots of this time dependent sesquiterpene synthesis were constructed with R.

4.5.2 Comparison of the volatile metabolic profiles of *F. graminearum* PH-1 wild-type and Δ*tri5* mutant

To study fungal cultures of gene disruption mutants, like Δ*tri5* mutant, it is necessary to get a better understanding of single metabolic pathways, which are related to fungal virulence. DON was shown to be a virulence factor during FHB disease (Bai et al., 2001). For the biosynthesis of DON, trichodiene is the volatile precursor, synthesized from FPP by the trichodiene synthase. The Δ*tri5* mutant has no active trichodiene synthase, therefore no trichodiene is expected in the volatile metabolic profiles of the mutant. Three experiments were performed for comparison of fungal cultures of *F. graminearum* PH-1 wild-type and Δ*tri5* mutant: the comparison of volatile metabolites produced by wild-type and mutant in plant tissue culture boxes (plastic boxes), the influence from cultivation in the light compared to the dark, cultivation in glass HS-vials compared to plant tissue culture boxes.
10^5 spores were used for inoculation of 25 mL PDA in plant tissue culture boxes. These plastic boxes (plant tissue culture boxes, so called Magenta vessels) were purchased from Sigma Aldrich (Vienna, Austria). They are made out of polycarbonate and have a polypropylene lid. A small hole in the center of the lid was drilled manually. It was closed with 1.3 mm gas-tight silicone/teflon septum for automated HS SPME and covered with a 4 inch laboratory film from Pechniney plastic packaging (Manesha, WI, USA). The plant tissue culture boxes were autoclaved before 25 mL sterile PDA (20 g L^{-1}) were put into each box. Two plastic boxes were placed in a self-made wooden rack which fitted to one tray holder (see figure 4.1), resulting in one fungal culture of PH-1 and Δ*tri5* for each experiment under each condition. The fungal cultures were not flushed with synthetic air prior to extraction. The accumulated amount of the non-flushed fungal cultures was measured every four hours (every six hours in the third experiment) from the same box resulting in high time resolution for the time-series experiments.

Figure 4.1: Fungal cultures of *F. graminearum* PH-1 wild-type and Δ*tri5* mutant in HS-vials and plant tissue culture boxes (septa manually adjusted) in wooden rack.

To cultivate fungal cultures in plant tissue culture boxes under conditions similar to daylight, self-made wooden boxes with 10 constantly distributed light emitting diodes (LEDs), emitting cold-light of 1600 mcd (milli Candela) each were used, see figure 4.2. Before each LED, resistors of 100 Ω were placed to reduce the voltage from the battery (5 V) to 3.2 V. The LEDs were run in parallel mode. The distance from fungal culture to LED was approximately 8 cm, therefore approximately 25000 lux were used for daylight simulation.

Figure 4.2: Wooden box with 10 LEDs in parallel mode was placed above fungal cultures in plant tissue culture boxes. The light was switched on for fixed time intervals, additionally the box prevented from uncontrolled influences from light because it was covered with black paper on top.

HS SPME was carried out essentially as described in chapter 4.3.1 with the following differences: Extraction was done for 52.5 min with an injection penetration of 57 mm at 22 °C. The fiber was baked out for 5 min in the needle heater with a penetration depth of 43 mm. The analytes were separated on an DB5-ms column at 40 °C for 2 min, then with 6 °C min^{-1} to 280 °C which was kept for one minute. They reached the interface to the MSD via a non-coated guard column. The scan range was set at m/z 45-400 after a solvent delay of 3 min.

4.5.3 Volatile metabolic profiles of wheat ears of different developmental stages and *F. graminearum*-infected ears

To collect mass spectra and RI values from plant volatiles for the in-house library for volatiles, healthy wheat ears of different developmental stages and *Fusarium*-infected wheat ears were analysed using HS SPME GC-MS. Furthermore, the DON and ergosterol content in infected wheat ears were determined using LC-MS/MS.

Treatment and sampling of wheat ears from cultivar 'Capo'

Wheat ears of cultivar 'Capo' were inoculated at anthesis with 1 mL of a *F. graminearum* conidial suspension (10,000 macroconidia mL^{-1}) by Marc Lemmens from the Institute of Biotechnology in Plant Production. All wheat ears were harvested on the same day, picking ears of various developmental stages, see figure 5.17 on page 109: 27 healthy wheat ears, including 8 wheat ears before, 9 during, 10 after flowering and 9 infected wheat ears. Immediately after harvest, the metabolic activity in wheat ears were quenched by immediate cooling with liquid nitrogen (one wheat ear was placed in 50 mL tubes and liquid nitrogen was put over the wheat ear), ground in a Retsch mill using a 50 mL beaker for 2 min at 30 Hz and stored at -80°C until extraction. 100 ± 2 mg of each sample were weighted-in per HS-vial.

Volatiles from ground wheat samples were extracted according to 4.3.2. An HP5-ms and the OPTIMA Wax column were used for chromatographic separation. The guard column leading to the MS interface was installed. The mass range of m/z 35-500 was scanned.

MetaboliteDetector was employed for data processing. The parameters for MetaboliteDetector were listed in 4.3.4 on page 57 and were not changed. Blank samples, where the

fiber was inserted into an empty HS-vial, were analysed together with biological samples. Compounds detected in blank samples were not considered for data evaluation. The list of compounds detected in the samples was exported from MetaboliteDetector to Microsoft Excel. The list was compared to raw data using MSD Chemstation and only compounds which were detected on both columns of different polarity were listed. Student's t-test was performed for annotated sesquiterpenes and sesquiterpenes, which could not be annotated according to the set criteria, if the metabolites was detected in at least 5 samples of one sample type.

Extraction of DON and ergosterol from wheat samples

100 ± 1 mg ground wheat samples were weighted into 1.5 mL tubes. 400 μL deionized water was added and samples were shaken at 22 °C for 90 minutes. Then 400 μL n-hexane/isopropanol (97/3, v/v) were added and samples were shaken for another 30 minutes. After centrifugation, the water phase was used for determination of DON and hexane/isopropanol phase was used for quantification of ergosterol.

Determination of DON using LC-MS/MS

The presence of DON in wheat samples was tested using LC-MS/MS by Heidi Schwartz from the Center for Analytical Chemistry. Aqueous extracts of wheat samples were measured using a 2000 QTrapLC-MS/MS system (AB Sciex, Foster City, CA) equipped with an APCI source. The chromatographic separation was performed on an Agilent 1100 HPLC system (Agilent, Waldbronn, Germany). A C8 Zorbax Eclipse column (150x4.6 mm, 5 μm) was used as stationary phase. The injection volume of the samples was 25 μL and 1 mLmin^{-1} flow rate was set using a gradient system. In this method, the composition of mobile phase A was 20 % aqueous methanol with 5 mM ammonium acetate, mobile phase B consisted of 90 % aqueous methanol with 5 mM ammonium acetate. The gradient started with 100 % A and was changed to 100 % B after 2 minutes. 100 % B was kept for 3 minutes, in the following 0.1 min it was switched to 100 % A again, which was kept until end of the method. The runtime of the method was 7.5 minutes. The samples were analyzed in multiple reaction monitoring (MRM) mode and in negative polarity. The qualifier and quantifier of MRM transitions for DON were set as follows: (quantifier) m/z 355.1 > 59.1 (CE:-30 eV); (qualifier) m/z 355.1 > 265 (collision energy (CE):-16 eV). The source temperature was 450 °C, the declustering potential for the transitions was -16 V and dwell time was 25 ms.

Determination of ergosterol using LC-MS/MS

Mehrdad Shams had established an LC-MS/MS method for the quantification of ergosterol using a 4000 QTrapLC-MS/MS system (AB Sciex, Foster City, CA) equipped with an APCI turbo V source, which he used to measure the ergosterol content of the infected wheat ears. At this time point of the thesis, the ergosterol method using GC-MS was not available. The stationary phase was a Phenomenex Gemini RP-C18 column (150x4.6 mm, 5 μm). The injection volume of the sample extract was 2 μL and the flow rate was set to 2 mLmin^{-1} using an isocratic system. In this method, the composition of mobile phase remained constant (100 % methanol) throughout the entire procedure. The runtime of the method was set to 5 minutes. UV detection was performed at 220 nm on the diode-array detector coupled to 1100 HPLC. Afterwards,the samples were analyzed in single reaction monitoring (SRM) mode and in positive polarity. The quantifier and qualifier of

SRM transitions for ergosterol were set as follows: (quantifier) m/z 379.3 > 105.1 (CE:60 eV); (qualifier) m/z 379.3 > 125.3 (CE:20 eV). The source temperature was 450 °C, the declustering potential was 50 V and dwell time was 100 ms.

4.5.4 Comparison of volatile metabolic profiles of different genotypes after infection with *F. graminearum*

Metabolic differences regarding volatiles between healthy and *Fusarium*-infected wheat ears of two genotypes, the cultivars 'Remus' (susceptible to *Fusarium* infection) and 'CM' (resistent against FHB) were studied.

Treatment and sampling of wheat ears of different genotypes

Wheat ears were inoculated at anthesis with 100 μL in total of a *F. graminearum* IFA65 conidial suspension (50,000 macroconidia mL^{-1}) applied in ten neighbouring spikelets. As a reference, wheat ears (10 spikelets per ear) were treated with water (healthy wheat ears). Ten spikelets per wheat ear were harvested immediately after treatment (0 h), 12, 24, 48 and 96 hours after treatment. Five wheat ears were harvested at each time point to have five biological replicates, each consisting of ten treated spikelets per wheat ear. All samples were shock-frozen in liquid nitrogen, ground in a Retsch mill and stored at -80°C until extraction. 100 ± 2 mg of each sample were weighted-in per HS-vial. The plant breeding and cultivation part, as well as the treatment and quenching was performed by Andrea Koutnik and Imer Maloku from the Insitute of Biotechnology in Plant Production.

HS SPME GC-MS method was performed according to 4.3.2. Only an HP5-ms column was used for chromatographic separation. One sequence contained the measurements of all wheat samples from one genotype, plus timely equally distributed measurements of blank samples and alkanes. Blank samples (empty HS-vials) were measured three times per measurement sequence. Alkane standards C_5-C_{10}, C_8-C_{20} and C_{21}-C_{40} were measured four times per sequence. Data processing was performed with MetaboliteDetector. Blank samples, where the fiber was inserted into an empty HS-vial, were analysed together with biological samples. The list of compounds detected was exported from MetaboliteDetector to Microsoft Excel. All signal intensities were divided by the sample weight (100±2 mg) and autoscaled to be equally weighted in statistical evaluation. The ward method was used for hierarchical clustering displayed in the heatmap. Single value decomposition (SVD) method was used for PCA.

4.6 Development and evaluation of online-derivatisation GC-MS methods

4.6.1 Standard measurements for built-up of an in-house library for non-volatile, polar metabolites

Besides the HS SPME methods for the volatile metabolites, a two-step, online-derivatisation GC-MS method was developed for the determination of non-volatile, polar metabolites in wheat ears. For the identification of non-volatile, polar metabolites, standard substances were purchased and collected from another metabolomics group. The mass spectra and RIs of these standard compounds were measured and collected in an in-house library for

non-volatile, polar metabolites.
For this purpose, standard solutions of single standards were prepared as described in chapter 4.1.2. Mixtures from Bielefeld were measured in the delivered concentration. Mass spectra and RI from these standard solutions and mixtures were measured on an HP5-ms column, manually inspected using MSD Chemstation and stored in an in-house library for non-volatile, polar metabolites. As described in 4.4.1, a user-defined library was created in MSD Chemstation, converted to '.msl' format and imported in MetaboliteDetector. The ions for quantification for each reference substance were selected in MetaboliteDetector and stored in the final '.lbr' file, the in-house library for non-volatile, polar metabolites.
Online-derivatisation and GC-MS analysis was carried out according to 4.3.3, with the following changes: 20 μL of standard solutions were dried and derivatised with 60 μL MOX and 100 μL MSTFA. No IS was spiked to the standard solutions. The GC instrument was run in pulsed splitless mode (30 psi for 2 min, then adjusting to constant flow of 1 mLmin^{-1} helium).

4.6.2 Qualitative analysis of non-volatile, polar metabolites from wheat ears under different extraction conditions

In a pilot experiment, the qualitative metabolic profile of non-volatile, polar metabolites from a wheat ear sample was determined under different extraction conditions. The number of detected compounds and the precision of each extraction method were used to decide on a suitable protocol for future metabolomics experiments using the same extract for GC-MS and LC-HRMS analysis.
The wheat ear sample consisted of residues from wheat ear powder from cultivar 'Remus' und 'Capo' during and after flowering. The frozen sample was homogenized in a 50 mL beaker, precooled with liquid nitrogen, for 2 min at an oscillating frequency of 30 Hz using the Retsch mill. Still placed in liquid nitrogen to prevent thawing, 100±2 mg were weighted in 1.5 mL tubes for each sample, which were frozen immediately in liquid nitrogen and kept at -80 °C until extraction. Six replicates were prepared for five sets of wheat ear samples (in total 30 samples).
Four sets of wheat ear samples were extracted with 1 mL extraction solution (MeOH:H2O 3:1 (v/v)) and agitated for 10 sec. Subsequently, one set of wheat samples was extracted for 15 min at 4 °C (on ice in a 4 °C laboratory), the second set at 22 °C (room temperature), the third set at 60 °C (in a thermomixer) and the forth set in an ultrasonication bath at 22 °C. The fifth set of wheat ear samples was extracted with 1 mL extraction solution (MeOH:H2O 3:1 (v/v)), with the addition of 0.1 % HCOOH, agitated for 10 sec and incubated in an ultrasonication bath for 15 min, according to (De Vos et al., 2007)). All samples were centrifuged for 4 min at 14 000 g (9500 rpm). On the bottom of the tube, the cellular pellet, which contained the proteins, was observed but not further analysed. 200 μL of the supernatant were transferred into a new 1.5 mL tube, 10 μL internal standard solution (500 mgL^{-1} valine-d8, succinic-d4-acid, $^{13}C_6$-glucose), 75 μL $CHCl_3$ and 150 μL H_2O were added for fractionation of the polar and the nonpolar phase. Samples were agitated and centrifuged for 2 min at 14 000 g. Only the polar phase was analysed using the online-derivatisation GC-MS method described in chapter 4.3.3. The fractionation step was therefore used as a cleaning step of the polar metabolites from the nonpolar metabolites. MetaboliteDetector was accustomed for the data evaluation. The settings are described in chapter 4.3.5. The RSD values of the integrated signal intensities of the quantification ions of detected metabolites were used as a precision measure for each

metabolite.

4.7 Application of online-derivatisation GC-MS methods

The basic priniciple of the two-step, online-derivatisation GC-MS method was used to develop target methods for specific fungal and plant sterols and for the phytohormone ACC and its degradation product 2-ketobutyric acid.

4.7.1 Target analysis of ergosterol, sitosterol, stigmasterol

The study of selected analytes is of special interest to collaborators, who can focus gene alterations on certain parts in the metabolic pathway or the inhibition/enhancement of important end products. Ergosterol is present in high amounts in the fungal membrane and not present in plants, animals or bacteria. It is considered as a fungal biomarker for fungal biomass. For plant biomass, β-sitosterol and stigmasterol are candidate biomarkers. These three sterols are structurally closely related and were considered to show the same extraction efficiency. The derivatisation conditions were optimized from the online-derivatisation GC-MS method for metabolic profiling of non-volatile, polar metabolites for the three sterols of interest.
The amount of ergosterol, sitosterol and stigmasterol was determined in *Fusarium*-infected maize seedlings in seven different maize cultivars. The cultivars were compared for their suitability for further studies using *F. graminearum* PH-1 for infection. The cultivation, harvesting and extraction were carried out by Gerlinde Wiesenberger from the Department of Applied Genetics and Cell Biology.

Cultivation, harvesting and extraction of maize seedlings of seven different cultivars

Seeds of seven different maize cultivars ('Fortress', 'Marcello', 'Eduardo', 'Gilberto', 'Ginko', 'Okato' and 'Pandoso') were sterilized. Five seeds per box were placed on veryMS agar in plant tissue culture boxes. Six boxes were incubated at 20 °C, 55 % relative humidity and 15/9 hours light (room E.2.4.151). Six other boxes were incubated at 55 % humidity, 15/9 hours light/dark and 20/10 °C temperature (climate chamber). Two out of six boxes were infected with 10^2 spores of *F. graminearum* PH-1 501 (no. 378 from Marc Lemmens from the Institute of Biotechnology in Plant Production) two days after seedling using 4 mL topagar (0.5 % LMP Agarose in water, autoclaved in aliquots, equilibrated at 35 °C). The plants were incubated for two weeks in the climate room and for three weeks in the climate chamber (due to slower growth). Pictures were taken after 12 days of all boxes.
For the extraction of the sterols, cultivation medium was carefully removed, the plants were rinsed with water and dried. Leaves and roots were cut into small pieces and the plant fresh weight was determined. 60 mL of pre-cooled acetone was added and mixed in a blender (mix 30 sec, wait 30 sec, mix 30sec). Extracts were centrifuged for 10 min at 4000 rpm at 4 °C. Aliquots of the supernatant were filled in 10 mL HS-vials and cooled at 4 °C until measurement.

Target analysis method for the sterols

Ergosterol, β-sitosterol and stigmasterol were purchased from Sigma Aldrich (Vienna, Austria). Stock solutions of 1 gL^{-1} were prepared individually in $CHCL_3$ and stored at 4 °C (ergosterol was stable for one month only, sitosterol and stigmasterol were stable for more than half a year). Calibration solutions of the target analytes were made in the following concentrations: 0.25, 0.5, 1, 2.5, 5, 10, 25, 50 mgL^{-1} in $CHCl_3$. The online-derivatisation GC-MS method, see 4.3.3 was adapted for these metabolites: 250 μL of each solution was dried in triplicate using microinserts in 2 mL glass vials and a constant flow of nitrogen. 50 μL MSTFA were added from the autosampler and samples were shaken for 60 min at 37 °C in the agitator. 50 μL of pyridine were added, agitated for 2 min and samples were kept for 10 min at 4 °C to allow sedimentation of particles. Then 1 μL of liquid sample was injected in the injector at 290 °C, holding a split/splitless glass liner in splitless mode. A constant flow of 1 $mLmin^{-1}$ helium) was applied. An HP5-ms column (30 m x 0.25 mm x 0.25 μm) from Agilent Technologies (Waldbronn, Germany) was used for chromatographic separation. The oven temperature started at 100 °C and was heated with 70 $°Cmin^{-1}$ to 310 °C, which was kept for 15 minutes. The MSD interface was kept at 310 °C throughout the run. After 6.5 minutes of solvent delay, the detector was used in scan mode (m/z 50-500) and single ion monitoring (SIM) mode with dwell times of 100 msec each: m/z 363 was for ergosterol with one trimethylsilyl group (1 TMS), m/z 394 for stigmasterol 1 TMS and m/z 396 for sitosterol 1 TMS. MSD ion source was kept at 230 °C, MSD quad at 150 °C. The MSD was autotuned before every sequence.

4.7.2 Target analysis of ACC and 2-ketobutyric acid

Another target GC-MS method was developed for the determination of ACC and 2-ketobutyric acid based on the online-derivatisation GC-MS method for metabolic profiling. ACC is the precursor for ethylene, a key phytohormone in defense-related response of the plant to biotic and abiotic stress. There are indications that not only plants and bacteria, but also fungi are able to synthesize ACC and even to degrade ACC to 2-ketobutyric acid. Genes for ACC synthase and ACC deaminase were found during sequencing of *F. graminearum* PH-1. These genes are transformed into bacterial cells and induced with methionine (in case of ACC synthase) or ACC (in case of ACC deaminase) to monitor their activity.

Development of target method for ACC and 2-ketobutyric acid and determination of matrix effects

First, the online-derivatisation GC-MS method for metabolic profiling of non-volatile, polar metabolites was used to determine ACC and KBA derivatives. Stock solutions of 2.5 gL^{-1} in water were prepared and stored at 4 °C. The following concentrations in 90 % aqueous methanol were used for the first studies of the linear range: 0.1, 0.25, 0.5, 1, 2.5, 5 and 10 mgL^{-1}. A standard solution of 5 mgL^{-1} ACC in 90 % aqueous methanol was used to spike different media (obtained from Gerlinde Wiesenberger from the Departement of Applied Genetics and Cell Biology), which were diluted with pure methanol to result as a 90 % aqueous solution: *Fusarium* minimal medium (FMM, see chapter 4.4.3), synthetic complete medium without isoleucine (SC-Ile), potato dextrose broth (PDB), malt extract (ME), M9 medium, yeast peptone dextrose medium (YPD). *E. coli* and *S. cerevisiae* cells were also studied for their matrix influences on standard measurement. Derivatsation

temperatures of 37 °C, 60 °C and 90 °C were tested. 40 μL MOX (20 mgmL^{-1} in pyridine) and 50 μL MSTFA, 50 μL MSTFA and 50 μL pyridine or 100 μL MSTFA were compared for the derivatisation efficiency of ACC and 2-ketobutyric acid. In addition, 25 μL MTBSTFA (100 °C for 1 h) were tested because these conditions were described as optimum for determination of ACC and other phytohormones in literature (Birkemeyer et al., 2003).
Finally, the online-derivatisation GC-MS method for metabolic profiling of non-volatile, polar metabolites was optimized for the determination of ACC and 2-ketobutyric acid in the following parameters: 50 μL MSTFA were added from the autosampler and samples were shaken for 30 min at 90 °C in the agitator. 50 μL of pyridine were added, agitated for 20 sec and samples were kept for 10 min at 4 °C to allow sedimentation of particles. Then 1 μL of liquid sample was injected in the injector, holding a split/splitless glass liner in pulsed splitless mode (30 psi for 2 min, then adjusting to constant flow of 1 mLmin^{-1} helium) at 250 °C. An HP5-ms column was used for chromatographic separation. The oven was kept at 50 °C for 2 min, heated with 10 °Cmin^{-1} to 150 °C, for clean-up heated with 70 °Cmin^{-1} to 325 °C which was kept for 15 minutes. The MSD interface was kept at 250 °C throughout the run. After 6 minutes of solvent delay, the detector was used in scan mode (m/z 50-300) and single ion monitoring (SIM) mode with dwell times of 100 msec each: m/z 55 and 130 were monitored for ACC 1TMS, m/z 128, 147, 202 for ACC 2TMS and m/z 147 and 231 for 2-butenoic acid 2TMS. SIM traces of m/z 128, 147 and 202, which are characteristic ions for ACC 2 TMS, were used for matrix evaluations. The recovery rate was determined by dividing the intensity of ACC 2TMS from the standard in medium by the intensity of ACC 2 TMS from standard in solvent. The following quantification ions were used once all optimization were included: the base peak of m/z 147 for ACC 2TMS and 2-butenoic acid 2TMS and the selective ions m/z 202 for ACC 2TMS and m/z 231 for 2-butenoic acid 2TMS, base peak m/z 55 and selective ion 130 for ACC 1 TMS.

Study of fungal ACC synthase and ACC deaminase biosynthesis products in bacterial cells

To study the biosynthesis products of ACC synthases and degradation products of ACC deaminases from *F. graminearum* PH-1, single genes were transformed in *E. coli* BL21 cultures by Clemens Schmeitzl from the Department of Applied Genetics and Cell Biology. Three cultures were transformed with one gene each encoding for an ACC synthase, named A1 (FGSG_05184), A2 (FGSG_07606), A3 (FGSG_13587) and two cultures were transformed with a single gene encoding for ACC deaminase, named D1 (FGSG_02678), D2 (FGSG_12669). Overnight cultures of each transformant were prepared in 3 mL LB (lysogeny broth) medium (containing ampicillin). 1 mL of this cultures was used to inoculate 50 mL LB medium (1 % (v/v) tryptone, 0.5 % yeast extract, without NaCl) to give an optical density (OD) of appr. 0.1. It was cultivated at 37 °C until an OD of 0.3 was reached. 1 mM IPTG was added and cultures were incubated for another 3 h at 30 °C. Cultures were centrifuged for 10 min at 3200 rcf at 4 °C and the dry pellet was kept at -80 °C.
Induction of ACC synthases: Four equal volumes of the supernatant were harvested and the following concentrations of methionine were added to induce ACC synthesis: 0, 0.15, 0.3 and 3 gL^{-1}. Water was used to balance volumetric differences. For the blank samples, LB medium (+IPTG) samples were spiked with the same concentrations of methionine.

Induction of ACC deaminases: Two equal volumes of supernatant were harvested and 0 and 0.5 gL^{-1} ACC were added. Blank samples contained LB medium (+IPTG) and 0 and 0.5 gL^{-1} ACC. Samples were taken immediately, 0.5 h, 1 h, 3 h and 24 h after induction. They were centrifuged and the supernatant was frozen at -80 °C until measurement. For measurement, the samples were diluted 1:10 in pure methanol to result in a 90 % aqueous methanol solution. 100 μL were dried under a constant flow of nitrogen. The optimized target analysis method for ACC and 2-ketobutyric acid derivatives was described in 4.7.2.

Chapter 5

Results and Discussion

5.1 Development and evaluation of HS SPME GC-MS methods

HS SPME GC-MS methods were developed for the determination of volatile metabolic profiles of *Fusarium* cultures, healthy and *Fusarium* infected wheat ears. Mass spectra and RIs from authentic standards, from NIST library entries with RIs from the NCWB and from metabolites, which could not be annotated, were used to built an in-house library for volatiles. The RI values of a list of standard substances were studied with respect to their accuracy and precision for the HS SPME GC-MS methods.
Analytical methods, such as HS SPME GC-MS, are necessary for metabolic profiling of volatiles which are involved in *Fusarium*::wheat interactions during FHB disease. Mycotoxin analysis of *F. graminearum* PH-1 revealed two important pathways for the biosynthesis of trichothecenes, where sesquiterpenes play crucial roles as volatile precursors for mycotoxins. The HS SPME GC-MS method was critically assessed for matrix effects influencing the extraction efficiency of target analytes. For selected sesquiterpenes, calibrations were made and LOD and LOQ were calculated. Still, the HS SPME GC-MS method could only be used for qualitative profiling of volatiles and relative comparison of the signal intensities of the sesquiterpenes.

5.1.1 Built-up of an in-house library for volatile metabolites containing mass spectra and RIs of authentic standards, annotated and unknown metabolites

As a first step to develop a HS SPME GC-MS method for the determination of volatile metabolites in fungal cultures and wheat ears, an in-house library for volatile metabolites to be used for compound annotation in MetaboliteDetector was generated. The in-house library was built consisting of mass spectra and RIs from measurements of authentic standards and library entries from Wiley 8^{th} and NIST/EPA/NIH Mass Spectral Library - Version 2008 which were detected in pilot experiments of fungal cultures and wheat ears. The first measured fungal cultures and wheat ear samples using HS SPME GC-MS were used to build up the in-house library of volatiles. The detected mass spectra of compounds were deconvolved and annotated using AMDIS and the NIST/EPA/NIH Mass Spectral Library - Version 2008. As a first step, only a mass spectral match of at least 80 % was set (no RI comparison was made). The annotated metabolites were manually inspected in MSD Chemstation for their chromatographic peak shapes and mass spectral peaks. Then,

RI values for the annotated metabolites were collected from NCWB and compared with the detected RI values. Only mass spectra with corresponding median RI values were added into the in-house library whose RI value range (of NCWB listed entries) covered the detected RI value.
It was a successive process of mass spectra and RI collection over the whole thesis. With each experiment additional compounds were detected and stored as annotated compounds or unknowns. As a first consequence, mass spectra and RIs from fungal volatiles were stored separately from those of wheat ears. The two organism specific libraries were finally combined to one in-house library for volatiles (in table A.3 it is indicated which compounds were of plant and/or fungal origin).
At the end of this thesis, a total of 296 mass spectra, corresponding to 296 compounds, see table A.3, were collected and RIs included (for HP5ms column RIs for all metabolite were available, but not for OptimaWax column). 139 of 296 mass spectra and RI were obtained from measurements of authentic standards, 116 mass spectra were obtained from NIST library and 41 mass spectra and RI were stored from unknown metabolites, which were repetitively detected in fungal cultures. Figure 5.1 shows how many substances of different chemical classes were included in the library. In blue, the total number of compounds (corresponding to one mass spectrum and RI each), in red, the number of mass spectra and RIs from measurements of authentic standards, and in green, the number of unknown metabolites is displayed. For alcohols, alkanes and esters, more than 75 % of the library entries are confirmed spectra and RIs from standard measurements. 70 % of the monoterpenoid spectra and RIs were confirmed with standards. For the other substance classes, this ratio is less than 50 %. Regarding the sesquiterpenoids, 45 % of the mass spectra and RI in the in-house library could not be annotated and are therefore named 'unknown' metabolites. In table A.3 the organisms (fungus and/or plant), which are able to synthesize this compound (described in literature or during our studies), is given.
The list of volatile compounds, which are included in the in-house library for volatile metabolites, also shows the limits of the HS SPME method regarding size and polarity of the analytes and the GC starting conditions. Pentane, is the first detectable alkane standard. The alkanes are apolar substances and their separation is dependent on their boiling points, not on their interaction with the stationary phase of the apolar HP5-ms column. Pentane has a boiling point of 35 °C and a vapour pressure of 428 mm Hg at 20 °C (data provided from supplier). The GC starting conditions in the oven are 35 °C or 40 °C (dependent on the organism). In both cases, this is the low limit of the HS SPME GC-MS method. The first eluting polar compound, which is reliably annotated is ethyl acetate, with a boiling point 77 °C of and a vapour pressure of 73 mm Hg at 20 °C (data provided from supplier). For polar substances, the interaction with the stationary phase of the HP5-ms column additionally influences their separation. According the the volatile classification from the WHO, see table 3.2, both compounds, pentane and ethyl acetate belong to the VVOCs. Compounds with RIs less than 500 (RI for pentane) can therefore not be detected using this HS SPME GC-MS method.
The latest eluting compounds in the chromatogram are the sesquiterpenes, which are SVOCs with boiling points up to 270 °C. Diterpenes were not detected in *Fusarium* cultures, but they were detected with a similar method in *Trichoderma* spp. (unpublished data). The alkane standard last detected with the fungal HS SPME GC-MS method is octadecane. Only compounds with RIs less than 1800 can therefore be detected using this method, which covers a few diterpenes, a few sesquiterpenoids and most sesquiterpenes. The HS SPME GC-MS method used for the determination of volatiles in wheat ears

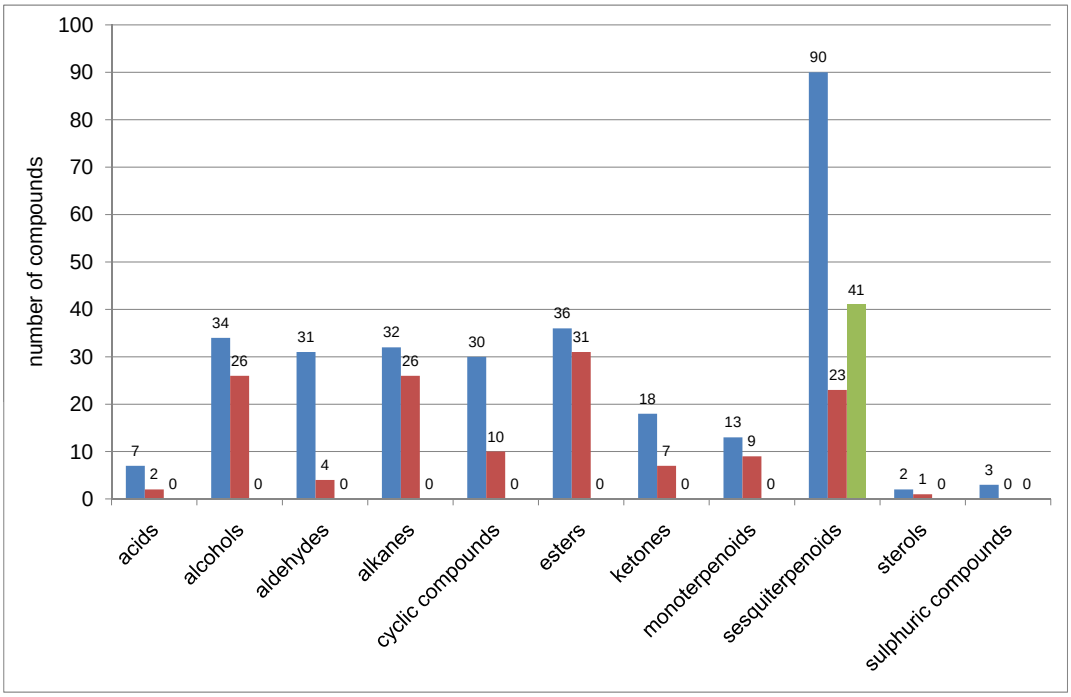


Figure 5.1: Number of compounds per chemical class included in the in-house library for volatile metabolites. The total number of compounds is displayed in blue, the number of standards measured in red, the number of 'unknowns' included in green.

is able to extract and detect triacontane as the latest eluting alkane standard. With this method (the important difference to the fungal HS SPME GC-MS method is the extraction temperature of 90 °C instead of 30 °C), compounds with RIs less than 3000 can be detected.
The HS SPME GC-MS methods were found suitable to be used for monitoring a few VVOCs, VOCs and a few SVOCs in living fungal cultures and frozen, ground wheat ears.

5.1.2 Compound annotations and identifications using the in-house library for volatiles and dealing with metabolites, which cannot be annotated, using MetaboliteDetector

Additionally to mass spectra and RI values from measurements of authentic standards using HS SPME, mass spectra and RI values from annotated metabolites, which were detected using AMDIS and NIST Wiley library from the first measured fungal cultures and wheat ear samples, were stored in the in-house library for volatiles to be used for targeted and non-targeted metabolic profiling of volatiles from the same organisms subsequently. The software tool employed for data processing was AMDIS for the first one and a half years of this thesis. The main drawbacks of AMDIS were: First, many false-positives were annotated with this software tool and manual inspection was very time-consuming. Second, it was not possible to integrate single, user-selected ions for annotated compounds, therefore, no quantification was achieved. Third, another software tool, named SpectCon-

nect, was necessary to deal with metabolites, which could not be annotated. AMDIS itself is not suitable for non-targeted data analysis. For all these reasons, MetaboliteDetector was accustomed for data processing for the second period of the thesis. Using MetaboliteDetector, the selection of single quantification ions was possible for all library entries in the in-house library. Furthermore, the user can choose to perform a target analysis or a non-targeted approach for metabolic profiling of all compounds detected. Still, the manual inspection of the detected compounds was necessary, but compared to AMDIS the number of false-positives had decreased. The settings in MetaboliteDetector were thoroughly set for the data processing of standard substances and fine tuned during the thesis. The final settings are listed in chapter 4.3.4.
In this thesis, the following criteria were set for annotations and identifications of volatile metabolites using MetaboliteDetector: For annotations of detected compounds, a mass spectral match higher than 80 % and a RI deviation of less than 20 RI units must be achieved. For identification, the mass spectrum and RI of the detected compound must fit with the mass spectrum and RI of an authentic standard with the same criteria. Annotated and identified metabolites were detected on both columns of different polarity. 'Fg' metabolites were not annotated to any substance or structural class, but they were reproducibly detected in *F. graminearum* cultures. Most of these metabolites were 'sesquiterpenes', which indicated a structural relationship with the sesquiterpenes according to their mass spectra.
One big drawback of MetaboliteDetector is that annotations with library entries can be made more than once per sample, which means that MetaboliteDetector annotates the same compound more than once in one sample if the mass spectral and RI match is above the set limit in both compounds. In these cases, the metabolites are named 'similar to...' and are not considered annotated metabolites.
This nomenclature will be used throughout the thesis to describe the detected compounds.

5.1.3 RI accuracy, precision and reproducibility

Besides the mass spectral information, the RI is of a compound is used for compound annotation or identification. The RI is the normalized retention time information, giving information about the chromatographic behaviour of a compound. In 1958 Kovats (Kováts, 1958) described how to determine RIs using alkanes for normalization. His calculations were based on isothermal measurements, but for a wide field of applications temperature programmed methods were used. Van Den Dool and Kratz (Van den Dool and Kratz, 1963) adapted the equation of Kovats index towards an application on temperature programmed methods, resulting in the linear temperature programmed retention index (LTPRI). Today, mainly LTPRIs are found in databases showing the wide spread use of this retention index system (Strehmel et al., 2008; Schauer et al., 2005; Kopka, 2006).
In this thesis, I monitored the RI values of 29 standard substances (6.25-625 mgL^{-1} in 50% aqueous acetonitrile), individually and in mixtures, over half a year using two GC-MS instruments equipped with HS SPME. In total, four columns were used for the chromatographic separation: three with apolar stationary phases, one with a polar stationary phase. The measured retention time values were used to calculate the KI according to Kovats and the LTPRI according to Van Den Dool and Kratz. The experimentally determined KI and LTPRI are listed in table 5.1. The experimental LTPRI values were compared with literature LTPRI values from the NCWB. This is exemplary shown in form of boxplots

for selected alcohols and sesquiterpenes using apolar and polar stationary phases, see figure 5.2. The boxplots show for each compound the median (horizontal line in the box), the 25 and 75 % percentiles (edges of the box) and the 1.5 interquartile range (whiskers) of its LTPRI values. Especially for the LTPRI values of the sesquiterpenes, there is no common intersection of the boxes and whiskers from the experimental and literature values. The experimental LTPRI values of the sesquiterpenes are generally lower than the corresponding literature values. In addition, the literature values show a higher deviation of the LTPRI values in comparison to the experimental values.

Hence, for the determination of the accuracy of the experimental RI values, the arithmetic mean (average) of the experimentally obtained KI/LTPRI values was compared with the median of the listed KI/KTPRI values in the NCWB. Using columns with an apolar stationary phase, accuracies of 95-102.5 % were achieved for the experimentally KI and LTPRI values. Using a polar stationary phase, 99.8-103.3 % accuracy were reached for the KI and LTPRI values of 29 standard substances. The differences between the literature and experimental LTPRI values of the sesquiterpenes, was therefore not significant.
The differences between the experimental KI and the LTPRI values were less than 5 RI units, as pointed out by Van Den Dool and Kratz as well. In general, the standard deviation σ, as a measure of the precision, of each standard substance over half a year was less than 5-12 RI units. According to literature, 5 units for methyl silicone stationary phases and 10 units for polyethylene glycol phases were considered acceptable (Zellner et al., 2008). The standard deviation of the experimental RI values varied more than the difference between KI and LTPRI, hence we concluded that both KI and LTPRI values can be used for compound annotation.

The following influences on the RI values were studied regarding reproducibility of the experimental LTPRI values: replacement of the guard column (independent from the chromatographic column), column ageing, heating rate, standard concentration measured (regarding quantitiy loaded on the column) and matrices such as cultivation medium for fungal cultures.
Within one month using the same column and instrument, the standard deviation was only 1-2 RI units. Replacement of the guard column was necessary during RI studies. However, after replacement, the new guard column, which is a non-coated and deactived column from the supplier supposed to show no interactions with the analytes, resulted in shifts of the LTPRI values towards higher values. The LTPRI values of apolar substances was shifted only 2-10 RI units, whereas the LTPRI values of polar substances was shifted 30-50 RI values. Therefore, the guard column was completely removed from the system to avoid unspecific interactions.
After many injections (approximately 1000 injections during half a year of measurements) and ageing of the column, the standard deviation of the experimentally obtained LTPRI values was higher, resulting in RI deviations of 5-12 units. The importance of ageing of a column was pointed out by Isidorov and Szczepaniak (2009) earlier.
In the following, the heating rate and the quantitation of the standard substances were tested for their influences on the standard deviation. Our data were in good agreement with Isidorov and Szczepaniak (2009), especially for the sesquiterpenes. Concerning the heating rate, the measurements were performed at 100 °C isothermal, and using a temperature program rate of 3 °C min^{-1}, 6 °C min^{-1} and 10 °C min^{-1}. The LTPRIs varied less than 5 RI units for all standard substances besides the sesquiterpenes, which varied 20 RI units between the heating rate of 3 °C min^{-1} and 10 °C min^{-1}. The lowest RI values

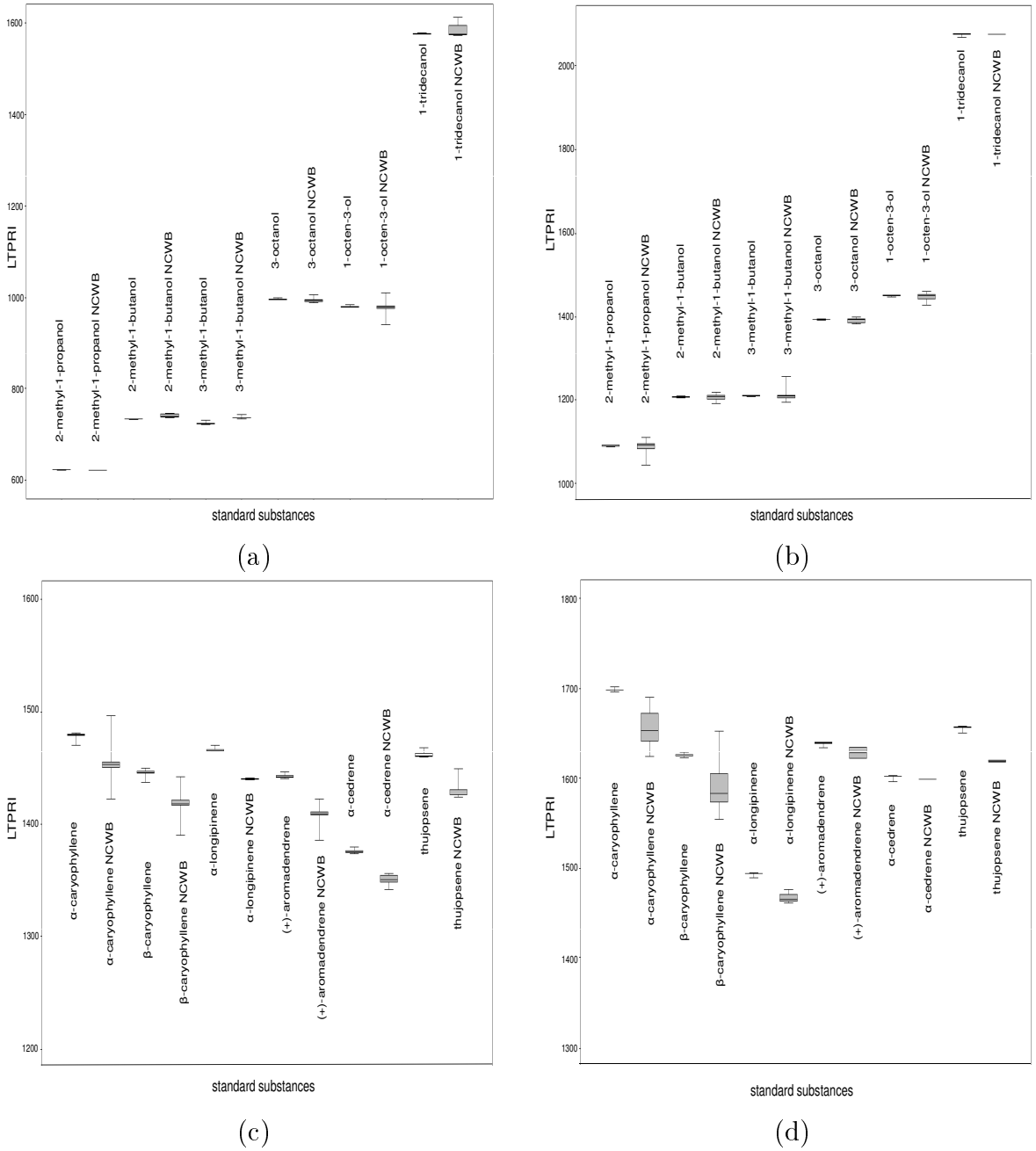


Figure 5.2: Boxplots, revealing the median value, the 25 and 75 % percentiles (lower and upper end of the boxes, respectively) and the 1.5 interquartile range (whiskers), of experimental RI values and literature values obtained from NCWB for selected alcohols and sesquiterpenes. RI values experimentally determined and from NCWB for selected alcohols separated on an apolar stationary phase (a), for selected alcohols on a polar stationary phase (b), for selected sesquiterpenes separated on an apolar stationary phase (c) and for selected sesquiterpenes on a polar stationary phase (d).

were obtained with the lowest heating rate, which fit best to the RI values from NCWB, see table 5.1. This suggests that the RI values listed in NCWB were obtained with low heating rates. In addition, the LTPRI values increased about 20 RI units if overloading of the column was achieved with high quantities of standard substances.
Matrices, like cultivation medium such as potato dextrose agar (PDA), did not enhance the standard deviation of LTPRI values suggesting that matrices like cultivation medium of fungal cultures did not increase in biological samples over time.

In the field of metabolomics using GC-MS instruments, compound (metabolite) annotations are always based on the mass spectral and RI information from the detected compound in comparison with a libary entry. Strehmel et al. (2008) concluded that an error percentage of 0.5-1 % was best for proper annotations. Regarding the RI values from standard substances and their absolute standard deviation obtained over half a year (5-12 RI units), this finding was in good agreement with our results. Using the software tool MetaboliteDetector, absolute RI deviations are used as limits for annotations. Since the latest eluting alkane standards are octadecane or triacontane, dependent on extraction temperature of the HS SPME method, the RI deviation should be set 18 or 30, corresponding to 1 %. Regarding annotations based on matches of detected compounds with library entries from NIST, a greater RI deviation might be necessary, especially for the sesquiterpenes. Our results showed that both KI and LTPRI values for the sesquiterpenes listed in NCWB were up to 3 % lower than the experimentally obtained RI values.

Table 5.1: Retention indices measured for 29 standard substances using 3 apolar (2 instruments) and 1 polar column over half a year. Kovats index (KI) and linear temperature programmed retention index (LTPRI) were calculated according to equation 3.2 and 3.3. The arithmetic mean (average) and absolute standard deviation (σ) were calculated for n repeats. LIT...median value of entries listed in NCWB, % accuracy...recovery of experimental (average) value - LIT value.

metabolite	CAS	LTPRI HP5 average	n	accuracy %	σ	LTPRI LIT HP5 range:median(n)	KI HP5	KI LIT HP5 range:median(n)	LTPRI Wax average	n	accuracy %	σ	LTPRI LIT Wax range:median(n)	KI Wax	KI LIT Wax range:median(n)
1-propanol, 2-methyl	78-83-1	625	33	100.4	5	622(2)	626	636(1)	1089	14	99.8	4	1043-1110: 1091(18)	1090	1092-1094: 1094(4)
2-ethylfuran	3208-16-0	700	98	99.7	9	691-730: 702(7)	700	701(1)	954	87	99.9	4	952-957: 955(3)	955	
1-butanol, 3-methyl	123-51-3	727	78	98.9	11	734-743: 736(9)	731	730-741: 735.5(2)	1209	85	100	4	1194-1255: 1208(24)	1209	1209-1211: 1211(4)
1-butanol, 2-methyl	137-32-6	735	42	99.5	5	736-746: 73(6)	738	743(1)	1206	99.9	16	3	1191-1217: 1206(8)	1206	
1,3,5-cycloheptatriene	544-25-2	795	22		2		796		1068	26		1		1070	
isobutylacetate	110-19-0	773	42	99.1	4	780(1)	775	764(1)	1014	16	100.7	2	1000-1029: 1007(5)	1016	1013(1)
2-(E)-hexenal	6728-26-3	857	82	100.4	10	803-867: 854(31)	860	849-855: 854(5)	1227	86	101.2	3	1200-1234: 1212(27)	1227	1218-1219: 1219(4)
m-xylene	108-38-3	874	88	100.9	5	861-898: 866(10)	877	862(1)	1149	94	100.6	3	1116-1158: 1142(12)	1150	1141-1143: 1142(4)
p-xylene	106-42-3	875	61	101.7	6	861(1)	877	8612-884: 866(12)	1142	15	100.5	1	1119-1192: 1137(11)	1144	1136(1)[a]
o-xylene	95-47-6	900	45	101	2	888-896: 891(13)	900	884(1)	1194	15	100.7	1	1159-1195: 1185(10)	1194	1185(2)
2-heptanone	110-43-0	892	86	100	5	849-900: 892(13)	892	890(1)	1188	89	100.7	3	1161-1193: 1183(19)	1188	1184-1185: 1184(2)
α-pinene	80-56-8	942	45	100.7	5	9007-943: 936(118)	944	909-942: 936(35)	1028	26	101.2	3	1008-1101: 1015(34)	1030	1012-1092: 1036(3)
1-octen-3-ol	3391-86-4	981	80	100.1	7	942-1012: 980(39)	982	964-980: 977(6)	1451	94	100	4	1428-1461: 145(31)	1452	1456(2)
3-octanone	106-68-3	989	29	100.2	2	971-994: 987(14)	990	985(1)	1262	18	100.3	1	1205-1272: 1258(19)	1263	
3-octanol	589-98-0	997	73	100.2	8	989-1007: 995(22)	997	994(1)	1394	93	100	3	1383-1400: 1394(13)	1394	1386(1)
β-myrcene	123-35-3	992	71	100.1	6	963-999: 991(99)	992	955-993: 990(34)	1166.894223	86	100.9	3	1146-1184: 1156(32)	1168	1145-1167: 1156.5(32)
m-cymol	535-77-3	1029	45	99.6	3	1033(1)	1031	1026(1)[a]	1278	26	100.2	4	1263-1279: 1276(3)	1280	
p-cymol	99-87-6	1032	51	100.7	5	1002-1033: 1025(102)	1034	992-1030: 1024(31)	1279	26	101.7	5	1234-1281: 1258(31)	1280	1265(1)[a]
o-cymol	527-84-4	1047	45	102.5	3	1020-1028: 1022(10)	1049	1002-1027: 1020(5)	1313	26	103.3	2	1254-1279: 1271(4)	1313	
limonene	138-86-3	1035	106	100.5	9	1005-1038: 1030(104)	1037	995-1044: 1029(30)	1208	26	101.1	2	1176-1218: 1195(43)	1209	1180-1217: 1198(2)
terpinolene	586-62-9	1096	42	100.8	4	1063-1095: 1088(85)	1096	1072-1098: 1088(24)	1294	26	102.2	3	1258-1290: 1266(26)	1294	1274(1)[a]
α-longipinene	5989-08-2	1376	45	101.8	6	1342-1356: 1351(11)	1376	1330-1334: 1332(2)	1494	26	101.9	5	1466(1)[a]	1494	
β-longipinene						1394-1405: 1398(7)		1398(1)					1561(1)[a]		
α-cedrene	469-61-4	1442	45	102.4	6	1386-1422: 1409(22)	1443	1408-1415: 1412(3)	1601	26	100.1	6	1599(1)	1601	
β-cedrene	546-28-1					1418-1435: 1420(9)		1424(1)					1596(1)[a]		
cedrene	11028-42-5					1449(1)									

[a] similar column but different coating thickness, inner diameter or length

Table 5.1: (continued)

metabolite	CAS	LTPRI HP5 average	n	accuracy %	σ	LTPRI LIT HP5 range:median(n)	KI HP5	KI LIT HP5 range:median(n)	LTPRI Wax average	n	accuracy %	σ	LTPRI LIT Wax range:median(n)	KI Wax	KI LIT Wax range:median(n)
thujopsene	470-40-6	1461	56	102.5	9	1424-1449: 1426(7)	1462	1429(1)[a]	1656	26	102.3	7	1619(2)[a]	1657	
aromadendrene	109119-91-7					1407-1491: 1439(38)		1419-1485: 1438.5(14)					1582-1670: 1649(5)		1631(1)
alloaromadendrene	25246-27-9					1453-1477: 1461(45)		1442-1467: 1459(11)					1618-1662: 1646(3)	1660(1)[a]	
(+)-aromadendrene	489-39-4	1466	48	101.8	6	1439-1441: 1440(3)	1467		1639	26	101.0	6	1622(1)[a]	1640	
(E,E)-α-farnesene	502-61-4					1456-1512: 1507(50)		1504-1522: 1508.5(6)					1720-1752: 1741.5(10)		1752-1755: 1754(4)
(E,E)-β-farnesene	18794-84-8	1430	32	95	12	1506(1)			1671	12		1		1672	
(Z,E)-α-farnesene	26560-14-5					1484-1496: 1490(6)							1725-1747: 1732.5(4)		
(Z,Z)-α-farnesene						1462(1)							1697(1)[a]		
(E,Z)-α-farnesene						1475(1)							1726(1)		
α-caryophyllene		1479	44	101.8	11	1422-1497: 1453(98)	1480		1699	79	102.7	5	1624-1690: 1654(20)	1697	
β-caryophyllene	87-44-5	1446	51	102	12	1390-1442: 1418(125)	1447	1390-1451: 1419(36)	1625	93	102.7	6	1555-1653: 1582(26)	1626	1575-1617: 1585(3)
1-tridecanol	112-70-9	1576	25	100	3	1572-1612: 1575(3)	1576		2076	94	100	10	2076(1)	2074	

[a] similar column but different coating thickness, inner diameter or length

5.1.4 Influences from the cultivation medium and flushing with synthetic air before HS SPME on the volatile metabolite pattern of *F. graminearum* PH-1 cultures

For metabolomics studies it is of high interest to detect as many metabolites as possible with one extraction and analytical instrument from different organisms without changing to metabolic state of the compounds. Regarding FHB disease, it is important to reveal metabolic pathways which are involved in the interaction between fungus and plant. However, as a first step, the interaction partners must be studied individually. Starting with the volatile metabolome of the fungus, the study of living fungal cultures brings some difficulties and restrictions in comparison to studies of dead, frozen material, such as a low extraction temperature, the avoidance of stress inducers such as a shortcut in nutrients or oxygen or the addition of solvents for quantification.
The fungal cultures were grown at 22 °C in the dark and extracted at 22 °C (at room temperature) or at maximum 30 °C (the lowest temperature possibly applied to the heater/agitator, which is better controlled than room temperature) to avoid monitoring responses after a heat shock in the volatile metabolome. The use of HS-vials for the cultivation of fungal cultures for one week should provide enough oxygen and the availability of 2 mL cultivation medium was enough to allow fungal growth until the edges of the HS-vials. No medium slants were used because nutrients should be provided at the same level at each point for the fungus.
For the cultivation of fungal cultures in HS vials three different media potato dextrose agar (PDA), Czapek Dox and *Fusarium* minimal medium (FMM), were used in a small study, to determine the number of volatile metabolites produced. The medium, which allowed the fungal cultures to synthesize and export the highest amount of volatiles, was chosen for further studies. For this purpose, fungal cultures of *F. graminearum* PH-1 were grown on 2 mL of each medium. PDA and FMM were used as solid media, Czapek Dox and FMM without agar were used as liquid media. All media contained sucrose which was reported to induce expression of *tri* genes best compared to other carbohydrates (Jiao et al., 2008). Data processing and evaluation using MetaboliteDetector and visual inspection of the chromatograms using MSD Chemstation showed that most metabolites were produced and exported to the HS of fungal cultures on PDA. Using PDA for the cultivation of *F. graminearum* PH-1, up to 60 metabolites were detected, whereas only 8 metabolites were detected in the HS above fungal cultures grown on Czapek Dox and 10-20 metabolites were detected in the HS of fungal cultures on liquid and solid FMM media. Therefore, PDA was chosen for cultivation of *F. graminearum* for all following experiments.

Moreover, the volatile metabolites synthesized from flushed and non-flushed fungal cultures were determined to see whether flushing induced a number of compounds to be produced of released to the HS.
F. graminearum PH-1 cultures were grown in HS-vials in two different ways: 'Flushed' fungal cultures were flushed six hours before extraction which caused the removal of all volatiles exported into the HS at this time point and the supply of fresh oxygen. Whereas 'non-flushed' cultures were not disturbed until extraction (no removal of HS constituents prior to extraction and no fresh oxygen). The experiment was carried out twice with three biological replicates for each sampling point.
The TIC chromatogram in figure 5.3 shows that most of the metabolites were produced from all cultures, independent from flushing. Some metabolites were detected at different

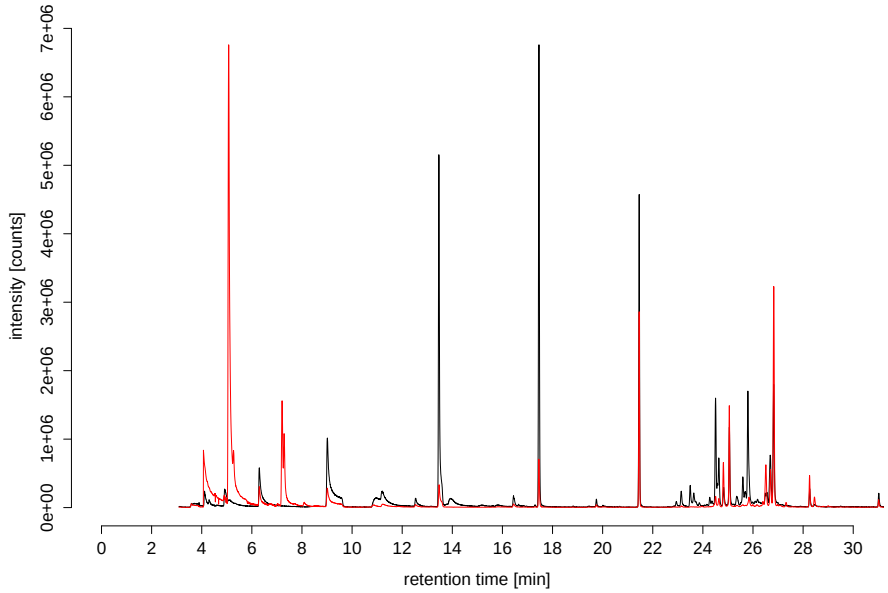


Figure 5.3: TIC chromatogram of flushed and non-flushed cultures of *F. graminearum* PH-1 at 131 h after inoculation.

time points (but the same metabolites were detected), others showed significantly differing intensities, especially for the early eluting metabolites. Esters like ethyl acetate (t_R 5.0 min) and alcohols like 3-methyl-1-butanol (t_R 7.2 min) and 2-methyl-1-butanol (t_R 7.3 min) were more abundant in non-flushed cultures than in flushed cultures. This accumulation of volatiles was expected in non-flushed cultures because the HS constituents were not removed before measurement. However, it is unclear how much of these metabolites was adsorbed by the growth medium and at which time point the fiber coating was saturated. Since the accumulated amount of volatiles, which was measured in the non-flushed cultures, cannot be directly compared to the time specific amount of volatiles in the flushed cultures, this study was used for a qualitative analysis of fungal cultures only.

The qualitative comparison of flushed and non-flushed fungal cultures revealed a total of 55 metabolites, see table 5.2. 17 metabolites were identified on an HP5-ms column, including esters, alcohols, ketones, cyclic compounds, mono- and sesquiterpenes. 21 metabolites were annotated. The annotated compounds were sesquiterpenes except for four metabolites, namely butanal, 2-methylfuran, 3-hydroxy-2-butanone and dimethyldisulfide. 17 detected metabolites could not be annotated, but all of them, besides one which could not be structurally related to any substance class, were assigned to the group of sesquiterpenes due to their molecular mass (m/z 202 or 204) and characteristic fragmentation pattern. The sesquiterpenes dominated the chromatogram with respect to their number. Most sesquiterpenes were found in higher intensities in flushed cultures than in non-flushed cultures of *F. graminearum* PH-1 suggesting that either, the sesquiterpenes were rapidly metabolized by the fungus after removal of all HS constituents and introduction of fresh oxygen, or that the accumulation of other volatiles (such as ethyl acetate and 3-methyl-1-butanol) in non-flushed cultures, hindered efficient extraction of the sesquiterpenes or even their release into the HS. Furthermore, there are limited active sites available on the DVB/CAR/PDMS coated fiber, this restriction might lead to discrimination of the volatiles at a certain time point when the overall amount of volatiles produced was en-

hanced. The availability of free binding sites by the DVB/CAR/PDMS fiber was tested for very high concentrations of standard solutions. Even at the g L^{-1} level no limitations were monitored. Hence, a limited number of binding sites was not the reason for this pattern.

Table 5.2: influence from flushing

metabolite	CAS	LTPRI HP5ms	t_R(min) HP5ms	quant. ions m/z
Butanal	123-72-8	608	5.04	44 57 72
Ethyl acetate[a]	141-78-6	610	5.08	61 70 88
Furan 2-methyl-	534-22-5	611	5.09	53 81 82
2-methyl-1-propanol[a]	78-83-1	622	5.27	55 56 74
2-Butanone-3-hydroxy-	513-86-0	712	6.79	43 45 88
3-methyl-1-butanol[a]	123-51-3	731	7.20	55 57 70
2-methyl-1-butanol[a]	137-32-6	739	7.41	55 57 70
Disulfide-dimethyl	624-92-0	743	7.50	45 79 94
isobutylacetate[a]	110-19-0	772	8.08	56 71 73
p-xylene[a]	106-42-3	870	10.80	91 105 106
2-methyl-1-butanol-acetate[a]	624-41-9	879	10.86	43 55 70
1-octen-3-ol[a]	3391-86-4	981	13.63	57 72 85
3-octanone[a]	106-68-3	987	13.83	57 72 99
β-myrcene[a]	123-35-3	990	13.90	69 91 93
limonene[a]	5989-27-5	1032	15.12	68 93 136
terpinolene[a]	586-62-9	1091	16.62	91 93 136
sesquiterpene1		1356	22.92	105 119 161
sesquiterpene2		1365	23.13	95 119 204
sesquiterpene3		1381	23.47	68 107 189
β-cubebene	13744-15-5	1384	23.62	105 120 161
sesquiterpene4		1398	23.84	119 161 204
sesquiterpene5		1413	24.15	119 161 204
α-longifolene	475-20-7	1408	24.62	94 107 161
α-cedrene[a]	469-61-4	1415	24.31	119 161 204
sesquiterpene6		1422	24.34	119 189 204
γ-patchoulene	508-55-4	1429	24.49	105 161 204
(+)-β-Gurjunene(Calarene)	17334-55-3	1442	24.60	105 119 161
sesquiterpene7		1436	24.62	93 107 108
α-himachalene	3853-83-6	1444	24.77	93 94 119
(+)-aromadendrene[a]	489-39-4	1445	24.81	91 105 161
(-)-Alloaromadendrene	25246-27-9	1462	25.18	91 105 107
γ-humulene[a]	26259-79-0	1469	25.32	93 107 189
(+)-Epi-bicyclosesquiphellandrene	54324-03-7	1469	25.32	91 105 161
α-humulene[a]	6753-98-6	1470	25.34	93 121 204
sesquiterpene8		1482	25.57	119 121 204
sesquiterpene9		1485	25.66	69 93 133
β-humulene[a]	116-04-1	1486	25.66	93 107 204
α-curcumene		1489	25.73	119 132 202
sesquiterpene10		1491	25.77	105 161 204
β-chamigrene	18431-82-8	1484	25.79	93 105 189
α-selinene	473-13-2	1491	25.82	107 121 189
sesquiterpene11		1495	25.85	131 187 202
α-(Z)-bisabolene	29837-07-8	1502	26.00	93 121 204
β-bisabolene	495-61-4	1507	26.11	69 93 121
calamanene	483-77-2	1510	26.15	128 159 202
sesquiterpene10_RI1510		1512	26.20	105 161 204
β-Himachalene	1461-03-6	1523	26.40	119 134 204
similar to α-curcumene	644-30-4	1527	26.48	119 132 202
sesquiterpene13		1530	26.54	134 161 204
similar to γ-bisabolene		1537	26.67	93 107 204
trichodiene	76231-80-6	1541	26.76	67 108 109
γ-bisabolene	53585-13-0	1545	26.94	93 107 204
α-(E)-bisabolene	25532-79-0	1551	26.96	93 121 204
Fgstrain3		1559	27.10	142 157 200
sesquiterpene14		1616	28.46	119 161 204

[a]identified metabolites

To conclude, both types of cultures, the flushed and non-flushed fungal cultures, revealed the same metabolite pattern, with differences only in the exact time points when the

metabolite was detected and the intensity, especially the early eluting compounds. Flushing can therefore be applied prior to HS SPME without disturbing the fungal growth or the biosynthesis of volatiles. The removal of HS constituents allows a defined study of those volatiles, which are produced at a certain time point, which is better defined than the accumulated amount of volatiles. During the thesis, both systems were used for metabolomics studies. The volatile metabolite pattern of flushed *F. graminearum* PH-1 cultures was subject to the following detailed study.

5.1.5 Qualitative, non-targeted characterization of the volatile metabolic profile of *F. graminearum* PH-1

Now that flushing has been shown to have no effect on the number of volatiles produced and PDA was shown to be most suitable for cultivation of fungal cultures to study volatile metabolites, a detailed, qualitative characterization of *F. graminearum* cultures using HS SPME GC-MS followed by non-targeted data processing was performed. The fungal cultures were grown for one week in the dark at 22 °C and measured in defined time intervals. The chromatographic separation was done using two columns with stationary phases of different polarity, namely an apolar HP5-ms column and a polar DB-Wax column. Only metabolites, which could be identified or annotated on both columns were included here. Additionally, metabolites detected on the HP5-ms column, which could not be annotated, were considered, but could not be attributed to chromatographic peaks on the DB-Wax column due to high mass spectral similarity and no available RI for comparison.
Figure 5.4 shows a characteristic total ion current (TIC) chromatogram of a *F. graminearum* PH-1 sample after 52 hours of cultivation on an HP5-ms column. Few metabolites eluted in the first 18 minutes on the HP5-ms column. Most metabolites eluted between 20 to 24 minutes and resulted in partly overlapping peaks. This dominating group of metabolites was the sesquiterpenes, mainly $C_{15}H_{24}$ with a molecular mass of m/z 204. Several sesquiterpenes with a sum formula of $C_{15}H_{22}$ and molecular mass of m/z 202 were detected in this retention time range as well.

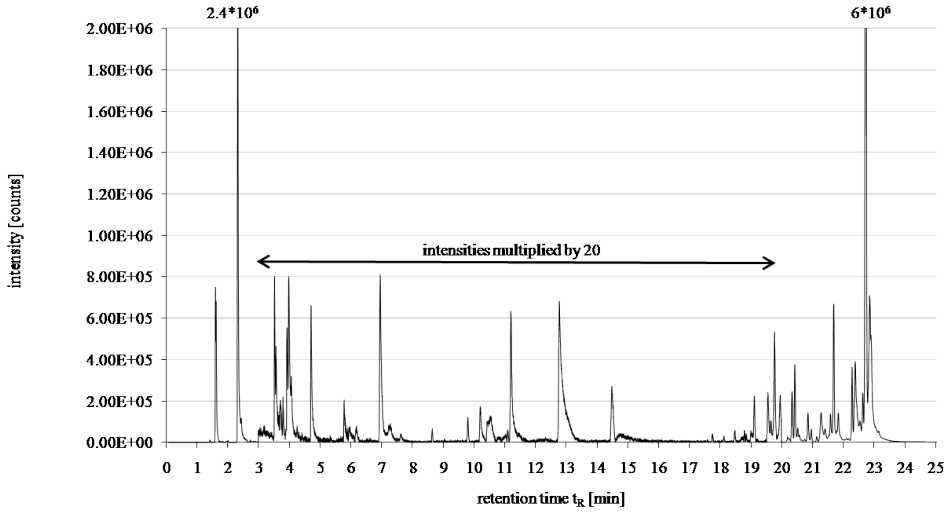


Figure 5.4: TIC chromatogram of *F. graminearum* PH-1 on HP5-ms column after 52 hours cultivation in the dark (signal of nutrition medium was subtracted). The intensities of the two most intense peaks (ethyl acetate at 2.3 min, trichodiene at 22.7 min) are labeled; from 3-20 minutes intensities of peaks were multiplied by a factor of 20 to visualize smaller peaks. Metabolites detected are listed in table 5.3.

Table 5.3: Results of non-targeted qualitative analysis of *F. graminearum* PH-1 using MetaboliteDetector. Metabolites are listed according to their elution order on HP5-ms column. Only identified or annotated metabolites which were detected on both HP5-ms and DB-Wax columns are listed. Metabolites, which could not be annotated were detected on HP5-ms column. LTPRIs of metabolites detected were compared to median of LTPRIs of NCWB, the bias was calculated. n.a. not available, **bold** ***m/z*** values were used for quantification of the respective compound.

	metabolite	CAS	Quant. ions *m/z*	t_R(min) HP5ms	LTPRI HP5ms	NCWB LTPRI HP5ms	RI bias HP5ms	LTPRI DB-Wax	NCWB LTPRI DB-Wax	RI bias DB-Wax
1	ethyl acetate[a]	141-78-6	45, **61**, 88	2.3	613	613	0	888	898[b]	10
2	1-propanol,2-methyl-[a]	78-83-1	55,56,**74**	2.5	623	622	1	1089	1091	2
3	alkane1		57,74,75	3.5	713	-	-			
4	Fg1		55,72,101	3.8	727	-	-			
5	1-butanol,3-methyl-[a]	123-51-3	**55**,57,70	3.9	734	736	2	1204	1208	4
6	1-butanol,2-methyl-[a]	137-32-6	**55**,57,70	4	737	739	2	1206	1206	0
7	disulfide,dimethyl-	624-92-0	46,79,94	4.1	742	741	1	1057	1078[b]	21
8	isobutylacetate[a]	110-19-0	**56**,71,73	4.7	774	780	6	1015	1007	8
9	m-xylene[a] p-xylene[a]	108-38-3 106-42-3	**91**,105,106	7	868	866 861	2 7	1110	1137 1142	27 32
10	2-heptanone[a]	110-43-0	**58**,71,114	7.6	890	892	2	1172	1183	11
11	α-pinene[a]	80-56-8	91,92,**93**	8.6	930	936	6	1015	1015	0
12	1-octen-3-ol[a]	3391-86-4	**57**,72,85	9.9	980	980	0	1442	1451	9
13	3-octanone[a]	106-68-3	**57**,72,99	10.1	987	987	0	1240	1258	18
14	β-myrcene[a]	123-35-3	**69**,91,93	11	990	991	1	1143	1156	13
15	p-cymol[a]	99-87-6	91,**119**,134	11.1	1025	1025	0	1253	1258	5
16	limonene[a]	5989-27-5	**68**,93,136	11.2	1028	1030	2	1172	1195	23
17	terpinolene[a]	586-62-9	**93**,121,136	12.8	1090	1088	2	1263	1266	3
18	α-longipinene[a]	5989-08-2	105,**119**,133	19.1	1358	1351	7	1456	1466	10
19	ylangene α-copaene	14912-44-8 3856-25-5	91,93,**105**	19.6	1389	1379[b]	1	1571	n.a.	-
20	β-cubebene	13744-15-5	91,105,**161**	19.6	1380	1388	8	1702	n.a.	-
21	sequiterpene25		105,119,204	19.7	1384	-	-			
22	sequiterpene26		105,119,120	19.8	1388	-	-			
23	α-longifolene	475-20-7	94,107,**161**	20.2	1408	1405	3	1560	n.a.	-
24	α-cedrene[a]	469-61-4	**119**,161,204	20.2	1410	1409	1	1560	n.a.	-
25	similar to α-cedrene		79,91,135	20.3	1411	-	-			
26	similar to α-longifolene		105,133,161	20.4	1415	-	-			
27	similar to thujopsene		93,95,108	20.4	1419	-	-			
28	sesquiterpene27		69,93,189	20.5	1424	-	-			
29	thujopsene[a]	470-40-6	**119**,123,204	20.6	1425	1426	1	1615	1619	7
30	sesquiterpene28		105,120,161	20.6	1427	-	-			
31	(+)-aromadendrene[a]	489-39-4	91,105,**161**	20.8	1439	1440	1	1601	1622	21
32	similar to thujopsene		93,107,189	20.8	1436	-	-			
33	(+)-β-gurjunene (calarene)	17334-55-3	105,119,**161**	20.9	1440	1437[b]	3	1654	n.a.	-
34	sesquiterpene29		121,136,189	21	1445	-	-			
35	sesquiterpene30		93,94,108	21.1	1453	-	-			
36	β-(*trans*)-farnesene[a]	18794-84-8	**69**,93,133	21.3	1460	1446[b]	14	1628	n.a.	-
37	Fg3		79,94,122	21.4	1467	-	-			
38	sesquiterpene31		119,189,204	21.5	1469	-	-			
39	(+)-epibicyclosesquiphellandrene	54324-03-7	91,105,161	21.5	1471	1487[b]	13	1597	n.a.	-
40	similar to β-chamigrene		105,119,121	21.6	1475	-	-			
41	β-chamigrene	18431-82-8	93,105,**189**	21.7	1480	1474[b]	6	1678	n.a.	-
42	α-selinene	473-13-2	93,**189**,204	21.9	1487	1495	8	1656	n.a.	-
43	ar-curcumene	644-30-4	119,**132**,202	21.9	1488	1483	5	1768	n.a.	-
44	similar to β-bisabolene		119,133,204	22.3	1509	-	-			
45	similar to α-chamigrene		121,136,137	22.4	1513	-	-			
46	sesquiterpene22		131,132,202	22.4	1515	-	-			
47	β-bisabolene	495-61-4	69,**93**,121	22.4	1515	1506	9	1721	n.a.	-
48	sequiterpene34		119,132,161	22.4	1516	-	-			
49	calamenene	483-77-2	128,**159**,202	22.5	1517	1517[b]	0	18003	n.a.	-
50	sequiterpene32		93,107,204	22.6	1522	-	-			
51	sequiterpene23		95,108,109	22.6	1527	-	-			
52	trichodiene	28624-60-4	67,108,**109**	22.7	1532	1533[b]	1	1761	n.a.	-
53	sesquiterpene24		91,119,204	22.9	1538	-	-			
54	γ-(*trans*)-bisabolene	53585-13-0	**93**,107,119	22.9	1539	1537	2	1752	n.a.	-
55	sesquiterpene33		135,161,204	22.9	1539	-	-			
56	sesquiterpene35		92,105,133	22.9	1540	-	-			
57	sesquiterpene14		119,120,161	22.9	1541	-	-			

[a] identified metabolites

[b] RI from literature ((Jelen et al., 1995; Joulain and König, 1998; Goodner, 2008))

Table 5.3 lists all metabolites detected in PH-1 cultures, cultivated at 22 °C in the dark. A total of 57 metabolites were found under the conditions described. 32 metabolites were annotated on both columns, 19 of them were identified. 22 metabolites, which were not annotated, were assigned to the group of sesquiterpenes, but were not further characterized. One metabolite was found closely related to 2,2,3,3-tetramethylbutane, which is an alkane, but it could not be annotated and is labeled as 'alkane1'. Two 'Fg' metabolites were not attributed to any group or substance, but were reproducibly detected in *Fusarium* cultures.
The LTPRIs of the detected metabolites were calculated by MetaboliteDetector and compared with median of the LTPRIs from NCWB (RI bias). Using the HP5-ms column with an apolar stationary phase, the RI bias was found 0 to 5 for 23 metabolites, 5 to 9 for eight metabolites and above 10, namely 13 and 14, for two metabolites. Using the DB-Wax with a polar stationary phase, there were hardly any literature LTPRI values available and the RI bias between experimentally determined and literature LTPRI values was higher: For eleven metabolites the RI bias was below or equal 10, for three below 20, an RI bias of 21 was determined for (+)-aromadendrene and dimethyldisulfide, 23 for limonene and 32 for p-xylene. The greater RI bias for the polar stationary phase was reported in literature before (Zhang et al., 2011). From this experiment, LTPRI values using a polar stationary phase were reported for the first time for 14 metabolites.
Table 1 demonstrates the dominating presence of sequiterpenes and their importance for the qualitative characterization of HS profiles of fungal cultures of *F. graminearum* PH-1. Many other detected metabolites could be derived from amino acids in the medium by transamination and conversion of the α-ketoacids to aldehydes and alcohols. Dimethyldisulfide is most likely a methionine degradation product, its formation by C-S lyases via methanethiol has been investigated in microbes involved in aroma formation of cheese (Yvon and Rijnen, 2001). The following 18 metabolites were reported in fungal cultures of *Fusarium* spp. before (see table A.1 in Annex): ethyl acetate, isobutyl acetate 3-methyl-1-butanol, 2-methyl-1-butanol, 2-methyl-1-propanol, p-xylene, 1-octen-3-ol, 3-octanone, limonene, α-pinene, terpinolene, α-cedrene, β-farnesene, β-chamigrene, ar-curcumene, β-bisabolene, thujopsene and trichodiene. Fungal volatiles were described to act as autoinducers, defenders against competing species and attracters to insects for spreading fungal spores. They can be directed to the following chemical groups: alcohols, aldehydes, ketones, lactones, C8-compounds, acids and esters, alkanes, alkenes, furans, cyclic and aromatic compounds, chlorogenic hydrocarbons, monoterpenes and -terpenoids, sesquiterpenes and -terpenoids and diterpenes. This list contains identified metabolites which were confirmed with authentic standards and annotations using the commercially available NIST Wiley library. These compounds represent primary and secondary metabolites. Therefore, their chemical diversity is high showing the great extraction variety of the HS SPME method.
To our best knowledge, 12 metabolites were annotated the first time for fungal cultures of *F. graminearum* using HS SPME GC-MS: 2-heptanone, β-myrcene, p-cymol, α-longipinene, ylangene/α-copaene, β-cubebene, α-longifolene, (+)-aromadendrene, (+)-epibicyclosesquiphellandrene, α-selinene, calamenene, γ-(*trans*)-bisabolene. Besides one ketone (2-heptanone) and two monoterpenes (β-myrcene, p-cymol) these metabolites are sesquiterpenes. Calamenene is the only sesquiterpene thereof with a molecular mass of m/z 202 ($C_{15}H_{22}$), the others have a molecular mass of m/z 204 ($C_{15}H_{24}$).

The fungal volatile metabolites are biosynthesized via various metabolic pathways, see chapter 2.2. The most important pathway regarding the dominating presence of sesquiter-

penes is the mevalonate pathway. However, we were also able to detect other metabolites derived from amino acids and fatty acids. The C8-compounds were reported to be sporulation inhibitors. However, no spores could be observed under the microscope in cultures of *F. graminearum* PH-1 168 h after inoculation when 1-octen-3-ol and 3-octanone were detected in the HS.

Little is known about the specific mechanisms and ecological roles of sesquiterpenes in fungi. Their principal targets could be cell membranes because they are very apolar. Loss of osmotic control as well as facilitated passage of other toxins through membranes by sesquiterpenes were reported (Kramer and Abraham, 2012). In principle, the information mediated by sesquiterpenes can be very specific due to their huge structural variety. *Fusarium oxysporum* strains are known to alter growth and morphology of antagonistic fungal strains due to the emission of volatile sesquiterpenes. In *Penicillium decumbens*, thujopsene was observed the main volatile sesquiterpene, which inhibited the growth of other fungal strains and itself by an autoregulatory function (Kramer and Abraham, 2012). It is of high interest that also natural antagonists of *Fusarium* such as *Trichoderma* produce a huge variety of sesquiterpenes (Stoppacher et al., 2010), partially overlapping with sesquiterpenes produced from *Fusarium*. They have a similar enzymatic machinery but different expression pattern and some specific end products, like trichodiene for *Fusarium*. Additionally, plants synthesize sesquiterpenes as part of their defense response which makes terpenoid synthesis of major interest concerning studies of interaction processes between plant::fungus.
The sesquiterpenes are also important precursors for trichothecene synthesis in *Fusarium* and *Penicillium*. Trichothecenes, like deoxynivalenol, are toxic secondary metabolites, mycotoxins, produced by pathogenic fungi. In addition to the volatile metabolic profile of *F. graminearum* PH-1 cultures, the presence of mycotoxins was revealed for one week old cultures.

5.1.6 Determination of mycotoxins in *F. graminearum* PH-1 cultures on PDA using LC-MS/MS

The presence of mycotoxins in the HS-vials where PH-1 cultures were grown and extracted was tested. The whole content of the HS-vials, including PDA and the fungal mycelium, were extracted with aqueous, acidic methanol solution. The detected mycotoxins using LC-MS/MS are listed in table 5.4. Besides the quantified mycotoxins listed, rubrofusarin and 15-decalonectrin were detected in all samples with peak areas of $1.5*10^4 \pm 4.1*10^3$ and $5.6*10^5 \pm 1.5*10^4$ respectively. For this two fungal metabolites, no standards were measured during the sequence, hence they were quantified based on their peak areas.
According to Brodhagen and Keller (2006), sporulation and mycotoxin production in *Fusarium* are both regulated by G proteins, which commonly regulate fungal development, stress response and expression of virulence traits. However, no sporulation was observed for *Fusarium* cultures in HS-vials when C8-compounds and mycotoxins were detected after one week, but high amounts of trichothecenes and few non-trichothecenes were detected.
Trichothecenes all share the same 12,13-epoxytrichothec-9-ene skeleton. The most important volatile metabolite, from the viewpoint of mycotoxin contamination by *F. graminearum*, is the sesquiterpene hydrocarbon trichodiene, only present in toxigenic strains of *Fusarium* (Jelen et al., 1995). Although it seems to be metabolized in various ways and amounts, the most abundant sesquiterpene detected in the PH-1 cultures was trichodiene

Table 5.4: Mycotoxins detected in HS-vials where *F. graminearum* PH-1 was cultivated on 2 mL PDA for one week in the dark (n=6).

mycotoxin	μgg^{-1} PDA
DON	1.64 ± 0.18
3-acetyl-DON	0.72 ± 0.07
15-acetyl-DON	31.32 ± 3.07
sambucinol	3.91 ± 1.56
culmorin	35.86 ± 4.94
5-hydroxy-culmorin	0.34 ± 0.14
15-hydroxy-culmorin	0.88 ± 0.50
15-hydroxy-culmoron	0.10 ± 0.08
butenolide	72.55 ± 6.06
aurofusarin	4.99 ± 2.10

suggesting a high biosynthesis rate of this important metabolite. DON and its derivatives 3-acetyl-DON and 15-acetyl-DON were found in the extracts of PH-1 cultures, with 15-acetyl-DON being the most abundant (32.3 μgg^{-1}). *Fusarium* cultures were also reported to produce additional nontrichothecene sesquiterpenes, e.g. sambucinol, which lack the epoxide moiety characteristic of trichothecenes but are likely also derived from trichodiene (McCormick et al., 2010). The presence of sambucinol was confirmed in this experiment. In addition, 15-Decalonectrin, which was reported to be an intermediate for 15-acetyl-DON synthesis in *F. graminearum* (McCormick et al., 2004), was detected. 35.9 μgg^{-1} culmorin and a few μgg^{-1} of its derivatives 5-hydroxy-culmorin, 15-hydroxy-culmorin and 15-hydroxy-culmoron, were found in PH-1 cultures. Their presence was reported in *F. culmorum* before (Kasitu et al., 1992).

Besides the sesquiterpene derived mycotoxins, 72.6 μgg^{-1} butenolide(4-acetamido-4-hydroxy-2-butenoic acid lactone), 5 μgg^{-1} aurofusarin and rubrofusarin were detected. Butenolide is derived from amino acids and its synthesis was reported in various strains of *Fusarium*. Recently, a gene involved in butenolide synthesis was described in *F. graminearum*(Harris et al., 2007).

Aurofusarin and Rubrofusarin biosynthesis was described by Frandsen *et al.* (Frandsen et al., 2006). Starting from mevalonate pathway, the activity of polyketide synthase (PKS) 12 is required to form rubrofusarin and finally aurofusarin via nor-rubrofusarin.

Having described the mycotoxins produced by *F. graminearum* PH-1 cultures, I was interested in the involvement of volatile precursors which can be detected using the HS SPME GC-MS method. In figure 5.5 the metabolic pathways with their most important precursors for trichothecene biosynthesis are shown. Farnesylpyrophosphate from the mevalonate pathway is converted to the nerolidyl carbocation via the farnesyl cation. For the biosynthesis of DON, the nerolidyl carbocation is converted to the bisabolyl cation, whereas for the biosynthesis of culmorin, the nerolidyl cation is converted to (Z,E)-humulyl cation. Trichodiene is the immediate product of a number of conversions from the bisabolyl cation via trichodiene synthase, see figure 5.6. The volatile precursor trichodiene, which is found in high abundances in PH-1 cultures, is directed to isotrichodiol, which is the precursor for 15-decalonectrin and sambucinol. Via 3,5-diacetyl-DON, 3-acetyl-DON and 15-acetyl-DON are produced and finally also DON is produced by further deacetylation.

In planta longifolene has been described as the precursor of longiborneol, resulting from the (Z,E)-humulyl carbocation. Longiborneol itself has been reported as the volatile

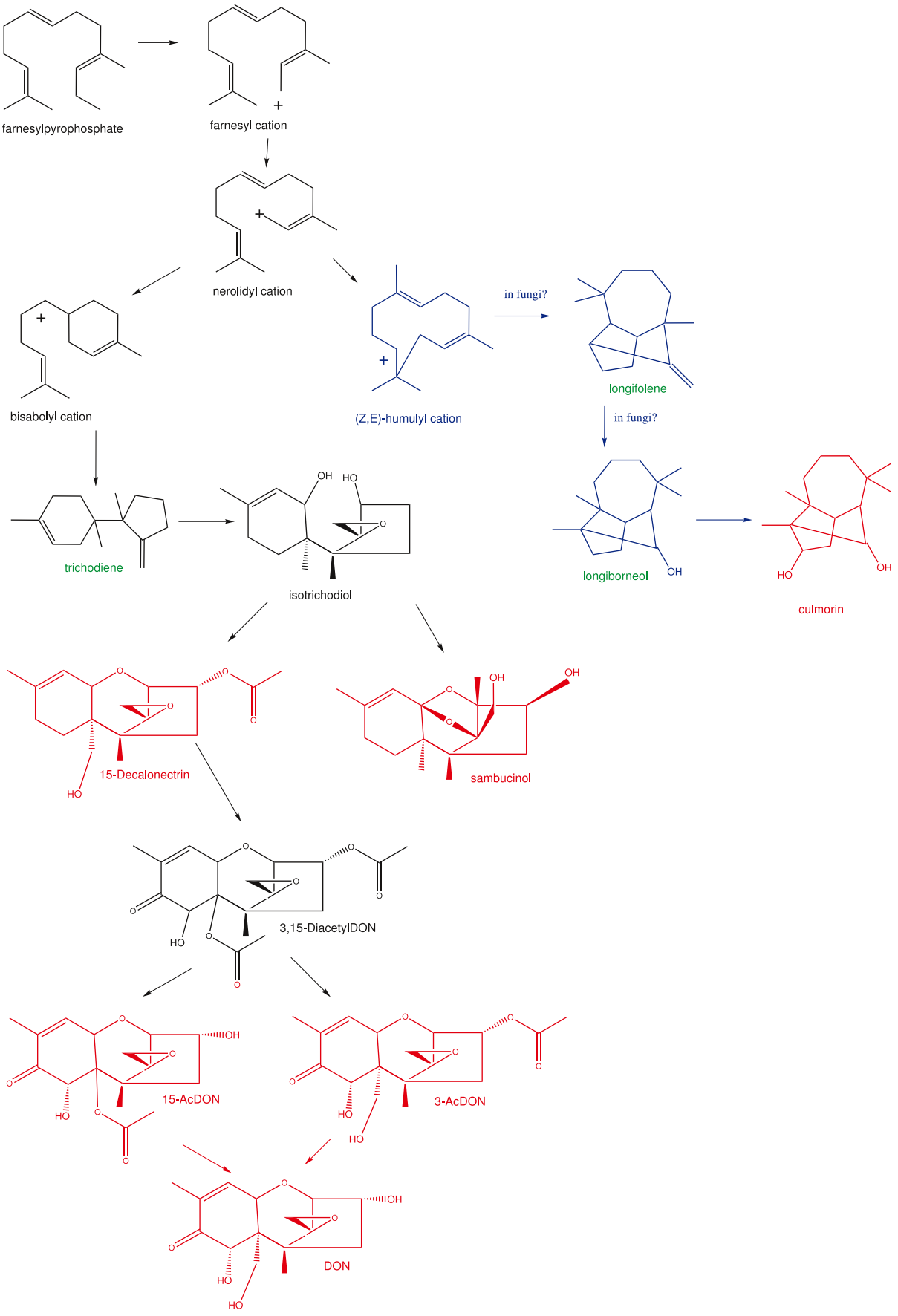


Figure 5.5: Biosynthesis of type B trichothecenes in *F. graminearum*, based on (Degenhardt et al., 2009; McCormick et al., 2010; Dewick, 2002b; Desjardins et al., 1993). Mycotoxins detected in *F. graminearum* PH-1 are marked in red, volatile precursors, which can be detected using HS SPME GC-MS method, are marked in green. DON biosynthesis is colored in black, culmorin biosynthesis is colored in blue.

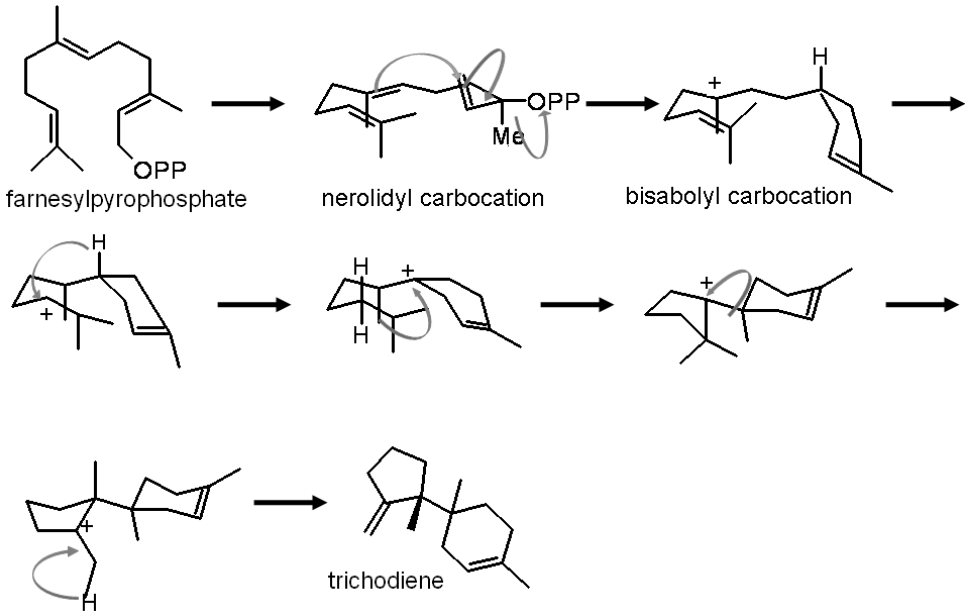


Figure 5.6: Biosynthesis of trichodiene from farnesylpyrophosphate (FPP) via nerolidyl carbocation and bisabolyl carbocation. The conversion from the bisabolyl cation to trichodiene is done by the trichodiene synthase (based on (Dewick, 2002b)).

sesquiterpenoid precursor of culmorin (McCormick et al., 2010), but it was not detected in PH-1 cultures. α-Longifolene, which could therefore also be a precursor for culmorin biosynthesis in fungi, was annotated in PH-1 cultures in this study and in *Fusarium sambucinum* strains that do not produce trichothecenes (Jelen et al., 1995). However, its role during culmorin biosynthesis is not clear.

Regarding terpene synthases, one gene encoding one enzyme leads to many different metabolic products. According to Dickschat et al. (2011), the synthesis of trichodiene, see figure 5.6, was accompanied by 15 additional sesquiterpene products which were all produced by trichodiene synthase from *Fusarium verticillioides* via the same farnesyl pyrophosphate-derived (R)-bisabolyl carbocation. The following sesquiterpenes were detected in our study as well and might therefore be synthesized via this pathway: trichodiene, β-bisabolene, γ-(*trans*)-bisabolene, β-(*trans*)-farnesene and ar-curcumene. Even all other sesquiterpenes found in *F. graminearum* could result from trichodiene synthase or, more than one terpene synthase contributes to the formation of the sesquiterpenes detected.

5.1.7 Quantification of fungal volatiles using HS SPME

Now that the importance of the volatile sesquiterpene precursors like trichodiene for the biosynthesis of mycotoxins (and therefore for the fungal virulence) was shown, absolute quantification of these metabolites would be of high interest. Absolute quantification is only achievable if an authentic standard is available for the calibration of this compound. In case of trichodiene, no reference standard is commercially available and no synthetic standard was at hand. This is a problem, which is often observed in the field of

metabolomics. For most of the metabolites and for the yet unknown metabolites, there are no authentic standards available. Therefore, it is state of the art for metabolomics applications, to do a relative quantification. There are different calibration techniques available leading to relative quantification. Furthermore, matrix effects need to be determined in any case. In the following, a number of relevant calibration techniques using HS SPME GC-MS in the field of metabolomics with special respect to the calibration of volatile compounds produced by living fungal cultures is given before matrix effects were determined for our experimental setup.

A summary of the calibration methods described in literature regarding their advantages and disadvantages is given in table 5.5. The SPME fibers are not uniformly sensitive to all compounds and they do not quantitatively extract compounds but only small amounts thereof. For the DVB/CAR/PDMS coated fiber, the fiber capacity is also limited for each adsorbent. A non-linear dependence between the amount of an analyte extracted by the fiber and its concentration in a sample was described for fibers with solid coating (Górecki T and Pawliszyn, 1999). Hence, relative GC peak areas do not properly reflect the true proportions of the components in the HS. If reproducibility is guaranteed, it is still possible to quantify in a relative way without the use of authentic standards for each compound.

Table 5.5: Advantages and disadvantages of calibration techniques for quantification of volatiles produced from living, fungal cultures using HS SPME

calibration method	**advantages**	**disadvantages**	**applications**
exhaustive - extraction	standard-free, applicable to all metabolites (known or unknown), for small sample volumes and analytes with high partition coefficients	not applicable for living organisms which reproduce extracted volatiles rapidly	(Deng et al., 2004; Hakkarainen, 2007; Tena and Carrillo, 2007)
diffusion-based calibration	standard-free, rapid field-sampling and quantification	diffusion coefficients unknown for DVB/CAR/PDMS-coated fiber, equilibrium extraction required	(Sukola et al., 2000)
standard addition	determination of matrix effects simultaneous with sample measurement	time-consuming, labour-intensive, not achievable for large sample number, spiking of living organism not possible without knowledge about interferences	(De Jager et al., 2007; Bao et al., 1999)
internal - standardisation	applicable for each sample, detection of instrumental drift and shift	cost-intensive if isotopically labelled standards are used, spiking of living organism not possible without knowledge about interferences	(Watson et al., 2000; Wang et al., 2008)
kinetic calibration using internal standards	no spiking of sample required because fiber is preloaded with standard, applicable for each sample, detection of instrumental drift and shift	cost-intensive if isotopically labelled standards are used, not applicable without knowledge about interferences in the headspace	(Musteata, 2009)
external calibration	most frequent used method, external standards do not influence living organism	matrix effects need to be adjusted to be comparable to biological sample, limited to target analysis	(De Jager et al., 2007; Watson et al., 2000; Rocha et al., 2001; Guadagni et al., 2011)

The most frequently published quantification technique for HS SPME uses exhaustive

extraction, the so called multiple headspace extraction (MHE). For this method the HS of a sample is extracted several times in a row until exhaustion. The concept of MHE is the exponential decline of volatiles during repeated extraction. Usually 2-4 serial extractions are enough to calculate the total amount of a volatile metabolite in the sample. In case of living organisms such as fungi, MHE is not achievable due to the fact that living organisms synthesize volatile metabolites continuously and very rapidly.
A standard-free kinetic calibration for aqueous samples has been proposed by Sukola et al. (2000) for rapid on-site sampling by SPME. It is based on the diffusion-controlled mass-transfer model and equilibrium extraction. With this calibration method, all analytes can be directly calibrated with only two samplings. Diffusion-based models are impracticable for solid coatings because diffusion coefficients are unknown for adsorbent materials. Additionally, this type of calibration method was described for equilibrium extraction using direct immersion which is not applicable for fungal cultures growing on solid medium with long equilibration times.
Traditional calibration methods in the field of metabolomics include external calibration, internal standardisation and standard addition. The standard addition approach involves spiking the sample matrix with known concentrations of the target analyte which originally has an unknown concentration of target analyte. The spiked sample matrices are analysed and calibration curves are constructed by plotting peak area responses against the spiked concentration of analyte. Extrapolation of the calibration curve to meet the y-axis gives the original concentration of the analyte in the unspiked sample. This calibration technique needs intensive sample preparation, particularly if the number of samples is high. It is often used to determine matrix effects. Standard addition is not possible for quantification of volatiles produced by living organisms because spiking of standard substances into the headspace is required. The addition of volatile substances can affect the living organism and changes the constitution of the headspace which is under investigation.
Internal standard calibration (Watson et al., 2000; Wang et al., 2008) requires the addition of a reference substance into the sample. It is critical that the internal standard is similar in analytical behaviour to the target analyte and it should not be present in the sample. For this reason isotopically labeled standard substances are preferred but cost-intensive. This approach helps to compensate for sample-to-sample variations during extraction, desorption and detector response. It is state-of-the-art in metabolomics applications to monitor chromatographic shifts and detector drifts from various origins over long time spans. The effects of the standard substance on the fungal culture and the HS constitution are unknown and need to be considered.
Kinetic-calibration was described for bioanalytical samples (Musteata, 2009) for *in vivo* sampling previous to equilibrium. In-fiber standardization, standard-free kinetic calibration, pre-equilibrium dominant desorption and diffusion-based interface model were discussed for this type of calibration (Vuckovic et al., 2010). Regarding in-fiber standardization, a standard substance was loaded prior to extraction of the sample, a new equilibrium was introduced and the volatiles bound to the fiber coating would dissociate partially during extraction of the sample. However, the constitution of the HS is influenced and in case of living fungi, the fungal culture is affected as well. The standard-free method overcomes this problem but two sequential extractions are not applicable for calibration of rapid changing biological systems such as fungal cultures. The dominant desorption method is suitable for homogenous samples only and requires multiple fibers and a reproducible analyte preloading procedure, which was discussed before. Diffusion rates are unknown for DVB/CAR/PDMS-coated fibers and a sampling time is rather long

to extract reproducible amounts of analytes from fungal cultures, hence, diffusion-based interface model is not an option for our study.
In the long run, external calibration is used for various extraction techniques including SPME. Standard solutions of the desired analyte are prepared over a range of concentrations (expected in the sample) and measured in parallel with the samples. A calibration curve is established plotting peak area responses over concentrations. A comparison of the sample's detector response to the calibration curve determines the amount of analyte in the unknown sample. This technique is usually applied for simple sample matrices that are homogeneous, do not vary in type or total number of compounds and where no interferences are expected. Still, there is no influence from the external calibration on the fungal culture, for it is measured in parallel in separate vials. Inconsistencies in quantification using HS SPME can arise from sampling, HS extraction (limited number of active sites on fiber surface, variations in extraction duration if extraction before equilibrium is performed) or measurement.
To date, no calibration methods to study fungal cultures on solid media using HS SPME are published. Wady et al. (2005) published a calibration method for one target analyte, methyl benzoate, as a marker for indoor moulds but HS sampling was performed over liquid extracts of contaminated samples not over living fungal cultures on solid media.
In this thesis, matrix effects were determined as a first step towards quantification.

Based on the results of qualitative characterization of the volatile metabolome, composition and amount of volatiles produced by *F. graminearum* PH-1 typically observed under the conditions described above was used to study matrix effects. Chemical and physical properties of compounds, such as the molecular weight, boiling point, molecular structure, solubility in the nutrition medium and affinity to be adsorbed on the fiber are responsible for different extraction duration and efficiency using HS SPME. According to these characteristics, representatives of the following chemical groups were defined for the study of matrix effects: the alcohols (3-methyl-1-butanol), ketones (3-octanone), monoterpenes (limonene) and sesquiterpenes (thujopsene). To clarify the extent of changes in the extraction efficiency, spiking experiments were carried out. First, the influence of the nutrition medium PDA on the extraction efficiency of target analytes was studied. Second, the influence of other volatiles on the signal intensity of single analytes was tested. This experiment included the influence from other volatiles on the sesquiterpene thujopsene and the influence from the sesquiterpenes on target analytes. Finally, we determined the matrix influence of the living fungal mycelium on volatiles. The volatiles were applied in concentrations equivalent to those detected in the fungal cultures.

Influence of nutrition medium PDA on the SPME efficiency of target analytes

Since the presented method employed SPME of volatiles directly from the HS of living fungal cultures on nutrition medium, the influence of PDA on the extraction efficiency was investigated in detail. To this end, external calibrations after addition of standard compounds into PDA containing or empty (reference) HS-vials were carried out. The calibration in the empty HS-vial was used to calculate the concentration detected in PDA containing HS-vials. The concentrations added to PDA versus the concentrations detected in the HS-vial were used for construction of recovery functions (see figure 5.7).

92 % extraction efficiency of thujopsene (displayed as the slope of the recovery line) was achieved over the concentration range tested (1-100 ng per HS-vial), which means that no

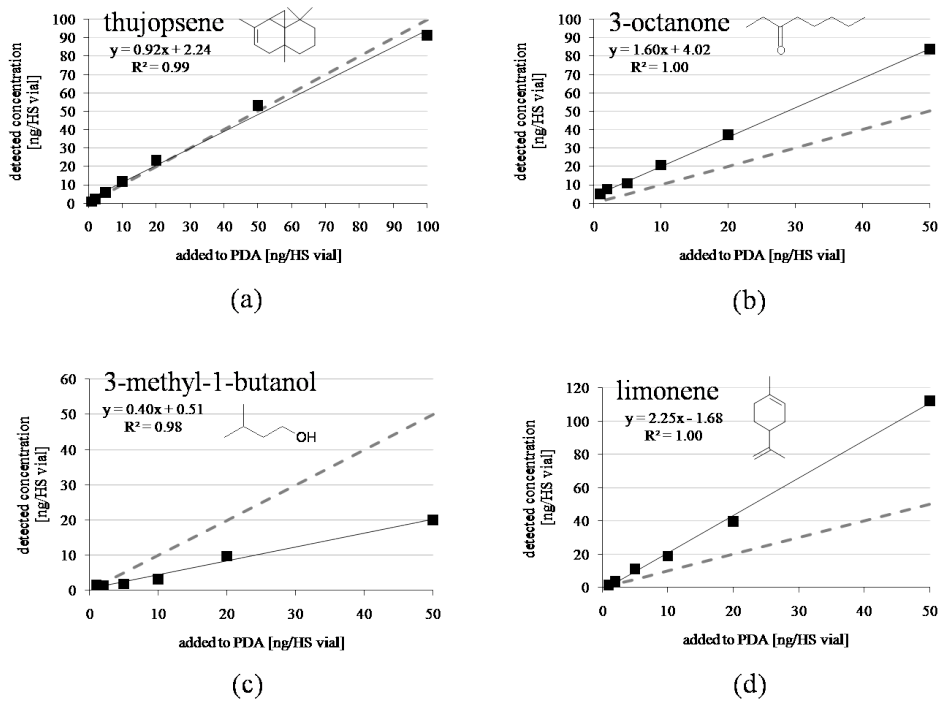


Figure 5.7: Influence of nutrition medium PDA on extraction efficiency of thujopsene (CV_{method} = 13 % relating to x_{mean} = 27 ng) , 3-octanone (CV_{method} = 4 % relating to x_{mean} = 15 ng), 3-methyl-1-butanol (CV_{method} = 18 % relating to x_{mean}= 15 ng) and limonene (CV_{method} = 7 % relating to x_{mean} = 15 ng). — expected relationship for 100 % extraction efficiency. Values represent the average of two separate experiments.

significant influence from PDA on the extraction of thujopsene was detected. The relative extraction efficiency of 3-methyl-1-butanol, representing alcohols, was concentration independently decreased to 40 %. Therefore, the underestimation of signal intensity for 3-methyl-1-butanol was proportional-systematic. A significant constant-systematic influence of 4 ng (displayed as the intercept of the recovery line) was detected for 3-octanone, ketone and C8-compound, which was not attributed to a background signal. Additionally, signal intensities of 3-octanone were overestimated by a factor of 1.6 due to a proportional-systematic influence of PDA. Equally, the signal intensity of limonene, a monoterpene, were overestimated by a factor of 2.3.

The extraction of limonene and 3-octanone were favored in presence of the nutrition medium. The addition of salt - nutrition media contain salts - was described to influence the extraction efficiency (Camara et al., 2006). It is assumed that the increase in the extraction efficiency of limonene and 3-octanone on PDA compared to the pure solvent can be attributed to the low solubility in agar. In contrast, 3-methyl-1-butanol might have participated in intermolecular interactions, which cause binding to the cultivation medium, leading to a decrease in extraction efficiency. The influence from cultivation medium on extraction efficiency of polar compounds was described for plant cultivation medium before (Maes et al., 2001). The results of this experiment show that matrix calibration on PDA was required. Hence, all further experiments were performed on PDA.

Influence of other volatiles on the extraction efficiency of the sesquiterpene thujopsene

As described in literature, SPME efficiency can depend on the relative amounts of the individual components in a given analyte mixture (Musteata, 2009). The relative composition of volatile mixtures produced by living fungal cultures is dependent on the cultivation time. This makes experiments even more complicated since matrix composition is changing over time. As a first step, three mixtures of volatiles previously annotated in fungal cultures were spiked to calibration solutions of thujopsene. Mixture 1 consisted of sesquiterpenes only. Mixture 2 contained various metabolites from *F. graminearum* PH-1 based on the results from qualitative analysis and availability of standard substances. Mixture 3 was the same as mixture 2 without 3-octanone, due to the late synthesis of this analyte by the fungus. The recovery functions showed that a change in HS constituents did not influence the extraction efficiency of thujopsene. Therefore, none of the three mixtures which were volatiles synthesized by fungal cultures of *F. graminearum* PH-1 affected the extraction efficiency of thujopsene, representing the sesquiterpenes, at concentration levels between 1 and 100 ng per HS-vial significantly.

Influence of sesquiterpenes on signal intensity of target analytes

To dissect effects of sesquiterpenes and PDA on extraction efficiency of target analytes of different chemical classes, the extraction efficiency of 3-methyl-1-butanol, limonene and 3-octanone was investigated individually. A constant amount of sesquiterpenes was added to each calibration solution of 3-methyl-1-butanol, 3-octanone and limonene for calibration in PDA containing and empty HS-vials. The resulting recovery lines of each target analyte are shown in table 5.6. The first spiking experiment revealed that signal

Table 5.6: Influence of sesquiterpenes and/or PDA on the extraction efficiency of 3-methyl-1-butanol, 3-octanone and limonene. Sesquiterpenes: (+)-α-longipinene, (-)-α-cedrene, (-)-thujopsene, (+)-aromadendrene and farnesene. Values represent the average of two separate experiments. CV_{method}...coefficient of variation of the recovery line [%], x_{mean}...arithmetic mean of the calibration range [ng], slope*100 ≙ % recovery.

	recovery line	CV_{method} [%]	x_{mean}[ng]
3-methyl-1-butanol			
in presence of sesquiterpenes in emtpy HS-vial	$y = 0.86 \cdot x - 1.0$	20	15
in presence of sesquiterpenes on PDA	$y = 0.35 \cdot x + 0.16$	23	15
3-octanone			
in presence of sesquiterpenes in emtpy HS-vial	$y = 1.2 \cdot x + 6.0$	13	27
in presence of sesquiterpenes on PDA	$y = 1.7 \cdot x + 5.0$	23	27
limonene			
in presence of sesquiterpenes in emtpy HS-vial	$y = 1.2 \cdot x + 0.7$	1.2	15
in presence of sesquiterpenes on PDA	$y = 1.5 \cdot x + 0.6$	16	15

intensity of 3-methyl-1-butanol was underestimated in presence of PDA. There was no significant influence from the sesquiterpenes detected. The presence of both, sesquiterpenes and PDA, showed that the effect of PDA was still significant, but the presence of sesquiterpenes was neglectable. The signal intensity of 3-octanone was enhanced by the sesquiterpenes as well as by PDA. In presence of both, sesquiterpenes and PDA, the effect of the sesquiterpenes was dominated by the effect of the nutrition medium and therefore, was neglected.
Unlike 3-methyl-1-butanol and 3-octanone, the signal intensity of limonene was slightly

enhanced in the presence of sesquiterpenes, whereas the first spiking experiment showed a significant signal enhancement in the presence of PDA. Here, the combination of both effectors, sesquiterpenes and PDA, revealed that the effect of sesquiterpenes overruled the matrix effect of PDA resulting in less signal enhancement of limonene by both effectors than only by PDA. For calibration of monoterpenes, matrix calibration alone was not sufficient for quantification but addition of sesquiterpenes to calibration solutions was impracticable due to changing concentrations of the sesquiterpenes during cultivation time.

Influence of the fungal mycelium on the extraction efficiency of volatiles

The influence of the fungal mycelium of *F. graminearum* on the extraction of volatiles was investigated by spiking selected VOCs to living fungal cultures. Recovery rates were evaluated only if signal intensity of spiked standard substance on PDA was twice as high as the signal intensity of this compound produced by the fungal culture and if precision (expressed as the relative standard deviation) of the three replicates was less than 30 %. Thujopsene was excluded from this evaluation due to coelution of sesquiterpenes produced by the fungus. They had overlapping retention times and similar fragment ions in the mass spectrum which did not allow proper deconvolution using MetaboliteDetector.
Recovery rates were determined by comparison of the signal intensities for each concentration of each analyte individually ($c_{fungal+added}/(c_{fungal}+c_{added})*100$). A recovery rate of 85 % was calculated for 100 ng 2-heptanone per HS-vial, 101 % for 2 ng α-pinene, 84% for 2 ng limonene, 94 % for 100 ng α-cedrene and 84 % for 100 ng (+)-aromdendrene. No significant influence of the fungal mycelium was detected for these analytes. However, the recovery rate of 100 ng 1-octen-3-ol per HS-vial was 61 % and for 100 ng 3-octanone even 181 %. Interaction of C8-compunds with the fungal mycelium was reported in literature in their role of fungal hormones or self-inhibitors regarding germination (Chitarra et al., 2004; Combet et al., 2006).

To conclude all observed matrix effects, the most critical step for external calibration is the use of a matrix calibration on PDA because the cultivation medium PDA was found detrimental for the extraction efficiency of three target analytes, namely 3-methyl-1-butanol, limonene and 3-octanone. Moreover, the presence of the sesquiterpenes influenced the extraction efficiency of limonene (representing the monoterpenes), while the fungal mycelium influenced the extraction efficiency of C8-compounds such as 3-octanone or 1-octen-3-ol. However, the dominating group of metabolites in the HS of fungal cultures, the sesquiterpenes, were not affected by the cultivation medium, the presence of other volatiles or the fungal mycelium. Therefore, external calibration for four commercially available sesquiterpenes was aimed for in the following.

Quantification of the sesquiterpenes

Based on the absence of matrix effects for the metabolite group of the sesquiterpenes, calibration lines were established for four commercially available sesquiterpenes and LOD, LOQ were estimated. For absolute quantification, each sesquiterpene of interest needs to be calibrated by the use of an authentic standard. The linear calibration range was determined for the sesquiterpenes between 1-50 ng per HS-vial (CV_{method}=13 % for thujopsene, 14 % for α-cedrene and (+)-aromadendrene, 17 % for α-longipinene). The slopes of the calibration lines of the different sesquiterpenes varied significantly, which could be explained because the quantification ions used (all of them were base peaks) contribute

different amounts to the sum of all ions in the spectrum based on the fragmentation pattern of the analyte or, due to different sensitivities of the DVB/CAR/PDMS coated fiber. The calibration range was constant over one week, but was not reproducible after a month or with freshly prepared standard solutions.
Furthermore, the reproducibility was determined at 100 ng per HS-vial. The intra-day precision was below 15 % (n=20 at 100 ng per HS-vial), the inter-day precision of four days (n=20 per day) was below 20 % for thujopsene, α-cedrene, α-longipinene and (+)-aromadendrene. It was not possible to reach relative standard deviations below 50 % for concentrations below 50 ng per HS-vials over longer time periods.
At last, extracted ion chromatograms at S/N ratios of 3 (to estimate the LOD) and 10 (to estimate the LOQ) corresponded to low signal intensities which could not be reliably deconvolved and annotated using MetaboliteDetector. A S/N ratio of 35 was necessary for reproducible compound annotation. Therefore, the LOD was set at a S/N ratio of 35, corresponding to 1 ng thujopsene or (+)-aromadendrene per HS-vial. The LOQ could not be determined reproducibly due to high standard deviations.
It is unclear whether the standard solutions were instable at this very low concentrations or the SPME fiber did not extract reproducible amounts of small concentrations, but all these problems regarding reproducibility at low concentrations make it impossible to absolutely quantify sesquiterpenes from the HS of fungal cultures in this very low concentration range.

Relative comparison of the signal intensities of the sesquiterpenes is possible in all applications regarding fungal cultures of *F. graminearum* PH-1 using the HS SPME GC-MS method. In this thesis, the volatile, metabolic profiles of different genotypes of *F. graminearum* were compared using their signal intensities, e.g. *F. graminearum* strains of the Northland population and the Δ*tri5* mutant.

5.2 Application of HS SPME GC-MS methods

The HS SPME GC-MS method was developed and evaluated for the targeted and non-targeted analysis of fungal and plant volatile metabolites. The limitations of the method were shown and discussed in the previous chapter. In the field of metabolomics, relative quantification is a major aim of each metabolomics experiment. In case of the fungal volatile metabolites using HS SPME, only relative quantification of the sesquiterpenes is feasible due to matrix effects observed for minor constituents. Dealing with these limitations, only qualitative analysis of volatile metabolic profiles from fungi and wheat is performed and only the signal intensities of the sesquiterpenes are compared on a relative level.
In the following, the study of different genotypes of *F. graminearum* could help to understand individual metabolic pathways involved in FHB disease. For this purpose, the HS profiles of *F. graminearum* cultures of the Northland population, which revealed a different mycotoxin pattern, and the Δ*tri5* mutant, lacking the *tri5* gene and therefore DON biosynthesis, are compared with the volatile metabolic profile of PH-1 cultures. Moreover, infected and non-infected wheat ears of different developmental stages and genotypes were characterized according to their volatile metabolic profiles.

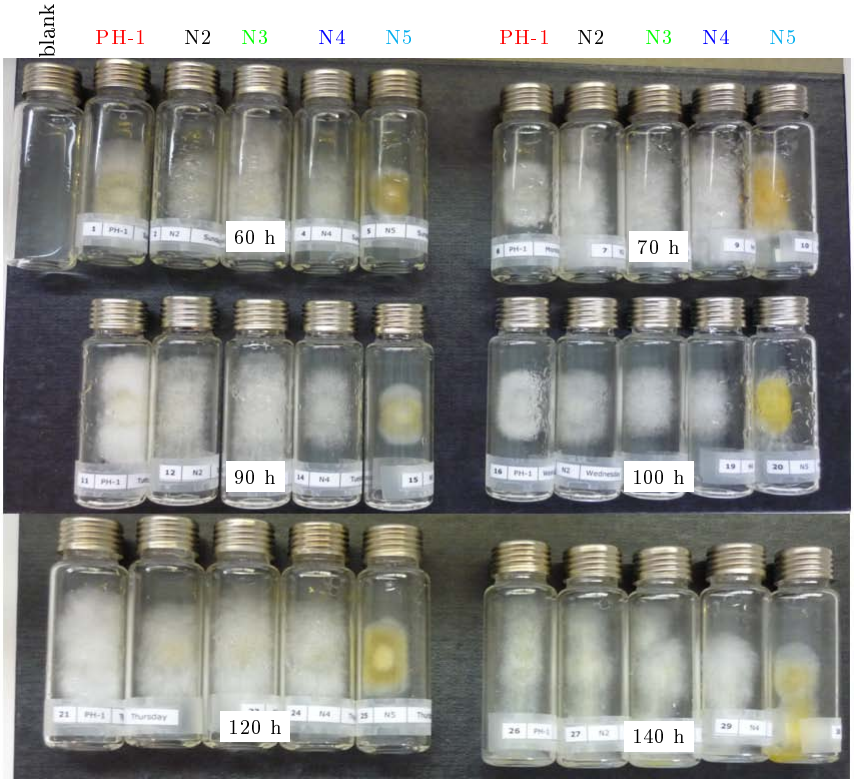


Figure 5.8: Fungal cultures of *Fusarium graminearum*: N2, N3, N4, N5 (Northland populations) and PH-1 (reference fungus). Fungal cultures were cultivated for 60, 70, 90, 100, 120 and 140 hours at 22 °C in the dark.

5.2.1 Qualitative, targeted and non-targeted characterization of four *Fusarium graminearum* strains of the Northland population

In Northern America new strains of *F. graminearum* were found and named the Northland population (Gale et al., 2010). They had been tested for their mycotoxin production by Michael Sulyok. No nivalenol, deoxynivalenol or other known type B-trichothecenes were detected using LC-MS/MS multimethod, but a 'new' trichothecene was detected, which will be published soon.
To check the presence of an active *tri5* gene for possible trichothecene synthesis, the presence of trichodiene was monitored over several time points after inoculation, namely 60, 70, 90, 100, 120 and 140 hours (see figure 5.8). As a reference (due to lack of authentic standard trichodiene), *F. graminearum* PH-1 was measured in parallel where trichodiene was previously detected. Furthermore, a non-targeted analysis was performed to monitor all other sesquiterpenes for the *Fusarium* strains using HS SPME GC-MS method.
The fungal cultures of PH-1 and the Northland population were cultivated at 22 ° in the dark in HS-vials. After 60 hours, the white mycelium covered already the greatest part of the cultiation medium, see figure 5.8. The cultures of N5 were retarded in their growth and showed a yellow coloured mycelium. This could be an indication that the fungal cultures of N5 were contaminated or not healthy. The fungal cultures were flushed with synthetic air six hours prior to extraction.

The TIC chromatograms of PH-1 and N2 cultures (representing the Northland population) at 140 h after inoculation are shown in figure 5.9. First of all, trichodiene was detected in the HS of all *F. graminearum* cultures, in PH-1 and all strains of the Northland population. Moreover, the *F. graminearum* strains synthesized metabolites from various chemical

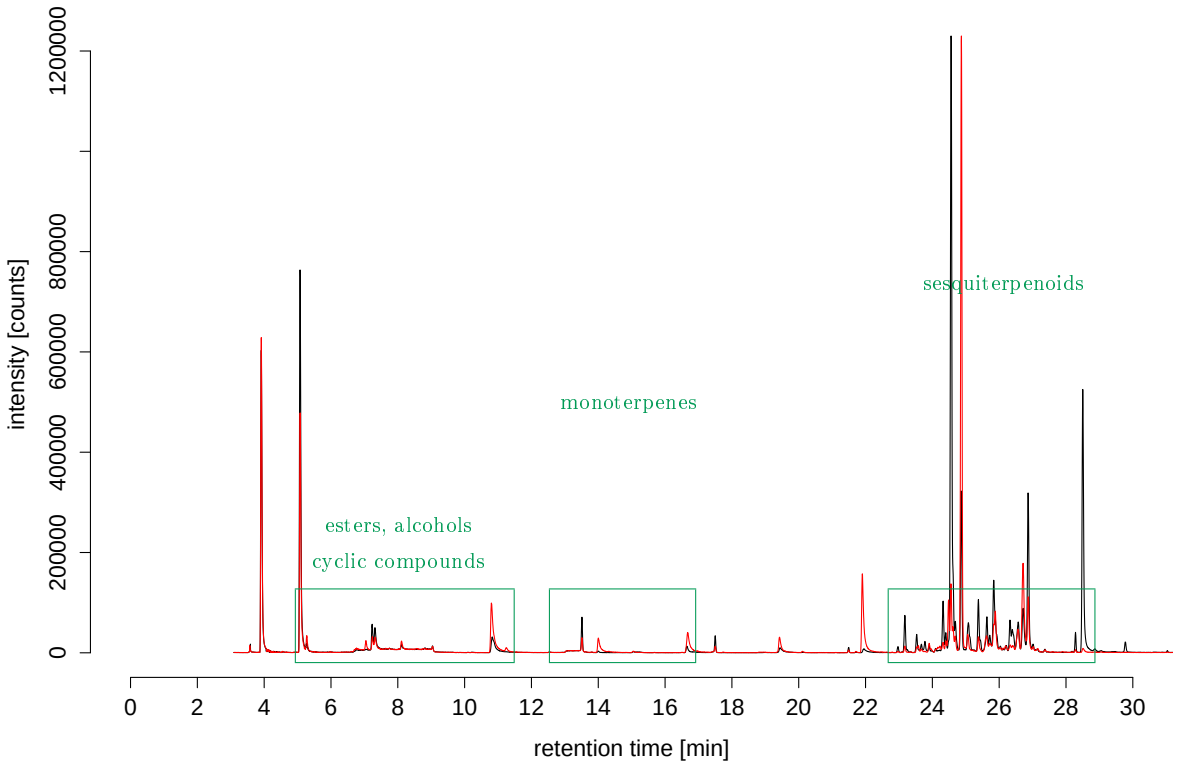
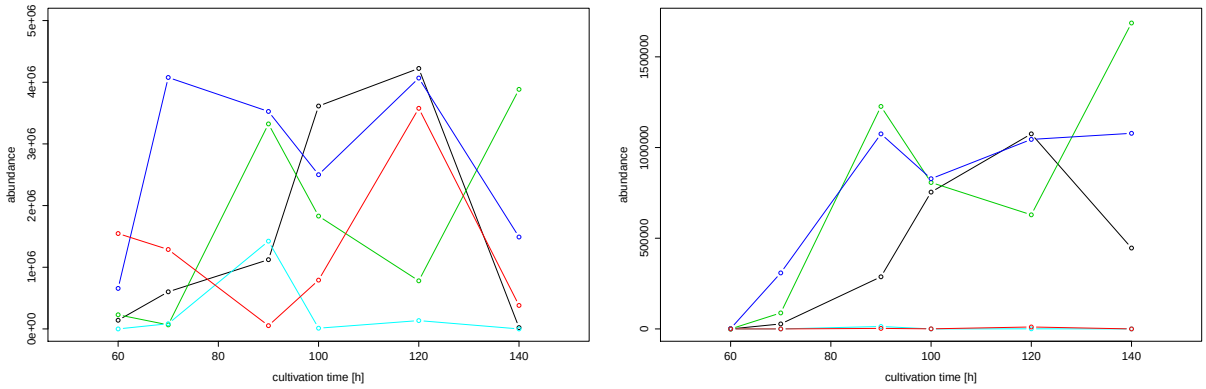


Figure 5.9: Total ion current (TIC) chromatogram of fungal cultures of **N2** and PH-1 at 140 h after inoculation.

groups: alcohols, esters, cyclic compounds, mono- and sesquiterpenoids.
A total of 73 metabolites were detected. 57 of them were described for PH-1 cultures before, see table 5.3. The *F. graminearum* strains of the Northland population produced these metabolites as well as 16 additional metabolites. Only one of these metabolites could be annotated as longiborneol using the NIST Wiley library. Longiborneol is an oxidised sesquiterpene (sesquiterpenoid) and was detected in cultures of the Northland population, but not in PH-1 cultures. Longiborneol eluted at 28.5 minutes on the HP5ms column, see figure 5.9. The additional 15 metabolites could not be annotated but were all attributed to the group of the sesquiterpenes because of their mass spectra were closely related to other sesquiterpenes.
The signal intensities of all strains over cultivation time are shown for trichodiene and longiborneol, see figure 5.10. The signal intensities of most metabolites were lower (or the metabolites were not detected at all)in fungal cultures of N5, which could be explained by the retarded growth and the possible contamination of these cultures (no additional volatile metabolites indicating a bacterial contamination were found in comparison to the other strains of the Northland population).

Trichodiene was produced by all five strains of *F. graminearum*, strains of the Northland population and PH-1, under the conditions tested. The strain N5, which synthesized significant amounts of trichodiene only at 90 hours, was visibly different to the others (see figure 5.8. The presence of trichodiene in all strains proves the presence of an active *tri5* gene, which was questioned due to the fact that no known trichothecene was produced by fungal cultures of the Northland population. Interestingly, high amounts of zearalenone



(a) trichodiene (b) longiborneol

Figure 5.10: Abundances of selected metabolites synthesized by fungal culture of -*F. graminearum* PH-1, -N2, -N3, -N4 and -N5 over cultivation time.(a) trichodiene (m/z 109), (b) longiborneol (m/z 85).

were detected using LC-MS/MS as well as the 'new' trichothecene.

Longiborneol was not synthesized by fungal cultures of the reference strain PH-1 but in significant amounts by cultures of N2, N3 and N4. This metabolite was also not detected in N5 cultures, a possible reason was discussed before. α-longifolene was previously found in the fungal cultures of PH-1, however, longiborneol was not detected for fungal cultures of PH-1 in this of other experiments. *In planta*, α-longifolene (sesquiterpene, sum formula $C_{15}H_{24}$) was described as a precursor for longiborneol (sesquiterpenoid, sum formula $C_{15}H_{26}O$). Longiborneol was reported to be the volatile precursor for culmorin biosynthesis (McCormick et al., 2010). When LC-MS/MS measurements of the strains of the Northland population were made, culmorin was not available as reference standard, therefore it was not searched for. This should be done since longiborneol was found in the HS of these cultures, but not in PH-1 cultures, which revealed great amounts of longiborneol. It could be that PH-1 synthesized culmorin faster from longiborneol than the strains of the Northland populations and that therefore the volatile intermediate was not detected in the HS of PH-1 cultures.
This experiment showed that the same volatile metabolites were produced by *F. graminearum* strains from Northland population in comparison to *F. graminearum* PH-1. All strains of the Northland population synthesized trichodiene in comparable amounts to the reference strain PH-1. Furthermore, 15 sesquiterpenes, which could not be annotated and the sesquiterpenoid longiborneol were detected in the HS of these fungal cultures.

5.2.2 Comparison of the volatile metabolic profiles of *F. graminearum* PH-1 wild-type and Δ*tri5* mutant

In the following study, the volatile metabolic profile of the Δ*tri5* mutant was compared with profile of *F. graminearum* PH-1. To be able to provide more nutrients during cultivation and to have a better time resolution between measurements (4 h instead of 6 to 16 hours), *F. graminearum* PH-1 wild-type and Δ*tri5* mutant were cultivated in plant tissue culture boxes. The fungal cultures were not flushed with synthetic air prior to extraction, therefore the accumulated amount of metabolites from these non-flushed cultures was monitored in three consecutive time-series experiments. Two questions should be answered with these experiments: first, if there was a difference in metabolic profiles of

F. graminearum PH-1 wild-type and Δ*tri5* mutant, and second, if there was an influence from light on the metabolic profiles. As a start, the metabolic profiles of the wild-type and the mutant, both cultivated in the dark in plant tissue culture boxes, were compared.

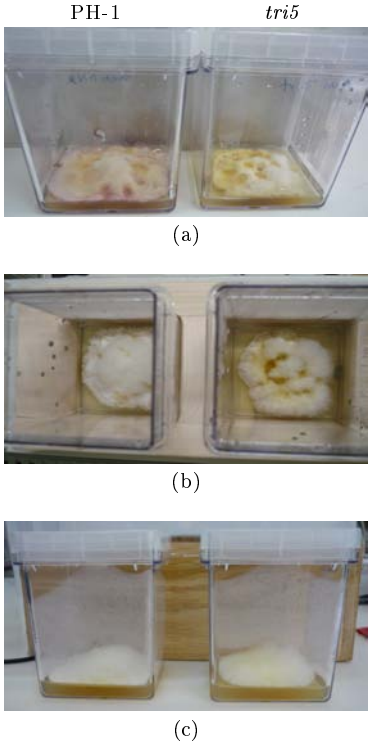


Figure 5.11: *F. graminearum* PH-1 wild-type and Δ*tri5* mutant cultivated in the dark for three repetitive experiments: first experiment (a), second experiment (b), third experiment (c). The same spore suspension was used for all experiments, resulting in an increase in spore age dependent on the experiment.

Visual inspection of the fungal cultures of *F. graminearum* PH-1 wild-type and Δ*tri5* mutant showed that the wild-type reached the edges of the plant tissue culture box after one week, whereas the mutant did not reach the edges (see figure 5.11). Still, the difference did not seem significant regarding the amount of fungal biomass.
Figure 5.12 shows the TIC chromatogram of fungal cultures of PH-1 and Δ*tri5* which were cultivated at 22 °C for 140 h in the dark. On a first glimpse, one can see that there are many peaks eluting from 18 to 22 minutes, which are synthesized by fungal cultures of the mutant only and from 24 to 28 minutes, there are metabolites which were only detected in the HS of fungal cultures of the wild-type.

Table 5.7: Results of targeted qualitative analysis of *F. graminearum* PH-1 wild-type and Δ*tri5* mutant using MetaboliteDetector. Metabolites are listed according to their elution order on HP5-ms column in two separate groups: identified and annotated metabolites. +...this metabolite was detected in the fungal culture, -...this metabolite was not detected in the fungal culture.

metabolite	PH-1	Δ*tri5*
2-methylfuran	-	+
ethyl acetate[a]	+	+
2-methyl-1-propanol[a]	+	+
3-methyl-1-butanol[a]	+	+
2-methyl-1-butanol[a]	+	+
3-methyl-butanal	+	+
p-xylene[a]	+	-

[a]identified metabolites

Table 5.7: (continued)

metabolite	PH-1	Δ*tri5*
α-pinene[a]	+	+
4-octanone	+	+
2-pentylfuran[a]	+	-
β-myrcene[a]	+	-
octanal	+	+
3-carene[a]	+	-
p-cymol[a]	+	-
limonene[a]	+	-
o-cymol[a]	+	-
α-terpinene[a]	+	-
2-propyl-1-pentanol[a]	+	+
terpinolene[a]	+	-
1-decanol[a]	-	+
acetic acid nonylester[a]	+	+
β-cubebene	+	-
α-cedrene[a]	+	-
γ-patchoulene	+	+
β-gurjunene	+	+
β-farnesene[a]	+	-
α-himachalene	+	-
α-humulene[a]	+	-
(+)-epibicyclosesquiphellandrene	+	-
β-chamigrene	+	-
α-selinene	+	-
ar-curcumene	+	-
α-chamigrene	+	-
β-bisabolene	+	-
calamenene	+	-
β-himachalene	+	-
trichodiene	+	(+)
γ-bisabolene	+	-
α-(E)-bisabolene	+	-

[a] identified metabolites

In general, small alcohols, aldehydes, ketones, cyclic and aromatic compounds as well as mono- and sesquiterpenes were synthesized by fungal cultures of *F. graminearum* PH-1 wild-type and Δ*tri5*mutant in the plant tissue culture boxes in the dark, see table 5.7. 1-Octen-3-ol and 3-octanone, which were usually detected in fungal cultures of PH-1 cultivated in HS-vials at appr. 100 h after inoculation, were not found in the plant tissue culture boxes. Since the tissue culture boxes are made out of polycarbonate, it could be that the C8-compounds interacted with the cultivation vessel and did not bind to the fiber coating.
Trichodiene was found in all experiments in fungal cultures of the wild-type PH-1, but the detected signal intensities varied between the three consecutive experiments. This high variation in signal intensity was not detected if the fungal cultures were cultivated in HS-vials, but it was detected in the plant tissue culture boxes. Furthermore, trichodiene, which was not expected in fungal cultures of the mutant because the mutant strain is a deletion mutant of the *tri5* gene, was detected in very low amounts in the HS of the Δ*tri5* mutant, see figure 5.13. Age of spores seemed detrimental for production of trichodiene in wild-type cultures (see figure 5.13) and small amounts of trichodiene were detected in fungal cultures of the mutant. Using polymerase chain reaction (PCR) technique, fungal cultures from the last experiment were analysed for the presence of the *tri5* gene. In the mutant strain, no *tri5* gene was detected. Therefore, the surprising detection of trichodiene by the fungal cultures of the mutant could be explained by the activity of a different terpene synthase in older fungal cultures which synthesized this sesquiterpene.

Many metablites, mostly mono- and sesquiterpenes, were detected in fungal cultures of

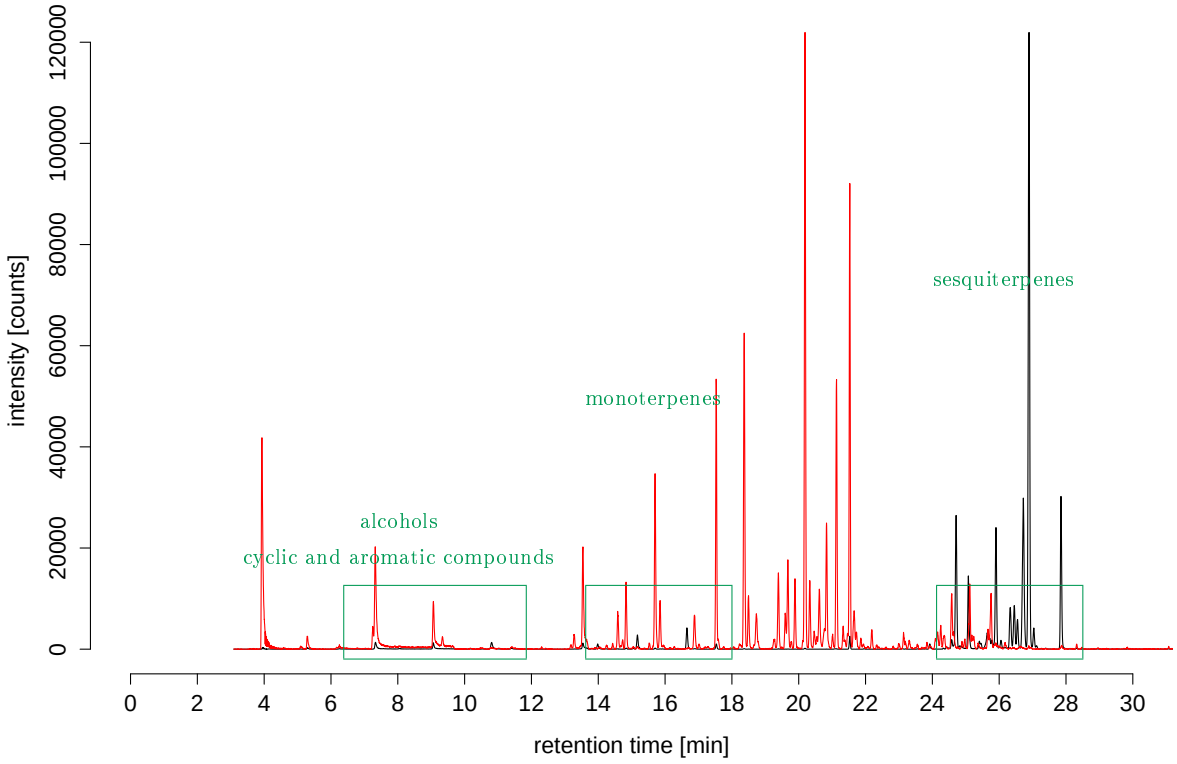


Figure 5.12: Total ion current (TIC) chromatogram of fungal cultures of *F. graminearum* PH-1 and Δ*tri5* at 140 h after inoculation in the dark.

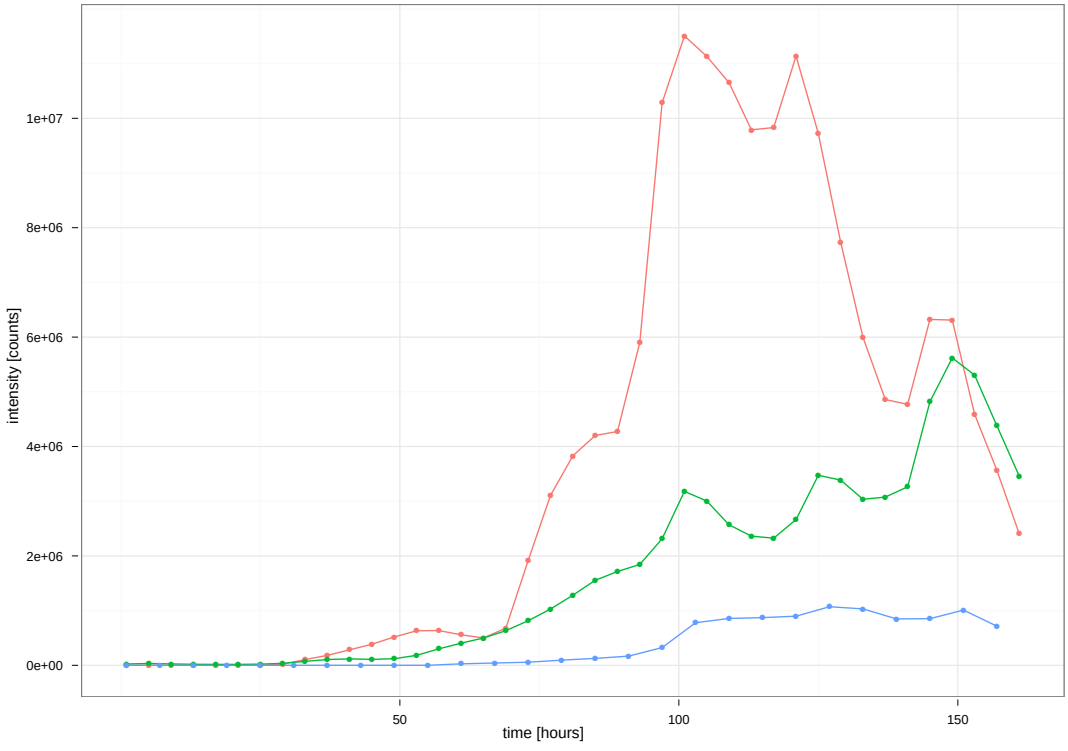


Figure 5.13: Signal intensities measured in three consecutive experiments for trichodiene in fungal cultures of *F. graminearum* PH-1 cultivated in the dark. –experiment 1, –experiment 2,–experiment 3

the wild-type only. Besides the identified and annotated sesquiterpenes, additional 14 sesquiterpenes were detected which could not be annotated, but were structurally related to the group of sesquiterpenes due to their molecular mass of m/z of 202 or 204 and their characteristic fragmentation pattern. Most sesquiterpenes produced by fungal cultures of *F. graminearum* PH-1 were not detected in fungal cultures of Δ*tri5*. This finding supports the hypothesis that trichodiene synthase (encoded by *tri5* gene) was able to synthesize a great variety of sesquiterpene products with trichodiene being the principle compound (Dickschat et al., 2011).
There were also metabolites which were detected in fungal cultures of the mutant Δ*tri5*strain only: five sesquiterpenes which could not be annotated, 2-methylfuran, 1-decanol and more than 20 metabolites which could not be annotated but were structurally related to alkanes or substances with alkyl residues due to their fragmentation pattern. These metabolites were found in comparable amounts at all time points during cultivation and measurements. It could be that, although they are quite domineering in the chromatogram (see figure 5.12), they should not be attributed to the fungal cultures but to some background signals. A small number of alkanes like tetradecane and pentadecane was detected in PH-1 cultures as well as in the cultivation blank and was therefore not considered biologically relevant.

Having studied the differences between metabolic profiles of *F. graminearum* PH-1 wild-type and Δ*tri5*mutant from cultures in the dark, the influence from cultivation in the light was under investigation. For cultivation in the light, wooden boxes with artifical light (LEDs with 25000 lux for daylight simulation) were placed over the plant tissue culture boxes. The same boxes were used to cover the plant tissue culture boxes for cultivation in the dark to protect them from light. Less condensed water was found in the plant tissue culture boxes where fungal cultures were cultivated in the light, see figure 5.14.

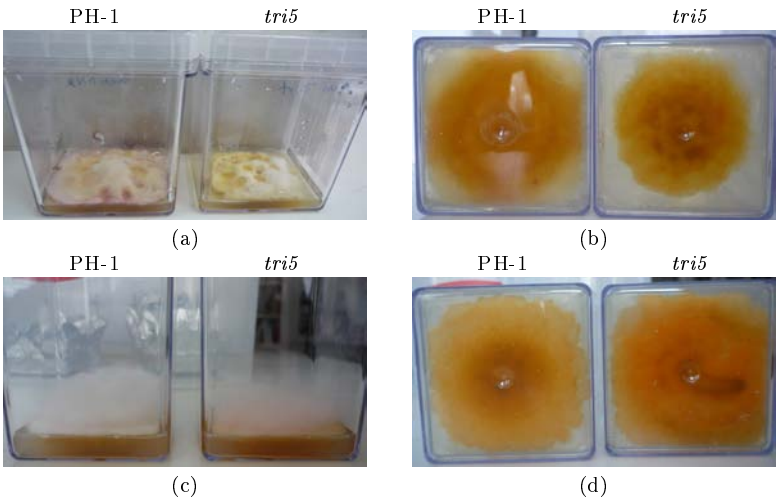


Figure 5.14: *F. graminearum* PH-1 wild-type and Δ*tri5*mutant were cultivated in the dark and in the light in parallel in experiment 1. (a) top view on cultures grown in the dark, (b) bottom view on cultures grown in the dark, (c) top view on cultures grown in the light, (d) bottom view on cultures grown in the light.

The TIC chromatogram in figure 5.15 shows that small alcohols, 2-methyl-1-propanol (t_R 5.3 min) and 2-methyl-1-butanol (t_R 7.3 min), were synthesized in higher amounts in PH-1 cultures grown in the light than in the dark, whereas the number and amount of

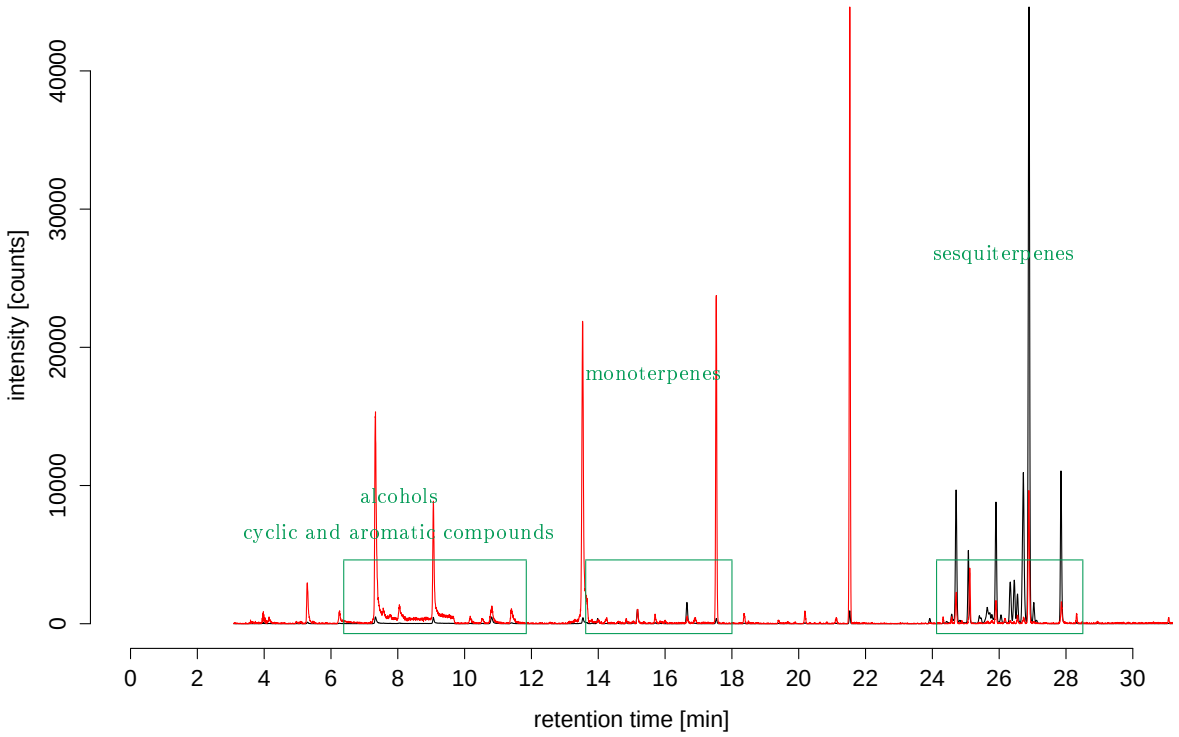


Figure 5.15: Total ion current (TIC) chromatogram of fungal cultures of *F. graminearum* PH-1 after 140 h cultivation in the dark and in the light .

sesquiterpenes was much lower. The only sesquiterpenes detected in fungal cultures of *F. graminearum* PH-1 exposed to light were (+)-β-gurjunene, α-selinene, γ-bisabolene and trichodiene. Most of the sesquiterpenes were not synthesized in the light and those which were produced, were found in much lower amounts (factor ≥ 50). In figure 5.16 the signal intensities of trichodiene in PH-1 cultures grown in the dark or in the light are shown as an example. There were no significant amounts of trichodiene detected in the cultures, which were exposed to light, in comparison to the high signal intensities of up to $6{\cdot}10^6$ (10^7 in one experiment) detected in cultures kept in the dark. This finding suggests that the *tri5* gene expression itself is sensitive to light, or, the enzyme trichodiene synthase (and other terpene synthases) is sensitive to light and less active if the fungal cultures are cultivated in the light.

There was no significant influence by light on the volatile profiles produced by cultures of the Δ*tri5* mutant, which did not synthesize many sesquiterpenes at all. Interestingly, fungal cultures of both genotypes revealed one metabolite only in the light. It was annotated as 1,2,4-trimethylbenzene. Jelen and Wasowicz (1998) described trimethylbenzenes for fungal cultures of *Fusaria* as frequently occurring hydrocarbons. Trimethylbenzene was detected as one of the main volatile compounds in the off-flavour of infected wheat (Olsson et al., 2000).

To sum up, the Δ*tri5* mutant cultures synthesized the same volatile metabolites as the PH-1 wild-type cultures regarding all substance classes, which were mainly the alcohols, aldehydes and esters, except for the mono- and sesquiterpenes. The lack of more sesquiter-

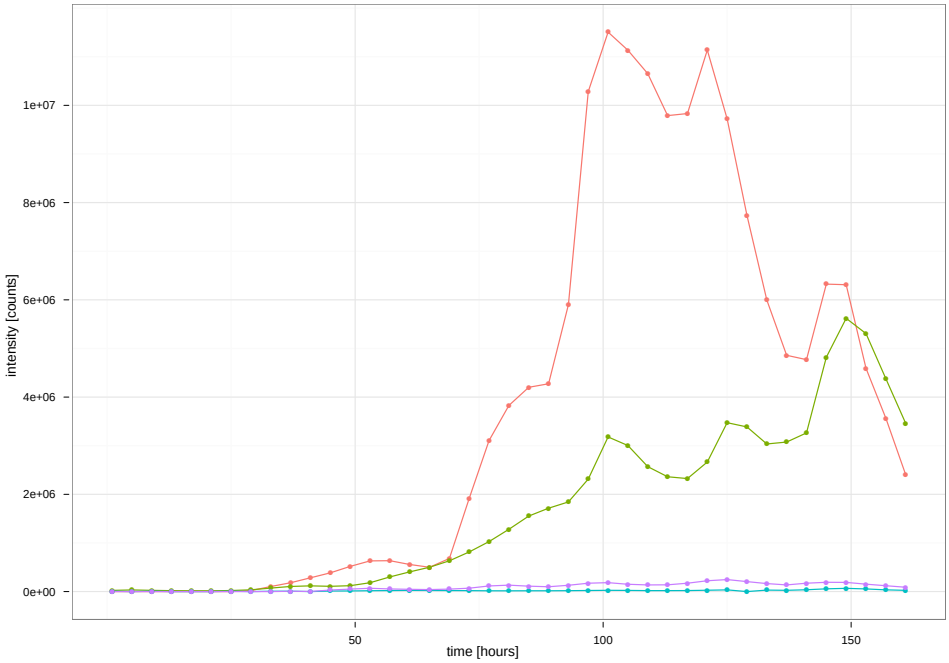


Figure 5.16: Signal intensities measured in two consecutive experiments for trichodiene in fungal cultures of *F. graminearum* PH-1 cultivated in the dark and in the light. –experiment1 dark, –experiment2 dark,–experiment1 light,–experiment2 light

penes than trichodiene is a strong indication that the trichodiene synthase is responsible for the biosynthesis of several sesquiterpenes. Possibly, the gene deletion of the *tri5* gene could also lead to a feedback inhibition of other enzymes, which are involved in mono- and sesquiterpene biosynthesis earlier, since only a few of the metabolites of these two substance classes were detected in the Δ*tri5* cultures. Moreover, the cultivation of PH-1 cultures in the light was detrimental for the biosynthesis of the sesquiterpenes.

Although more cultivation medium and space were provided to the fungal cultures in the tissue culture boxes compared to the HS-vials, the time series experiments were limited to one week because then the edges of the boxes were reached by the fungal mycelium and the metabolic profile showed a decrease in the volatile production and no appearance of later synthesized metabolites. The change of the cultivation vessel (from HS-vial to plant tissue culture box) did not improve our possibilities to study the volatile metabolic profiles of fungal cultures.
Throughout the thesis many repetitive experiments to study the fungal volatiles were carried out in HS-vials and only few were carried out in the plant tissue culture boxes. The VOC profiles were very consistent regarding the detected number of compounds and signal intensities if the cultivation was performed in HS-vials. To conclude, studies regarding the volatile metabolic profiles of *Fusarium* spp. should be carried out in HS-vials in the dark to receive reproducible VOC profiles with a high number of compounds.

5.2.3 Comparison of volatile metabolic profiles of wheat ears of different developmental stages and *Fusarium*-infected wheat ears

In the next step to study volatile metabolites which are involved during FHB disease, the plant volatiles were investigated. The plant tissue culture boxes were used to cultivate healthy plant seedlings of *Arabidopsis* and *Brachypodium* spp. to study the volatile metabolic profiles of growing seedlings. *Arabidopsis* and *Brachypodium* spp. could serve as model plants to study the interaction between fungus and plant in living systems. However, the extraction temperature of 22 °C or 30 °C was too low for the HS SPME of plant volatiles. No volatiles or not enough of them was bound to the fiber coating to result in detectable peaks. Furthermore, wheat ears, as the final targets (hosts) regarding FHB disease, are too big to be cultivated in the plant tissue culture boxes. At 90 °C the successful extraction of volatiles from the HS of frozen, ground grape vine leaves in HS-vials was reported and evaluated in the metabolomics group (Weingart et al., 2012). Therefore, it was used for the study the volatile metabolome of wheat ears in the following.

In the first experiment to detect volatiles from wheat ears, wheat ears from cultivar Capo were studied regarding their developmental stage and metabolic changes after *Fusarium*-infection. The purpose of this experiment was to enhance the in-house library for volatiles with mass spectra and RI values of annotated metabolites detected in non-infected and infected wheat ears. Moreover, I tried to find significant differences in the metabolic pattern of plant volatiles dependent on their developmental stage or *Fusarium* infection. Since all volatile metabolites except for the sesquiterpenes were shown to be influenced by matrix effects (see chapter 5.1.7), I focused on annotated sesquiterpenes and those metabolites, which could not be annotated but showed a mass spectrum similar to the sesquiterpenes and a corresponding RI value, for statistical analysis.

It was decided after visual inspection which wheat ears belonged to which flowering state, dependent on the presence of anthers and their color, see figure 5.17: before flowering (no anthers), during flowering (yellow anthers), after flowering (white anthers). The compounds detected in non-infected wheat ears of different developmental stages and infected wheat ears using two columns of different polarity for chromatographic separation, are listed in table 5.8. A total number of 8 wheat ears before, 9 during, 10 after flowering and 9 infected wheat ears was measured. Table 5.8 lists the number of samples in which each metabolite was detected.

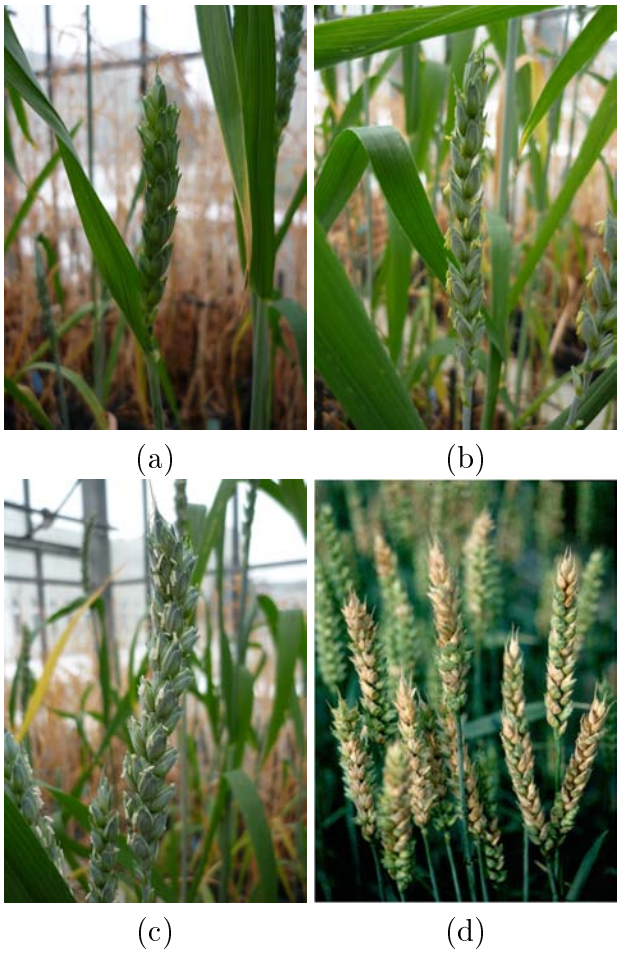


Figure 5.17: Non-infected wheat ears at different developmental stages (a) before flowering, (b) during flowering, (c) after flowering and (d) *F. graminearum*-infected wheat ears. Pictures were taken from Remus wheat ears; picture (d) was taken 14 days after inoculation.

Table 5.8: List of annotated and identified metabolites obtained in non-infected wheat ears at different developmental stages (before, during and after flowering) and *Fusarium*-infected wheat ears using HS SPME and two columns of different polarity. The number of samples for each sample type in which each metabolite was detected is given. Metabolites are sorted in elution order on HP5-ms column. RIs and t_Rs were determined on HP5-ms and Optima Wax columns respectively. Measurement and data processing were carried out according to chapter 4.3.2 and 4.3.4.

metabolite	CAS	RI HP5	t_R HP5	RI Wax	t_R Wax	Ion (m/z)	before flow. (n=8)	during flow. (n=9)	after flow. (n=10)	infected (n=9)
2-ethylfuran[a]	3208-16-0	702	5.24	1193	11.74	81	8	9	10	6
pentanal	110-62-3	704	5.73	927	6.03	58	3	2	3	3
hexanal	66-25-1	803	8.77	1091	9.04	56	8	9	10	9
3-furaldehyde	498-60-2	830	9.59	1469	18.62	95	7	8	8	6
2-(*trans*)-hexenal[a]	1335-39-3	857	10.41	1256	13.16	83	8	8	10	8
1-hexanol[a]	111-27-3	873	10.88	1370	16.13	56	8	9	10	9
2-heptanone[a]	110-43-0	894	11.55	1246	12.89	58	8	9	10	9
heptanal	111-71-7	904	11.86	1186	11.39	70	8	9	10	9
(*trans*,*trans*)-2.4-hexadienol[a]	111-28-4	917	12.25	1787	25.67	133	8	9	10	9
hexadecanoic acid	57-10-3	954	13.35	1666	22.76	98	4	8	5	4
benzaldehyde[a]	100-52-7	966	13.79	1521	19.86	106	8	9	10	9
1-octen-3-ol[a]	3391-86-4	982	14.32	1461	18.43	57	8	9	10	9
3-octanone[a]	106-68-3	986	14.43	1612	21.54	99	8	9	10	9
2-pentylfuran[a]	3777-69-3	994	14.68	1232	12.55	81	8	9	10	9
octanal	124-13-0	1005	15.04	1345	15.24	57	6	9	10	9
benzylalcohol	100-51-6	1041	16.14	1697	23.50	108	1	6	2	0
1-octanol[a]	111-87-5	1072	17.09	1580	21.28	56	8	8	10	9
nonanal	124-19-6	1106	18.12	1400	16.91	57	8	9	10	9
phenylethylalcohol[a]	60-12-8	1121	18.56	1967	29.54	91	8	9	9	9
1-nonanol	143088	1163	19.77	1567	20.85	56	7	7	9	9
(*trans*,*trans*)-2.4-nonadienal	5910-87-2	1195	20.39	1675	23.40	81	8	9	10	9
2.4-(*trans*,*trans*)-decadienal	25152-84-5	1219	21.36	1670	23.11	81	8	9	10	9
2-undecanone	112-12-9	1290	23.31	1617	22.06	58	8	9	10	9
3-octen-1-ol	18185-81-4	1345	24.33	1498	19.31	81	3	2	4	3
Vanillin	121-33-5	1411	26.43	2626	41.07	151	8	9	10	9
α-longifolene	475-20-7	1422	26.69	1588	21.50	161	0	0	0	8
(+)-β-gurjunene	17334-55-3	1445	27.28	2449	38.35	161	0	0	0	9
2-tridecanone	593-08-8	1469	27.78	1830	26.82	58	8	9	10	9
β-himachalene	1461-03-6	1527	29.24	1777	26.0	119	0	0	0	2
α-curcumene	644-30-4	1491	28.39	1846	27.16	132	0	0	0	3
calamenene	483-77-2	1494	28.46	2340	35.88	159	8	9	6	9
tridecanal	10486-19-8	1507	28.78	2091	32.01	57	8	9	10	9
β-bisabolene	495-61-4	1517	28.99	1735	24.82	69	0	0	0	7
trichodiene	76231-80-6	1538	29.50	1544	20.13	109	0	0	0	9
1-tridecanol[a]	112-70-9	1576	30.38	1838	26.69	70	3	0	2	5
longiborneol	465-24-7	1616	31.28	3292	33.85	85	0	0	0	9
2-pentadecanone	2345-28-0	1692	32.88	2044	31.15	58	8	9	10	9
tetradecanoic acid	544-63-8	1757	34.26	2724	42.53	73	8	9	10	9

[a]identified metabolites

The annotated metabolites belong to different chemical classes: furans, acids, alcohols, ketones, aldehydes, aromatic compounds, GLVs (green leaf volatiles), C8-compounds and sesquiterpenes and -terpenoids. Hexadecanoic acid and benzylalcohol were found in wheat ears during flowering, hexadecanoic acid was also detected in wheat ears after flowering. Both metabolites could not be detected in significant numbers in wheat ears before flowering or in infected wheat ears. Four of the 38 annotated and identified metabolites were not found in at least five samples of any sample type, which indicates a random appearance (not a significant appearance) of this metabolite. Besides these metabolites, the listed compounds in table 5.8 were found either in all non-infected wheat ears or in infected wheat ears. This means that none of the volatile alcohols, aldehydes, ketones, GLVs or C8-compounds was synthesized at a specific developmental stage in healthy wheat ears. Moreover, they were also produced in non-infected and infected wheat ears. Based on this qualitative comparison, no metabolites seem to be involved during FHB disease, except for the sesquiterpenes. Besides calamenene, the six sesquiterpenes and -terpenoid, which were found in at least five samples of one sample type, were found in infected wheat ears only, not in healthy wheat ears, suggesting that they were synthesized from the fungus or as defense related compounds by the plant. With this experimental setup their origin cannot be further defined.
On addition to the 38 annotated and identified metabolites (on both stationary phases), 261 metabolites were detected but could not be annotated according to the chosen criteria (mass spectral match higher 80 % and RI deviation of 30 units) using the HP5-ms column. Eight of these metabolites showed a characteristic sesquiterpene fragmentation pattern, only seven of them were present in more than five samples of at least one sample type. Out of the six annotated sesquiterpenes and the detected sesquiterpenoid (longiborneol), only four sesquiterpenes and longiborneol were detected in at least 5 samples of one sample type. Only sesquiterpenes, which were detected in at least five samples of one sample type were used in a data matrix for further statistical analysis. First, the non-infected wheat ears of different developmental stages were compared with respect to find significant differences in the sesquiterpenes. Since only calamene was successfully annotated, Student's t-tests were performed for calamene. There was a significant difference in the signal intensities of calamene of wheat ears before and during flowering (p-value <0.05). The signal intensities of calamenene in wheat ears during and after flowering were comparable. None of the sesquiterpenes, which could not be annotated, revealed significantly differing signal intensities dependent on the developmental stage.
This leads me to the comparison between healthy and *Fusarium*-infected wheat ears. Again, only the sesquiterpenes were statistically analysed. In case of α-longifolene, β-gurjunene, β-bisabolene, trichodiene and longiborneol no t-tests are necessary to see that these sesquiterpenes and -terpenoid were only detected in the *Fusarium*-infected wheat ears. As discussed before, they could results from fungal or plant biosynthesis, except for trichodiene which was attributed to fungal biosynthesis according to literature. In addition, five of the sesquiterpenes, which could not be annotated, were detected only in the infected wheat ears, but not in the non-infected wheat ears. Two sesquiterpenes were found in all wheat ears, independent from infection, in comparable amounts and also the signal intensity of calamene did not significantly change after infection.

Additionally to the volatile metabolic profiles of *Fusarium*-infected wheat ears, ergosterol and DON were determined. Ergosterol was taken as a marker for fungal biomass, whereas the presence of DON indicated the production of several mycotoxins with DON being the most prominent for *F. graminearum*-infection. The volatile precursor trichodiene and

the end product DON were shown to be both present in infected wheat ears. Table 5.9 shows the coappearance of the volatile precursor trichodiene and the end product DON in *Fusarium*-infected wheat ears as well as the coexistence of α-longifolene and longiborneol in the very same wheat ears.

The amount of ergosterol was determined quantitatively as a marker for fungal biomass

Table 5.9: Abundances of trichodiene (m/z 109), α-longifolene (m/z 161) and longiborneol (m/z 85) detected in *Fusarium*-infected wheat ears using HS SPME GC-MS using an HP5-ms column. The presence of DON and amount of ergosterol were determined using LC-MS/MS.

metabolite	column	sample1	sample2	sample3	sample4	sample5	sample6	sample7	sample8	sample9
ergosterol (ng/mL)		736	0	100	236	372	1958	1254	0	5540
trichodiene	HP5	$3.20{\cdot}10^6$	$8.66{\cdot}10^5$	$3.16{\cdot}10^6$	$1.74{\cdot}10^6$	$1.04{\cdot}10^7$	$6.85{\cdot}10^6$	$3.79{\cdot}10^7$	$8.84{\cdot}10^5$	$5.33{\cdot}10^7$
	Wax	$5.55{\cdot}10^6$	$9.09{\cdot}10^5$	$4.88{\cdot}10^6$	$4.38{\cdot}10^6$	$5.84{\cdot}10^7$	$2.48{\cdot}10^6$	$4.26{\cdot}10^7$	$8.95{\cdot}10^4$	$1.25{\cdot}10^7$
DON		present	present	present	present	present	present	present	present	present
α-longifolene	HP5	$3.48{\cdot}10^5$	$1.23{\cdot}10^5$	$3.59{\cdot}10^5$	$2.15{\cdot}10^5$	$8.56{\cdot}10^5$	$8.72{\cdot}10^5$	$1.60{\cdot}10^6$	0	$2.31{\cdot}10^6$
	Wax	$7.65{\cdot}10^5$	$1.68{\cdot}10^5$	$4.05{\cdot}10^5$	$6.73{\cdot}10^5$	$2.40{\cdot}10^6$	$3.94{\cdot}10^5$	$1.09{\cdot}10^6$	0	$9.17{\cdot}10^5$
longiborneol	HP5	$2.50{\cdot}10^5$	$8.75{\cdot}10^4$	$3.64{\cdot}10^5$	$1.32{\cdot}10^5$	$9.68{\cdot}10^5$	$5.45{\cdot}10^5$	$3.08{\cdot}10^6$	$1.04{\cdot}10^5$	$3.16{\cdot}10^6$
	Wax	$2.55{\cdot}10^5$	$1.04{\cdot}10^5$	$9.79{\cdot}10^5$	$2.24{\cdot}10^5$	$3.10{\cdot}10^6$	0	$2.85{\cdot}10^6$	0	$9.99{\cdot}10^5$

using LC-MS/MS. The amount of ergosterol did correlate for most samples, but not for sample 5 and 6, with the amount of trichodiene detected. This finding suggests that in general, the amount of volatile precursors for mycotoxin production is directly correlated with the amount of fungal biomass. This was just a pilot experiment to check the presence of important metabolites, hence it was analysed on a qualitative basis only for DON. All *Fusarium*-infected samples contained trichodiene and DON. Both metabolites were attributed to be fungal metabolites because they were not reported for plants before. α-longifolene was reported to be the precursor of longiborneol *in planta* (McCormick et al., 2010), which in succession is the precursor for culmorin biosynthesis. Whether α-longifolene and longiborneol were produced by wheat or by *Fusarium* could not be determined in this experiment. Having studied the fungal volatiles of PH-1 cultures individually, no longiborneol was detected in the HS above PH-1 cultures on PDA. This suggests that either longiborneol was synthesized from wheat or the fungus requires wheat as a matrix for a higher longiborneol production than on the cultivation medium PDA. This could be tested in labeling experiments or separate HS analysis of wheat and fungus and the detection of culmorin in parallel. Both experimental setups, for labeling and separate HS analysis of two organisms, are not available at the metabolomics platform now. For the time being, there was no culmorin standard available and could therefore not be determined to compare the detected amount of culmorin in infected wheat ears with the amount of culmorin produced by *F. graminearum* PH-1 on PDA.

5.2.4 Comparison of volatile metabolic profiles of different genotypes after infection with *F. graminearum*

The last experiment regarding metabolic profiles of volatiles from wheat ears included wheat ears of two different genotypes, cultivar 'Remus' is susceptible to *Fusarium*, cultivar 'CM' is resistent against FHB disease. The wheat ears were treated with water (healthy), with DON or infected with *F. graminearum* PH-1. In the following, the detected volatile metabolites of healthy wheat ears and the *Fusarium*-infected wheat ears

of the cultivars CM and Remus are described and statistically evaluated.

Table 5.10 lists all detected metabolites in wheat ears of CM and Remus, which were treated with water (healthy) or infected with *F. graminearum* PH-1. Only metabolites which were not present in the fiber blank and at least in 3 of 5 biological replicates at one sampling point were considered for statistical evaluation. The detected metabolites include alcohols, aldehydes, ketones, sulphuric and cyclic compounds, alkanes, mono- and sesquiterpenes (and one sesquiterpenoid, namely longiborneol). The sesquiterpenes were found in wheat ears after *Fusarium* infection only. They were not detected in healthy wheat ears at any sampling point. Some sesquiterpenes were found in one genotype only: β-Cubebene, sesquiterpene1, sesquiterpene 4, sesquiterpene27, a metabolite which is similar to ylangene (sesquiterpenes, which were found in PH-1 cultures frequently, but could not be annotated), β-farnesene, β-humulene and β-himachalene were only detected in wheat ears of the cultivar Remus, whereas e.g. (-)-alloaromadendrene, β-bisabolene and sesquiterpene10 were found in wheat ears of the cultivar CM only. This difference in the biosynthesis of the sesquiterpenes could be a result of a different plant defense response. Since wheat ears of the cultivar CM are resistent against FHB disease, the interaction with the fungus is different to the response of a susceptible cultivar like Remus. However, whether the differentially produced sesquiterpenes are a result of fungal or plant biosynthesis is unclear.
Besides pentadecane, which was detected in wheat ears of the cultivar CM only, the alkanes were found in wheat ears of both genotypes independent from the treatment or infection. Several small, volatile alcohols, aldehydes, ketones, sulphuric and cyclic compounds were specifically synthesized by wheat ears of the cultivar CM (independent from the treatment), but not by wheat ears of the cultivar Remus, e.g. 2-butanone, pentanal, 2-ethylfuran, dimethyldisulfide, (E)-2-pentenal, toluene, 3-furaldehyde, 1-hexanol, 2-heptanone, heptanal and pentadecane. This includes two of three GLVs, namely (E)-3-hexen-1-ol and 5-hexen-1-ol, which were detected in wheat ears of the cultivar CM only, whereas (E)-2-hexenal, the third detectd GLV, was detected in wheat ears of both genotypes.

Table 5.10: List of annotated and identified metabolites in wheat ears of CM and Remus harvested 0, 12, 24, 48 and 96 h (sampling points) after *Fusarium*-infection (inf) or water treatment (healthy). Detected metabolites are listed according to elution order on H5-ms column. +...this metabolite was detected at least in 3 of 5 replicate samples at least at one sampling point, -...this metabolite was detected in less than 3 of 5 samples at any sampling point.

	metabolite	CM healthy	Remus healthy	CM inf	Remus inf
1	dimethylsulfide	+	+	+	+
2	propanal-2-methyl-	+	-	+	-
3	2-butanone	+	-	+	-
4	butanal	+	-	+	-
5	furan-3-methyl-	+	-	+	-
6	furan-2-methyl-	+	-	+	-
7	2-methyl-1-propanol[a]	+	-	-	-
8	butanal-3-methyl-	+	+	+	+
9	butanal-2-methyl-	+	+	+	+
10	3-pentanone[a]	-	-	+	-
11	pentanal	+	-	+	-
12	furan-2-ethyl[a]	+	-	+	-
13	disulfidedimethyl	+	-	+	-
14	E-2-pentenal[a]	+	-	+	-
15	toluene	+	-	+	-
16	hexanal	+	+	+	+
17	furan-2-ethyl-5-methyl-	+	+	+	+

[a] identified metabolites

Table 5.10: (continued)

	metabolite	CM healthy	Remus healthy	CM inf	Remus inf
18	3-furaldehyde	+	-	+	-
19	furfural	-	-	+	-
20	E-2-hexenal[a]	+	+	+	+
21	E-3-hexen-1-ol[a]	+	-	+	-
22	5-hexen-1-ol[a]	+	-	+	-
23	p-xylene[a]	-	-	+	-
24	1-hexanol[a]	+	-	+	-
25	2-heptanone[a]	+	-	+	-
26	Z-2-heptenal[a]	+	-	-	-
27	2-heptanol[a]	+	+	+	+
28	heptanal	+	-	+	-
29	E,E-2,4-heptadienal	-	-	+	-
30	E,E-2,4-hexadienol[a]	+	+	+	+
31	benzaldehyde[a]	+	+	+	+
32	1-octen-3-ol[a]	+	+	+	+
33	2-pentyl-furan[a]	+	+	+	+
34	decane[a]	+	-	+	-
35	m-sylvestrene	-	-	-	-
36	octanal	+	-	+	+
37	(+)-4-carene	+	+	+	+
38	2-propyl-1-pentanol[a]	+	+	+	+
39	benzylalcohol[a]	-	-	+	+
40	1-octanol[a]	-	-	+	-
41	acetaldehyde	-	-	-	-
42	undecanea	-	-	+	-
43	nonanal	+	+	+	+
44	phenylethylalcohol[a]	+	+	+	+
45	1-nonanol	+	+	+	+
46	octanoic acid	+	+	+	+
47	Fgstrain2	+	+	+	+
48	dodecane[a]	+	+	+	+
49	decanal	+	+	+	+
50	β-cyclocitral	+	+	+	+
51	tridecane[a]	+	+	+	+
52	sesquiterpene1	-	-	-	+
53	α-longipinene[a]	-	-	+	+
54	ylangene	-	-	+	+
55	similar to ylangene	-	-	-	+
56	β-cubebene	-	-	-	+
57	tetradecane[a]	+	+	+	+
58	vanillin	+	+	+	+
59	sesquiterpene4	-	-	-	+
60	dodecanal	+	+	+	+
61	α-longifolene	-	-	+	+
62	sesquiterpene7	-	-	+	+
63	sesquiterpene27	-	-	-	+
64	sesquiterpene28	-	-	+	+
65	(+)-β-gurjunene	-	-	+	+
66	sesquiterpene29	-	-	+	+
67	(-)-alloaromadendrene	-	-	+	-
68	β-farnesene[a]	-	-	-	+
69	β-chamigrene	-	-	+	+
70	β-humulene[a]	-	-	-	+
71	ar-curcumene	-	-	+	+
72	sesquiterpene10	-	-	+	-
73	pentadecane[a]	+	-	+	-
74	β-bisabolene	-	-	+	-
75	sesquiterpene22	-	-	+	+
76	similar to β-bisabolene	-	-	+	+
77	tridecanal	+	+	+	+
78	calamanene	-	-	+	+
79	β-himachalene	-	-	-	+
80	trichodiene	-	-	+	+
81	γ-E-bisabolene	-	-	+	+
82	α-Z-bisabolene	-	-	+	+
83	Fgstrain3	+	+	+	+
84	1-tridecanol[a]	+	+	+	+
85	longiborneol	-	-	+	+
86	tetradecanal	+	+	+	+
87	tetradecanoic acid	+	+	+	+

[a] identified metabolites

Table 5.10: (continued)

	metabolite	CM healthy	Remus healthy	CM inf	Remus inf
88	octadecane[a]	+	+	+	+
89	pentadecanoic acid	-	-	+	+
90	nonadecane[a]	+	+	+	+
91	hexadecanoic acid	+	+	+	+
92	eicosane[a]	+	+	+	+
93	heneicosane[a]	+	+	+	+
94	docosane[a]	+	+	+	+
95	tricosane[a]	+	+	+	+
96	tetracosane[a]	+	+	+	+
97	pentacosane[a]	+	+	+	+

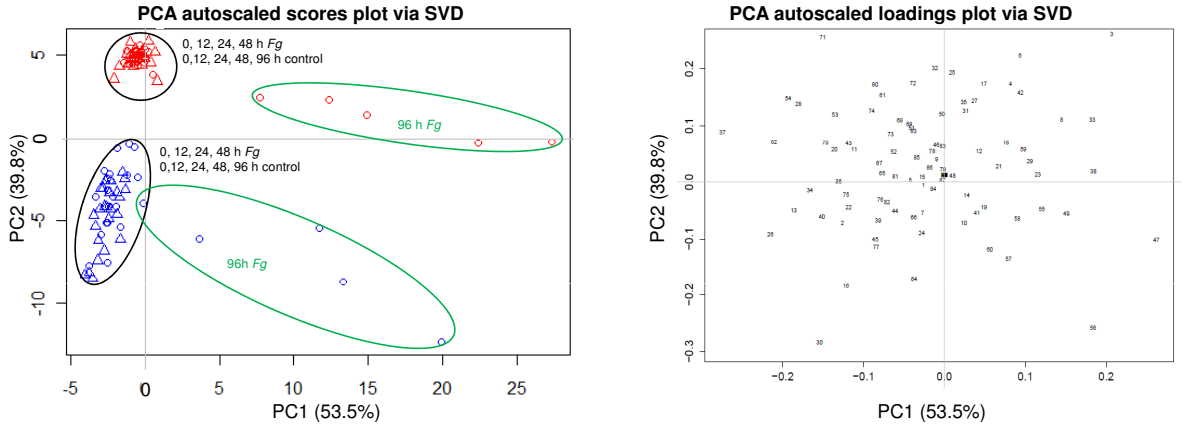
[a]identified metabolites

To get a first impression, which metabolites show the greatest variation in wheat ears of CM and Remus and after *Fusarium* infection, a PCA was performed with the autoscaled signal intensities of the detected metabolites, see figure 5.18. The score plot (a) revealed a clear separation of four clusters: PC1, which describes 53.5 % of the total variance of the data, distinguishes the samples, which represent *Fusarium*-infected wheat ears 96 h after infection, from a main cluster including all other samples, which were measured from one genotype. PC2, describing 39.8 % of the total variance of the data, clearly separates the samples of cultivar CM from the samples of cultivar Remus. The two main and very narrow clusters adhere to healthy wheat ears sampled at 0, 12, 24, 48 and 96 h after treatment and to infected wheat ears at 0, 12, 24 and 48 h after infection. Each main cluster contains the samples of one genotype. From the PCA we learned that the volatile metabolic pattern of the two genotypes allowed successful separation of the samples according to their genotype. Additionally, the *Fusarium*-infected samples, which were harvested 96 h after infection, could be separated from all other time points of sampling and the healthy wheat ears. This was achieved for both genotypes. The loadings plot (b), which shows a much smaller scaling range on the x- and y-axis, could not reveal the important information which metabolites were most important regarding the successful separation of the four clusters. Hence, to determine the metabolites which were synthesized in significantly different signal intensities due to different genotypes and *Fusarium* infection, a heatmap was built, see figure 5.19.

The heatmap contains the information from hierarchical clustering of the samples and the metabolites in one plot. On the x-axis, the dendrogram of the metabolites is displayed, on the y-axis, the dendrogram of the samples is shown. The samples were sucessfully separated according to their genotype, CM or Remus. In addition, one small cluster of eight samples (three from cultivar CM and five from cultivar Remus) representing the *Fusarium*-infected wheat ears, was separated from the main clusters of the individual genotypes, including the healthy wheat ears at all sampling points and the *Fusarium*-infected wheat ears at 0, 12, 24, and 48 h after infection.

The heatmap shows the differences in the autoscaled signal intensities of the detected metabolites in a color pattern according to the color key (from -3 to 9). The metabolites, whose difference in signal intensity between the samples was highest in a positive or negative direction, were painted in green or red respectively. The metabolites with the lowest difference in signal intensity between the samples were painted in black. In the cluster of *Fusarium*-infected wheat ears which were harvested 96 h after inoculation, the signal intensities of the sesquiterpenes, longiborneol (sesquiterpenoid) and p-xylene (aromatic compound) were signficantly higher than in the healthy wheat ears or *Fusarium*-infected wheat ears at earlier sampling points. Going back to table 5.10, it can be seen that these metabolites were not detected in healthy wheat ears at all. p-Xylene was found in fungal

cultures of *F. graminearum* PH-1 in earlier studies and it was described for infected wheat ears in literature (Presicce et al., 2006) It was discussed before that several sesquiterpenes were specifically produced by wheat ears of one genotype.
To conclude, the sesquiterpenes were found the dominating group in the HS of *Fusarium* cultures and they were detected in *Fusarium*-infected wheat ears of different genotypes. This leads to the assumption that the volatile sesquiterpenes (and -terpenoids) play an important role in fungal virulence and plant defense response. Trichodiene and longiborneol, a sesquiterpene and a -terpenoid, are two volatile precursors for the biosynthesis of trichothecenes, DON and culmorin respectively. They are crucial for the fungal virulence regarding the production of mycotoxins. Other detected sesquiterpenes could be synthesized by the fungus or the plant and might therefore be attributed to the fungal virulence or the plant defense.



(a) (b)

Figure 5.18: (a) Score and (b) loadings plot using PCA of the detected metabolites in healthy and *Fusarium*-infected wheat ears of the cultivars CM and Remus using an HP5-ms column.

Many more metabolites than the volatiles are involved during FHB disease and have to be studied. Using GC-MS, the non-volatile, small, polar metabolites (mainly of primary metabolism) can be detected. This method and its applications will be discussed in the following.

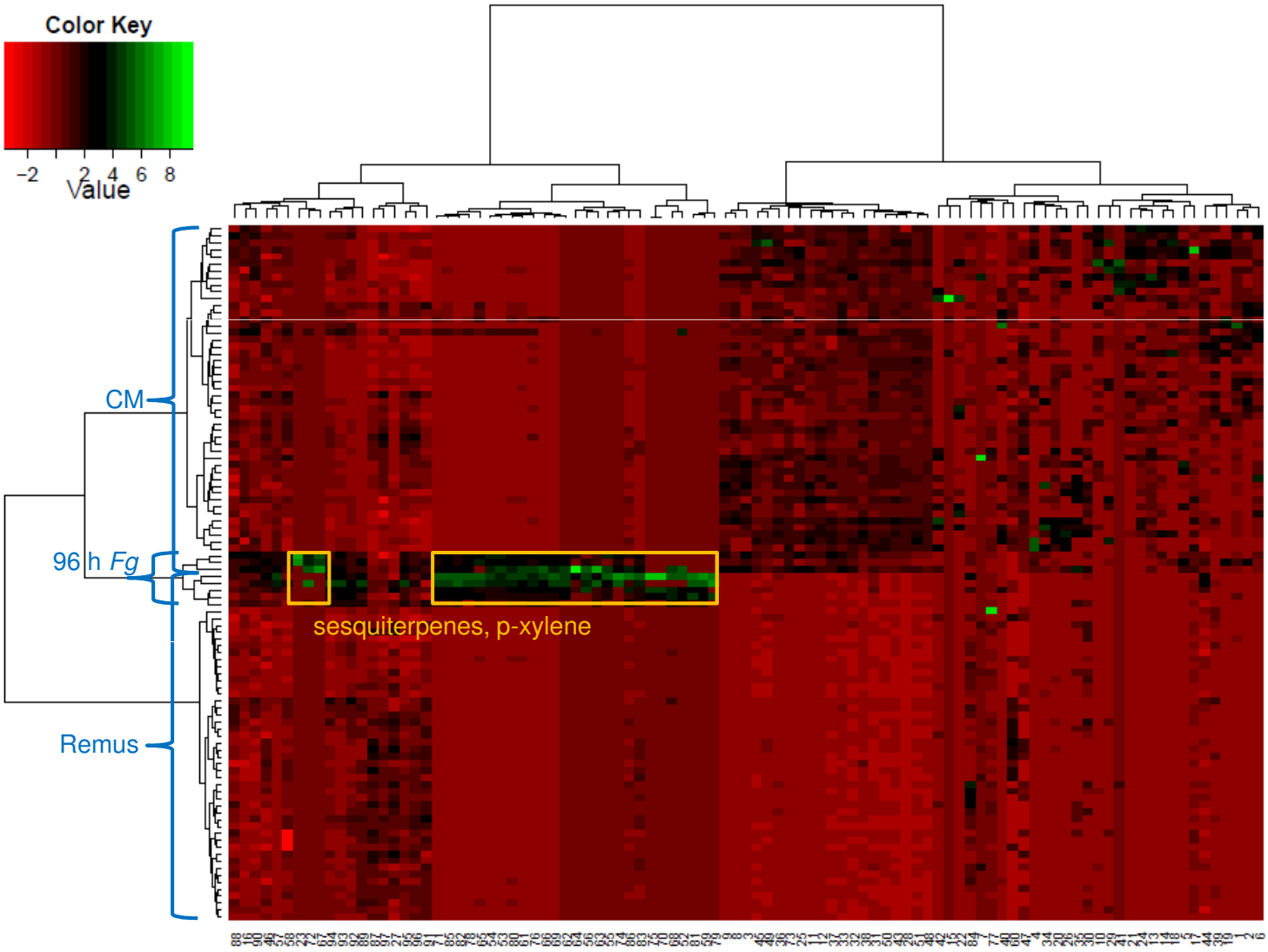


Figure 5.19: Heatmap of the detected metabolites in healthy and *Fusarium*-infected wheat ears of the cultivars CM and Remus using an HP5-ms column. The numbers on the x-axis represent detected metabolites, see table 5.10.

5.3 Development and evaluation of online-derivatisation GC-MS methods

For the determination of non-volatile, polar (mainly primary) metabolites in healthy and *Fusarium*-infected plants, a two-step online-derivatisation GC-MS method was developed and evaluated in healthy wheat ears. Its basic concept was used to develop target analysis methods for three sterols (ergosterol, β-sitosterol, stigmasterol) and a phytohormone and its degradation product (1-aminocyclopropane-1-carboxylic acid and 2-ketobutyric acid, respectively).
The primary metabolites are essential for growth and function of a cell and the biosynthesis of secondary metabolites. It seems that during pathogen attack, the biosynthesis of primary metabolites is shifted towards defense-related compounds (secondary metabolites) (Hamzehzarghani et al., 2005). Therefore, the study of primary metabolites should be accompanied to metabolic studies of secondary metabolites.
The metabolomics platform at the Center for Analytical Chemistry is established to gain metabolic profiles from one biological sample using derivatisation GC-MS for the determination of non-volatile, polar metabolites, HS SPME GC-MS for monitoring the volatile metabolome and LC-HRMS for screening large, mid- to nonpolar (secondary) metabolites. Hence, an extraction protocol must be established which allows extraction of various types of metabolites simultaneously. In table 3.4 on page 31 metabolomics protocols are listed which were used for the similar purposes and which are commonly used in the metabolomics society. For the extraction of non-volatile, polar metabolites aqueous methanol (with or without chloroform) is generally employed. The addition of chloroform is necessary to separate the nonpolar from the polar metabolites. On the one hand, this step is necessary to analyse the nonpolar fraction individually from the polar fraction, on the other hand, the removal of the nonpolar metabolites reduces the number of metabolites which are detected simultaneously and can therefore be used as a cleaning step for polar metabolites.
The cell pellet which is a left over after the extraction of the metabolites, can be used for proteomics and RNA analysis (Weckwerth et al., 2004). In this thesis, I concentrated on the study of the volatile metabolites using HS SPME GC-MS and the non-volatile, polar (mainly primary) metabolites using online-derivatisation GC-MS.

5.3.1 Standard measurements for built-up of an in-house library for non-volatile, polar metabolites

Primary metabolism, which is highly conserved in cells of all organisms including animals, bacteria, plants and fungi, reveals a set of non-volatile, polar metabolites, which are directly involved in growth and development of the cells (Alberts et al., 2008). Many of these compounds are commercially available, which allows standard measurements for the built-up of in-house libraries.
During this thesis, I collected mass spectra and RIs of 147 standard substances. The measurement of single standards revealed different numbers of derivatives dependent on the completeness of the derivatisation using MSTFA and the detection of stereoisomers by methoximation. The list of all standard substances and its detected derivatives using MOX and MSTFA for derivatisation can be found in table A.4.
In addition to the mass spectra and RIs, which were obtained individually for single standard solutions, mass spectra and RIs from 11 mixtures of standard solutions (each

containing 1 mM of each of 10 standard compounds in water, see chapter 4.1.2) were measured. Figure 5.20 shows the number of standard compounds, attributed to different chemical classes, measured for the in-house library throughout the thesis. The mass spectra and RIs from the compounds' detected derivatives are stored in the in-house library for non-volatile, polar metabolites. Most standard compounds were attributed to alkanes (31), followed by acids (24) and amino acids (24). Between five to twelve compounds were measured from each group of sugars, phenylpropanoids, amines, amino acid derivatives, sugar alcohols and sugar phosphates. Less than five compounds from the groups of bases, lactones, nucleosides, nucleoside-mono-phosphates, phytohormones, sterols and triosephosphates were purchased and measured. Four internal standards were bought for pilot experiments to do a qualitative analysis of non-volatile, polar metabolites from a wheat ear sample.

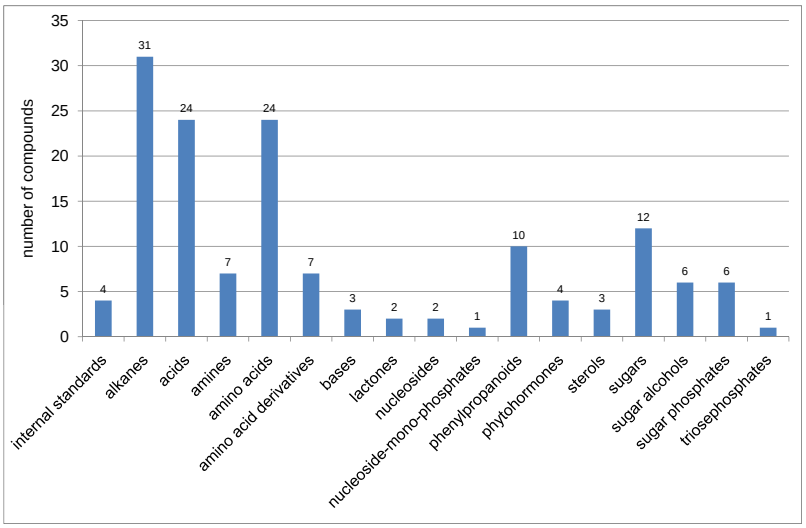


Figure 5.20: Number of standard compounds measured per chemical class whose mass spectra and RIs of derivatives are included in the in-house library for non-volatile, polar metabolites.

5.3.2 Qualitative analysis of non-volatile, polar metabolites from wheat ears under different extraction conditions

To study the non-volatile, polar metabolites, which are mainly results of primary metabolism, in addition to the volatile metabolome (using HS SPME) and the secondary metabolites (using LC-MS), a two-step derivatisation GC-MS method was developed for the metabolomics platform. The extraction protocol is used for the simultaneous extraction of non-volatile, polar metabolites and large, mid- to nonpolar secondary metabolites for subsequent analysis using GC-MS and LC-HRMS, respectively. Different extraction temperatures, the use of an ultrasonication bath and the addition of formic acid to the extraction solvent were compared with respect to the number of compounds detected and the precision of the extraction method (shown as root-mean-square, RMS, values). Additionally, the influence of the extraction temperature, sonication and acidification of the solvent was determined for the detected metabolites (displayed in RMS values).

The wheat sample used was a mixture of 'Remus' and 'Capo' wheat ears sampled during and after flowering, which was prepared for this pilot experiments regarding different extraction conditions for the extraction of non-volatile, polar metabolites. In figure 5.21 the TIC chromatogram of this wheat ear sample, measured on an HP5-ms column, which was extracted with acidified, aqueous methanol (MeOH:H_2O 3:1 (v/v) + 0.1 % HCOOH) in an ultrasonication bath, is shown. The identified metabolites are listed in table 5.11. A total of 72 metabolites could be successfully identified using the in-house library for non-volatile, polar metabolites in MetaboliteDetector. Four metabolites are the derivatives of three internal standards: valine-d8 (2 derivatives), succinic acid-d4 and nonadecanoic acid methylester. The other metabolites are derivatives from 56 compounds including primarily acids, amino acids, monosaccharides, disaccharides and several sugar phosphates. Putrescine, an amine, was detected under all extraction conditions, whereas serotonine, also an amine, was extracted at 22 °C, with the use of an ultrasonication bath and also with acidified aqueous methanol, but not at 4 °C or 60 °C. There were several other metabolites, which could not be extracted at 4 °C or 60 °C, but at 22 °C: pyruvic acid, lactic acid, valine-d8 (IS), glycine 2TMS (not fully derivatised), isoleucine 2TMS (not fully derivatised), maleic acid, uracil, malic acid, methionine, phenylalanine 1TMS (not fully derivatised), cysteine, phosphoenolpyruvic acid, asparagine 4TMS (fully derivatised), xylose, ornithine/arginine/citrullin and ribulose-5-phosphate. Ornithine cannot be distinguished from arginine and citrulline due to artefact formation of arginine and citrulline to ornithine during silylation using MSTFA, see figure 3.5 on page 34.
Shikimic acid, a phytohormone, was extracted at 4 °C and 60 °C, but not at 22 °C (independent from the use of an ultrasonication bath or the addition of HCOOH). The same finding could be observed for threonine 2TMS (not fully derivatised) and glycine 3TMS (fully derivatised). Interestingly, spermidine, a phenylpropanoid, could be extracted with acidified aqueous methanol and the use of an ultrasonication bath only.
A total of 52 metabolites was extracted at 4 °C, 54 metabolites at 60 °C, 68 metabolites at 22 °C, 67 metabolites at 22 °C using an ultrasonication bath (phenylalanine 1TMS (not fully derivatised) was not extracted using ultrasonication) and 69 of 72 metabolites at 22 ° using ultrasonication and the addition of HCOOH to the extraction solvent.
To conclude, the highest number of metabolites was extracted at 22 °C, the use of an ultrasonication bath did not influence this finding, whereas the addition of HCOOH to the extraction solvent allowed the additional extraction of a phenylpropanoid (spermidine).

Regarding the precision of the signal intensities of the detected metabolites, see figure 5.22, none of the conditions could achieve RSDs for all signal intensities of the metabolites below 30 %. There were more than 10 metabolites whose RSD values of their signal intensities was higher 30 %, independent from extraction condition. The RSDs of signal intensities from metabolites extracted at 4 or 60 °C were generally higher than the RSDs of the signal intensities obtained from the same metabolites at 22 °C. The highest number of metabolites showed RSD values for their signal intensities at 5-10 and 10-20 % if the extraction was performed at 22 °C using an ultrasonication bath (with or without the addition of HCOOH). The use of HCOOH in the extraction solvent was shown to extract a phenylpropanoid (spermidine) additionally, which is of high value to the method. Therefore, the extraction using acidified aqueous methanol (MeOH:H_2O 3:1 (v/v) + 0.1 % HCOOH) at 22 °C in an ultrasonication bath was chosen for future experiments in our metabolomics group.
The RSD values of the integrated signal intensities of the quantification ions of the detected metabolites were inspected more closely for the chosen extraction method. It was shown that the signal-to-noise (S/N) ratios varied between 10 and 1800 for the integrated

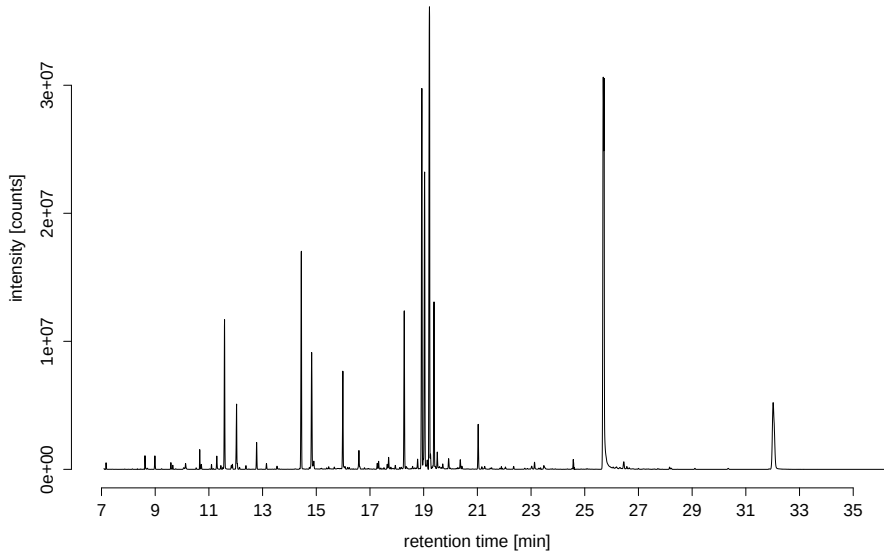


Figure 5.21: TIC chromatogram of a wheat sample on an HP5-ms column, extracted at 22 °C in the ultrasonication bath with acidified, aqueous methanol (MeOH:H_2O 3:1 (v/v) + 0.1 % HCOOH). The signal of derivatisation blank was subtracted from the signal of the sample.

quantification ions for the detected metabolites. The S/N ratios of the metabolites, whose precision was lower 30 %, were less than 60, except for four metabolites: leucine 1TMS (S/N 87), isoleucine 1TMS (S/N 113), proline 2TMS (S/N 406) and methionine 2TMS (104). All four metabolites are amino acids and not fully derivatised. The fully derivatised metabolite was only found for isoleucine. The RSD values for the signal intensities obtained with other extraction methods were also very high for these four metabolites (except for the extraction using ultrasonication, the RSD values of signals from leucine 1TMS and isoleucine 1TMS were below 30 %). Whether these high RSD values are a result of inappropriate derivatisation conditions or matrix effects during extraction should be checked by standard addition with the authentic standards in future. Metabolites, whose quantification ions showed S/N ratios higher 60, were reproducibly extracted with MeOH:H_2O 3:1 (v/v) + 0.1 % HCOOH at 22 °C using ultrasonication for 15 min and detected with the online-derivatisation GC-MS method.

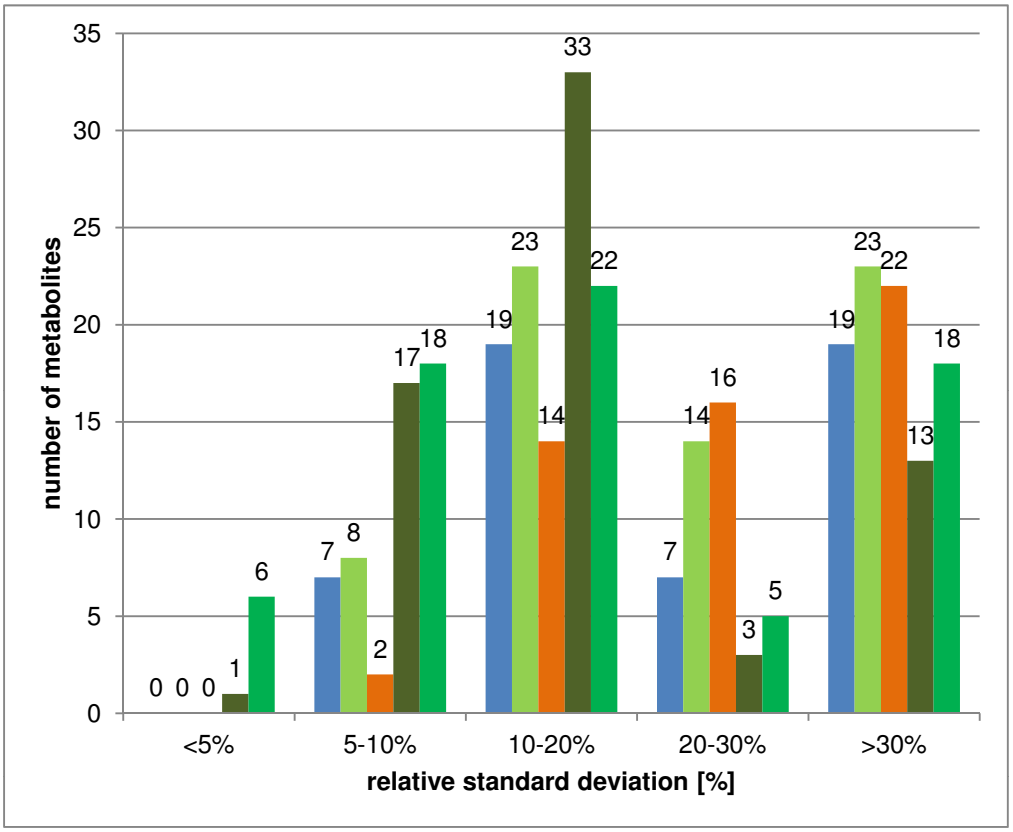


Figure 5.22: Precision, displayed as the relative standard deviation, of signal intensities of detected metabolites (n=6) using different extraction conditions: 4 °C, 22 °C, 60 °C, 22 °C using ultrasonication, with HCOOH in extraction solution and in ultrasonication bath.

Table 5.11: List of non-volatile, polar metabolites detected in a wheat ear sample, which was extracted under different conditions: using aqueous methanol (MeOH:H_2O 3:1, (v/v)) for 15 min at 4 °C, at 22 °C, at 60 °C, in an ultrasonication bath (US) or with acidified aqueous methanol (MeOH:H_2O 3:1 (v/v) + 0.1 % HCOOH) in an ultrasonication bath (US+HCOOH). The metabolites are listed according to their elution order on the HP5-ms column. The average and relative standard deviation (RSD) for each metabolite under each extraction condition are listed (n=6). MOX indicates the number of methoxime groups which were introduced during methoximation, TMS indicates the number of trimethylsilyl groups attachted during silylation. Main peaks (MP) and by-peaks (BP) label the detected stereoisomers of sugars. n.d., not detectable.

Metabolite	average RI	average RT (Min)	quantification ion	average 4°C	RSD 4°C	average 22°C	RSD 22°C	average 60°C	RSD 60°C	average 22°C+US	RSD 22°C+US	average 22°C US+HCOOH	RSD 22°C US+HCOOH
Pyruvic acid 1MOX 1TMS	1062	8.15	174	n.d.	-	$9.3{\cdot}10^4$	5.96	n.d.	-	$9.7{\cdot}10^4$	8.03	$7.5{\cdot}10^4$	7.89
Lactic acid 2TMS	1073	8.33	147	n.d.	-	$1.2{\cdot}10^5$	18.39	n.d.	-	$1.1{\cdot}10^5$	29.10	$1.1{\cdot}10^5$	10.77
Valine-d8 1TMS[a]	1090	8.62	80	n.d.	-	$5.5{\cdot}10^6$	31.00	n.d.	-	$4.6{\cdot}10^6$	19.73	$4.7{\cdot}10^6$	14.91
Alanine 2TMS	1113	8.99	116	$9.7{\cdot}10^3$	25.55	$3.4{\cdot}10^6$	24.76	$1.5{\cdot}10^4$	22.68	$3.5{\cdot}10^6$	17.03	$3.2{\cdot}10^6$	13.92
Glycine 2TMS	1130	9.24	102	n.d.	-	$1.6{\cdot}10^5$	13.23	n.d.	-	$1.5{\cdot}10^5$	17.64	$1.3{\cdot}10^5$	15.60
3-methyl-2-ketobutanoic acid 1MOX 1TMS	1156	9.65	73	$6.3{\cdot}10^3$	10.43	$9.9{\cdot}10^5$	11.99	$1.4{\cdot}10^4$	68.79	$8.8{\cdot}10^5$	10.13	$1.2{\cdot}10^6$	11.72
Leucine 1TMS	1164	9.76	86	$3.3{\cdot}10^2$	223.61	$1.5{\cdot}10^5$	42.25	$1.6{\cdot}10^3$	86.69	$1.3{\cdot}10^5$	23.22	$1.0{\cdot}10^5$	54.68
Isoleucine 1TMS	1184	10.08	86	n.d.	-	$1.9{\cdot}10^5$	29.47	n.d.	-	$1.8{\cdot}10^5$	14.03	$1.3{\cdot}10^5$	56.04
Valine-d8 2TMS[a]	1223	10.65	152	n.d.	-	$9.9{\cdot}10^6$	34.57	n.d.	-	$1.1{\cdot}10^7$	14.05	$9.7{\cdot}10^6$	15.63
Serine 2TMS	1267	11.29	116	$1.6{\cdot}10^4$	16.78	$1.4{\cdot}10^6$	24.05	$2.0{\cdot}10^4$	23.00	$1.3{\cdot}10^6$	11.64	$1.1{\cdot}10^6$	16.92
Isoleucine 2TMS	1283	11.53	158	$3.6{\cdot}10^3$	66.43	$4.2{\cdot}10^5$	40.35	$4.8{\cdot}10^3$	54.76	$4.9{\cdot}10^5$	6.96	$4.6{\cdot}10^5$	22.91
Phosphoric acid 3TMS	1287	11.58	299	$2.8{\cdot}10^5$	9.63	$2.8{\cdot}10^7$	8.43	$3.4{\cdot}10^5$	6.88	$2.8{\cdot}10^7$	9.50	$2.1{\cdot}10^7$	3.45
Threonine 2TMS	1305	11.84	117	$2.4{\cdot}10^3$	27.39	n.d.	-	$2.9{\cdot}10^3$	31.56	n.d.	-	n.d.	-
Proline 2TMS	1308	11.88	142	$9.9{\cdot}10^3$	54.93	$1.3{\cdot}10^6$	46.19	$1.3{\cdot}10^4$	58.92	$1.4{\cdot}10^6$	10.74	$1.5{\cdot}10^6$	31.60
Maleic acid 2TMS	1314	11.96	147	n.d.	-	$1.6{\cdot}10^4$	145.22	n.d.	-	$1.0{\cdot}10^4$	157.22	$4.5{\cdot}10^4$	14.74
Glycine 3TMS	1318	12.02	174	$3.2{\cdot}10^4$	24.16	n.d.	-	$4.1{\cdot}10^4$	16.24	n.d.	-	n.d.	-
Succinic-d4-acid 2TMS[a]	1319	12.03	147	$7.9{\cdot}10^3$	223.61	$2.2{\cdot}10^7$	9.98	$8.1{\cdot}10^3$	244.95	$2.1{\cdot}10^7$	3.61	$2.0{\cdot}10^7$	8.32
Glyceric acid 3TMS	1345	12.38	292	$2.4{\cdot}10^3$	9.90	$2.6{\cdot}10^5$	8.17	$3.5{\cdot}10^3$	11.83	$2.6{\cdot}10^5$	9.43	$2.4{\cdot}10^5$	5.70
Uracil 2TMS	1351	12.45	241	n.d.	-	$6.9{\cdot}10^3$	91.51	n.d.	-	$5.4{\cdot}10^3$	111.60	$3.0{\cdot}10^3$	155.36
Fumaric acid 2 TMS	1354	12.49	245	n.d.	-	$7.7{\cdot}10^4$	13.28	$1.0{\cdot}10^3$	52.91	$7.3{\cdot}10^4$	13.48	$6.1{\cdot}10^4$	11.23
Serine 3TMS	1375	12.78	204	$1.2{\cdot}10^4$	48.64	$2.5{\cdot}10^6$	52.30	$2.0{\cdot}10^4$	94.19	$2.8{\cdot}10^6$	19.68	$2.9{\cdot}10^6$	20.66
Threonine 3TMS	1402	13.14	218	$1.7{\cdot}10^3$	49.96	$2.8{\cdot}10^5$	43.69	$2.5{\cdot}10^3$	81.86	$3.2{\cdot}10^5$	14.24	$3.2{\cdot}10^5$	13.77
Aspartic acid 2TMS	1433	13.54	160	$7.0{\cdot}10^3$	75.67	$4.9{\cdot}10^5$	87.03	$8.5{\cdot}10^3$	69.99	$3.0{\cdot}10^5$	19.06	$2.9{\cdot}10^5$	14.85
Beta-Alanine 3TMS	1439	13.61	174	$3.5{\cdot}10^2$	86.80	$5.8{\cdot}10^4$	7.58	$6.5{\cdot}10^2$	12.11	$5.5{\cdot}10^4$	9.72	$4.9{\cdot}10^4$	4.34
Homoserine 3TMS	1465	13.93	218	$2.2{\cdot}10^5$	7.86	$6.7{\cdot}10^3$	95.59	$2.9{\cdot}10^5$	10.69	$5.6{\cdot}10^3$	112.94	$7.8{\cdot}10^3$	77.83
Malic acid 3TMS	1505	14.44	233	n.d.	-	$2.7{\cdot}10^7$	9.38	n.d.	-	$2.7{\cdot}10^7$	8.08	$2.6{\cdot}10^7$	4.32
Methionine 2TMS	1533	14.78	176	n.d.	-	$1.6{\cdot}10^5$	69.47	n.d.	-	$2.0{\cdot}10^5$	44.58	$2.2{\cdot}10^5$	30.32
Pyroglutamic acid 2TMS	1537	14.83	156	$1.7{\cdot}10^5$	7.60	$1.9{\cdot}10^7$	11.96	$2.1{\cdot}10^5$	10.06	$1.9{\cdot}10^7$	9.36	$1.8{\cdot}10^7$	4.48
gamma-amino-butyric acid 3TMS	1543	14.91	174	$1.7{\cdot}10^4$	66.93	$1.2{\cdot}10^6$	23.69	$1.9{\cdot}10^4$	37.48	$1.2{\cdot}10^6$	12.75	$1.1{\cdot}10^6$	3.85
Phenylalanine 1TMS	1558	15.08	120	n.d.	-	$1.5{\cdot}10^4$	223.61	n.d.	-	n.d.	-	$1.4{\cdot}10^4$	49.77
Cysteine 3TMS	1572	15.24	218	n.d.	-	$1.6{\cdot}10^4$	56.48	n.d.	-	$1.7{\cdot}10^4$	16.47	$9.6{\cdot}10^3$	16.84
2-ketoglutaric acid 1MOX 2TMS MP	1590	15.46	198	$3.7{\cdot}10^3$	11.01	$4.2{\cdot}10^5$	14.47	$5.1{\cdot}10^3$	20.40	$4.1{\cdot}10^5$	10.75	$3.3{\cdot}10^5$	5.24
Phosphoenolpyruvic acid 3TMS	1620	15.81	369	n.d.	-	$7.0{\cdot}10^3$	47.08	n.d.	-	$6.5{\cdot}10^3$	27.25	$6.3{\cdot}10^3$	45.56
Glutamic acid 3TMS	1636	15.99	246	$9.8{\cdot}10^4$	46.51	$1.2{\cdot}10^7$	25.00	$1.3{\cdot}10^5$	36.04	$1.3{\cdot}10^7$	10.28	$1.2{\cdot}10^7$	7.04
Phenylalanine 2TMS	1643	16.07	218	$2.5{\cdot}10^3$	38.20	$3.0{\cdot}10^5$	23.69	$3.4{\cdot}10^3$	27.43	$3.3{\cdot}10^5$	14.93	$3.4{\cdot}10^5$	29.36
Asparagine 4TMS	1652	16.17	188	n.d.	-	$4.2{\cdot}10^4$	115.81	n.d.	-	$5.1{\cdot}10^4$	85.31	$6.4{\cdot}10^4$	38.51

[a] internal standard

Table 5.11: (continued)

Metabolite	average RI	average RT (Min)	quantification ion	average 4°C	RSD 4°C	average 22°C	RSD 22°C	average 60°C	RSD 60°C	average 22°C+US	RSD +US	average 22°C US+HCOOH	RSD 22°C US+HCOOH
Xylose 1MOX 4TMS BP	1674	16.42	103	n.d.	-	$7.7{\cdot}10^{3}$	223.61	n.d.	-	$8.4{\cdot}10^{3}$	244.95	$2.1{\cdot}10^{4}$	113.25
Xylose 1MOX 4TMS MP	1681	16.50	103	n.d.	-	$4.1{\cdot}10^{4}$	29.55	n.d.	-	$4.6{\cdot}10^{4}$	57.06	$8.4{\cdot}10^{4}$	80.50
Arabinose 1MOX 4TMS	1687	16.57	103	$1.6{\cdot}10^{3}$	15.22	$9.3{\cdot}10^{4}$	8.86	$2.1{\cdot}10^{3}$	18.42	$1.1{\cdot}10^{5}$	34.65	$1.0{\cdot}10^{5}$	28.72
Asparagine 3TMS	1689	16.59	116	$1.0{\cdot}10^{4}$	45.58	$1.3{\cdot}10^{6}$	22.70	$1.4{\cdot}10^{4}$	33.65	$1.4{\cdot}10^{6}$	10.26	$1.3{\cdot}10^{6}$	8.53
Ribose 1MOX 4TMS	1696	16.67	103	$1.3{\cdot}10^{3}$	28.28	$1.8{\cdot}10^{5}$	17.05	$1.6{\cdot}10^{3}$	21.94	$1.5{\cdot}10^{5}$	18.48	$1.3{\cdot}10^{5}$	21.66
Xylitol 5TMS	1751	17.27	217	$3.5{\cdot}10^{3}$	11.00	$3.7{\cdot}10^{5}$	15.08	$4.2{\cdot}10^{3}$	17.53	$3.6{\cdot}10^{5}$	9.99	$3.3{\cdot}10^{5}$	6.96
Putrescine 4TMS_Agmatine 4TMS	1756	17.32	174	$5.3{\cdot}10^{3}$	37.50	$6.0{\cdot}10^{5}$	21.34	$6.9{\cdot}10^{3}$	20.42	$5.7{\cdot}10^{5}$	11.53	$1.2{\cdot}10^{6}$	6.20
Glutamine 3TMS	1791	17.70	156	$6.9{\cdot}10^{3}$	61.20	$1.0{\cdot}10^{6}$	30.52	$1.2{\cdot}10^{4}$	82.62	$1.1{\cdot}10^{6}$	16.41	$1.1{\cdot}10^{6}$	16.86
Shikimic acid 4TMS	1830	18.12	204	$1.6{\cdot}10^{3}$	57.39	n.d.	-	$2.4{\cdot}10^{3}$	20.38	n.d.	-	n.d.	-
Glyceric acid-3-P 4TMS	1836	18.16	299	$1.0{\cdot}10^{3}$	19.81	$1.2{\cdot}10^{5}$	21.52	$1.4{\cdot}10^{3}$	29.02	$1.1{\cdot}10^{5}$	14.46	$9.2{\cdot}10^{4}$	13.16
Ornithine 4TMS_Arginine 4TMS_Citrulline 4TMS	1839	18.20	142	n.d.	-	$8.1{\cdot}10^{3}$	223.61	n.d.	-	$5.0{\cdot}10^{3}$	244.95	$2.8{\cdot}10^{4}$	57.30
Citric acid 4TMS	1846	18.28	273	$9.9{\cdot}10^{4}$	11.19	$1.1{\cdot}10^{7}$	14.22	$1.3{\cdot}10^{5}$	18.38	$1.1{\cdot}10^{7}$	9.84	$1.0{\cdot}10^{7}$	7.09
Fructose 1MOX 5TMS BP	1911	18.93	103	$2.5{\cdot}10^{5}$	12.26	$2.9{\cdot}10^{7}$	12.12	$3.2{\cdot}10^{5}$	11.33	$2.8{\cdot}10^{7}$	7.31	$2.7{\cdot}10^{7}$	5.73
Fructose 1MOX 5TMS MP	1921	19.04	103	$1.7{\cdot}10^{5}$	13.29	$2.0{\cdot}10^{7}$	13.86	$2.2{\cdot}10^{5}$	13.76	$1.9{\cdot}10^{7}$	8.04	$1.9{\cdot}10^{7}$	7.11
Glucose 1MOX 5TMS MP	1931	19.13	319	$6.2{\cdot}10^{3}$	18.46	$6.2{\cdot}10^{5}$	11.03	$8.0{\cdot}10^{3}$	20.37	$6.6{\cdot}10^{5}$	6.62	$6.0{\cdot}10^{5}$	9.45
Galactose 1MOX 5TMS BP	1939	19.21	319	$4.0{\cdot}10^{5}$	6.51	$3.6{\cdot}10^{7}$	7.28	$4.7{\cdot}10^{5}$	10.14	$3.6{\cdot}10^{7}$	5.03	$3.5{\cdot}10^{7}$	3.58
Lysine 4TMS	1943	19.25	174	$4.2{\cdot}10^{3}$	11.80	$5.5{\cdot}10^{5}$	26.12	$5.7{\cdot}10^{3}$	27.87	$5.2{\cdot}10^{5}$	18.10	$5.0{\cdot}10^{5}$	16.76
Glucose 1MOX 5TMS BP	1957	19.38	319	$8.8{\cdot}10^{4}$	10.27	$9.5{\cdot}10^{6}$	16.73	$1.2{\cdot}10^{5}$	21.40	$9.1{\cdot}10^{6}$	10.16	$8.8{\cdot}10^{6}$	8.56
Tyrosine 3TMS	1961	19.43	218	$3.6{\cdot}10^{3}$	20.89	$3.5{\cdot}10^{5}$	19.11	$4.0{\cdot}10^{3}$	25.47	$3.4{\cdot}10^{5}$	9.12	$3.4{\cdot}10^{5}$	8.29
Mannitol 6TMS	1969	19.51	205	$4.3{\cdot}10^{3}$	12.95	$4.4{\cdot}10^{5}$	19.95	$5.0{\cdot}10^{3}$	20.22	$4.1{\cdot}10^{5}$	8.12	$7.2{\cdot}10^{5}$	7.70
Gluconic acid 6TMS	2045	20.23	333	$3.0{\cdot}10^{2}$	28.38	$3.2{\cdot}10^{4}$	18.32	$3.2{\cdot}10^{2}$	46.21	$2.8{\cdot}10^{4}$	14.02	$2.6{\cdot}10^{4}$	15.48
Myoinositol 6TMS	2132	21.03	305	$2.5{\cdot}10^{4}$	9.61	$2.6{\cdot}10^{6}$	17.72	$3.0{\cdot}10^{4}$	16.94	$2.5{\cdot}10^{6}$	9.58	$2.5{\cdot}10^{6}$	8.76
Ribulose-5-P 1MOX 5TMS MP	2164	21.32	357	n.d.	-	$7.5{\cdot}10^{3}$	62.30	n.d.	-	$5.2{\cdot}10^{3}$	59.40	$6.8{\cdot}10^{3}$	69.61
Nonadecanoic acid methyl ester[a]	2230	21.90	74	$1.4{\cdot}10^{4}$	19.70	$1.5{\cdot}10^{6}$	16.69	$2.7{\cdot}10^{2}$	25.96	$1.4{\cdot}10^{6}$	7.86	$1.5{\cdot}10^{6}$	9.76
Spermidine 5TMS	2283	22.35	144	n.d.	-	n.d.	-	n.d.	-	n.d.	-	$2.0{\cdot}10^{5}$	10.98
Fructose-6-P 1MOX 6TMS	2363	23.02	315	$1.5{\cdot}10^{3}$	10.24	$1.9{\cdot}10^{5}$	27.04	$2.3{\cdot}10^{3}$	23.72	$1.6{\cdot}10^{5}$	14.43	$1.3{\cdot}10^{5}$	14.52
Glucose-6-P 6TMS	2376	23.13	387	$5.2{\cdot}10^{3}$	11.05	$5.6{\cdot}10^{5}$	24.38	$7.0{\cdot}10^{3}$	19.13	$5.3{\cdot}10^{5}$	13.13	$4.4{\cdot}10^{5}$	12.96
Myoinositol-P 7TMS	2473	23.90	318	$5.0{\cdot}10^{2}$	9.31	$6.6{\cdot}10^{4}$	23.42	$6.8{\cdot}10^{2}$	11.72	$6.1{\cdot}10^{4}$	12.06	$3.4{\cdot}10^{4}$	13.86
Serotonine 4TMS	2490	24.04	174	n.d.	-	$3.5{\cdot}10^{3}$	94.46	n.d.	-	$6.7{\cdot}10^{3}$	36.30	$5.6{\cdot}10^{3}$	68.09
Adenosine 4TMS	2672	25.42	230	$3.2{\cdot}10^{1}$	194.10	$1.6{\cdot}10^{4}$	63.57	$3.6{\cdot}10^{2}$	48.82	$3.9{\cdot}10^{3}$	145.75	$8.7{\cdot}10^{3}$	70.27
Saccharose 8TMS	2709	25.69	361	$6.2{\cdot}10^{5}$	28.86	$5.1{\cdot}10^{7}$	10.68	$5.8{\cdot}10^{5}$	10.00	$5.2{\cdot}10^{7}$	10.69	$5.0{\cdot}10^{7}$	11.02
Cellobiose 1MOX 8TMS BP	2805	26.37	361	$2.1{\cdot}10^{2}$	91.95	$5.3{\cdot}10^{4}$	35.51	$3.4{\cdot}10^{2}$	59.84	$4.5{\cdot}10^{4}$	60.67	$3.6{\cdot}10^{4}$	59.17
Maltose 1MOX 8TMS BP	2818	26.46	361	$1.7{\cdot}10^{4}$	13.97	$2.0{\cdot}10^{6}$	16.18	$3.6{\cdot}10^{4}$	58.07	$1.8{\cdot}10^{6}$	12.17	$1.1{\cdot}10^{6}$	9.80
Maltose 1MOX 8TMS MP	2847	26.66	361	$3.4{\cdot}10^{3}$	17.22	$4.3{\cdot}10^{5}$	14.66	$7.8{\cdot}10^{3}$	58.87	$3.6{\cdot}10^{5}$	14.78	$1.6{\cdot}10^{5}$	15.62
Melibiose 1MOX 8TMS MP	2953	27.37	361	$3.6{\cdot}10^{2}$	16.99	$4.4{\cdot}10^{4}$	18.74	$6.1{\cdot}10^{2}$	43.46	$3.7{\cdot}10^{4}$	12.86	$3.2{\cdot}10^{4}$	36.79
Adenosine-5-P 5TMS	3118	28.47	315	$1.5{\cdot}10^{2}$	50.04	$2.8{\cdot}10^{4}$	19.75	$5.2{\cdot}10^{2}$	20.19	$2.6{\cdot}10^{4}$	19.98	$1.2{\cdot}10^{4}$	57.69

[a] internal standard

5.4 Application of online-derivatisation GC-MS methods

The two-step, online-derivatisation GC-MS method for metabolic profiling of non-volatile, polar metabolites was adjusted for the targeted analysis of selected sterols to serve as plant and fungal biomarkers and a phytohormone and its degradation product.

5.4.1 Target analysis of ergosterol, sitosterol, stigmasterol

Fungal and plant specific sterols, namely ergosterol as a fungal membrane compound and β-sitosterol and stigmasterol as phytosterols, can be used as biomarkers for the fungal and plant biomass, respectively. The ratio of ergosterol to sitosterol shall be used as an indicator for the infection degree of a *Fusarium*-infected plant.

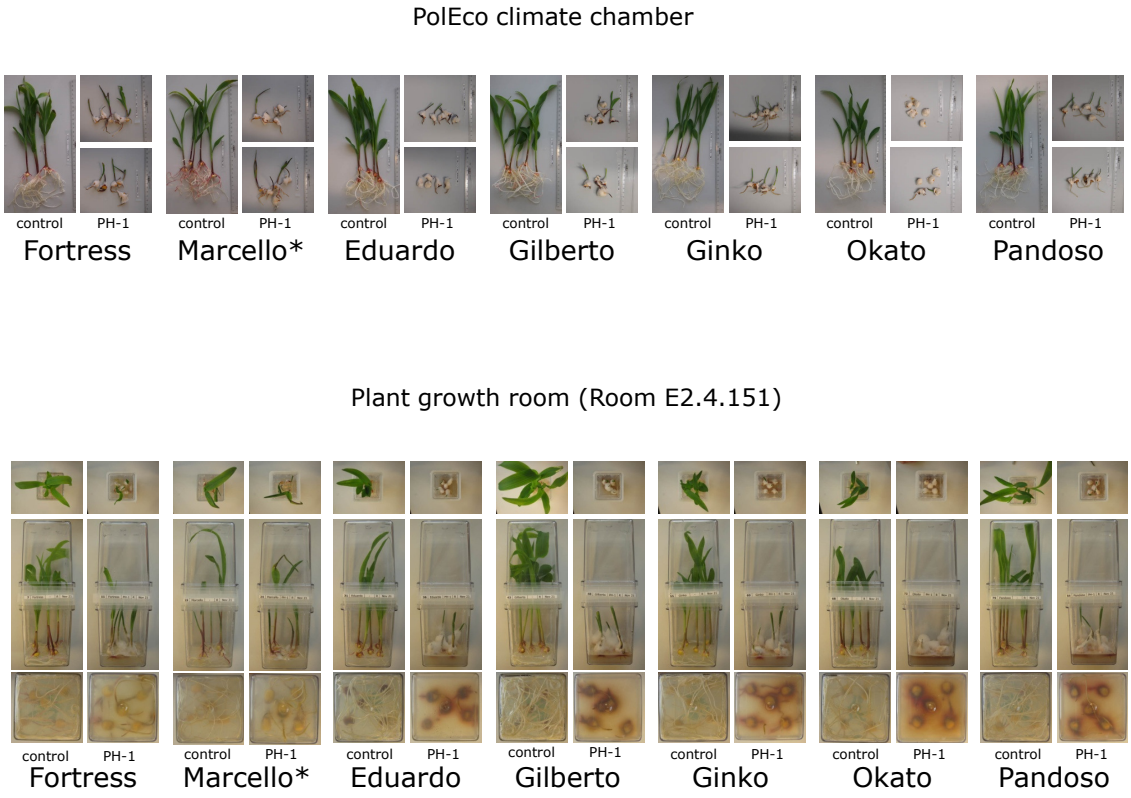


Figure 5.23: Photographs from seven maize cultivars (control and infected with *F. graminearum* PH-1) which were cultivated in the climate chamber PolEco or in a climate room (provided by Gerlinde Wiesenberger).

In this study, maize seedlings of seven different cultivars were cultivated *in-vitro* in plant tissue culture boxes in a climate chamber (PolEco) or in a plant growth room (Room E2.4.151) and infected with *F. graminearum* PH-1 two days after germination to find one or two cultivars which can be used as model plants for future experiments. The infected model plants must reveal a typical infection pattern regarding growth inhibition and ergosterol/sitosterol content, which is representative for other cultivars.
Pictures were taken of 12 day old plants, see figure 5.23 to display the growth inhibition after fungal infection. Visual inspection of these maize seedlings shows that all the infected maize seedlings were covered with white fungal mycelium of PH-1 and that plant

growth was strongly inhibited. The infected seedlings were brownish and small, revealing much less plant biomass than the healthy control plants. The amount of phytosterols was therefore expected to be much less in the infected seedlings than the healthy plants. In addition, no ergosterol was expected in extracts of the control plants, but significant amounts should be present in extracts of the infected seedlings.

The fresh weight of plants was determined before extraction with acetone. Aliquots of the acetone extracts were dried under a constant flow of nitrogen. The dried residues of control samples were green and pink before they were resuspended in the derivatisation reagent MSTFA. This could be a anthocyanin, cyanidin, which is synthesized by maize. This colour was not observed for extracts of the infected seedlings. They had brownish coloured residues.

Apart from the visual inspection where *Fusarium*-infection could clearly be observed, the amount of ergosterol, sitosterol and stigmasterol was determined using GC-MS. The extraction efficiency was assumed to be equal for all three sterols since they are structurally very closely related. The online-derivatisation GC-MS method for metabolic profiling of non-volatile, polar metabolites was adapted and shortened with respect to the analysis of the three sterols of interest: The methoximation step was omitted because the sterols have no carbonyl groups which could be methoximated, the residual amount of solvent (to have enough volume for the injection) was filled with pyridine. The GC method was shortened such as that the starting temperature was set at 100 °C and rapidly heated to 310 °C, which was kept for 15 min. At 310 °C, the three sterols could be separated very well, see figure 5.24. The calibration range was set according to the expected concentrations of target analytes from previous experiments and acceptable linearity. The calibration lines are listed in table 5.12.

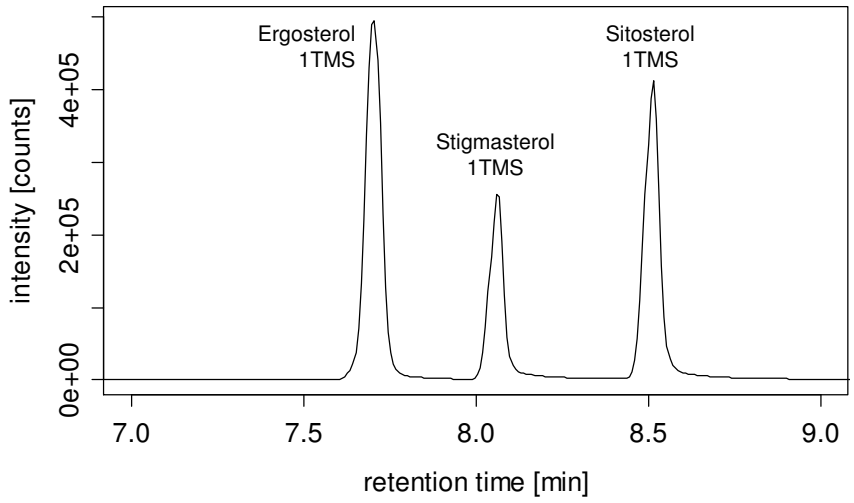
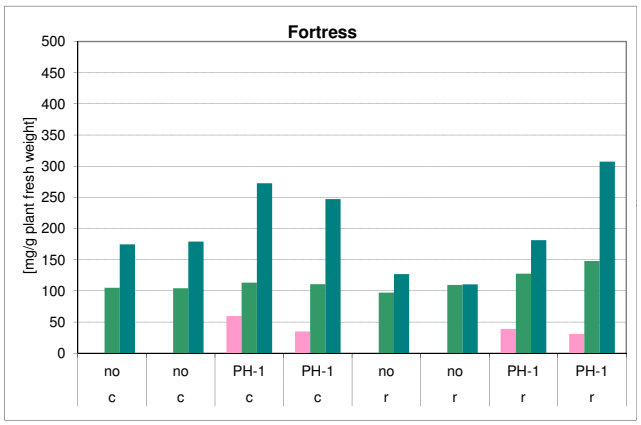
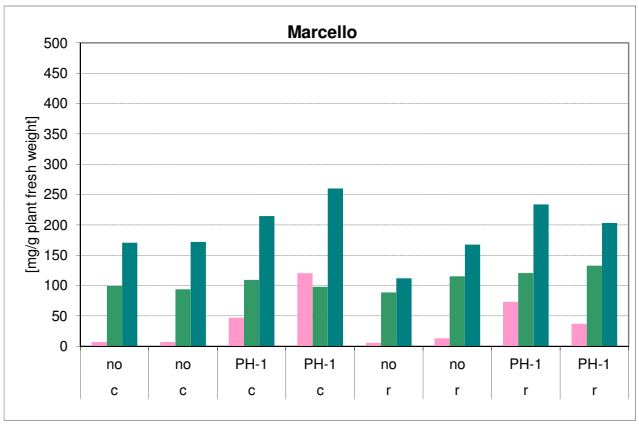


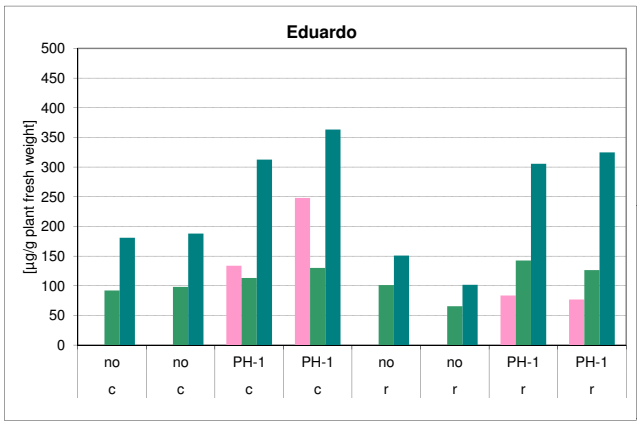
Figure 5.24: SIM chromatogram of a 50 mgL^{-1} standard solution containing ergosterol, sitosterol, stigmasterol. Selected ions, which were monitored, were m/z 363 for ergosterol 1TMS, m/z 394 for stigmasterol 1TMS, m/z 396 for sitosterol 1TMS.



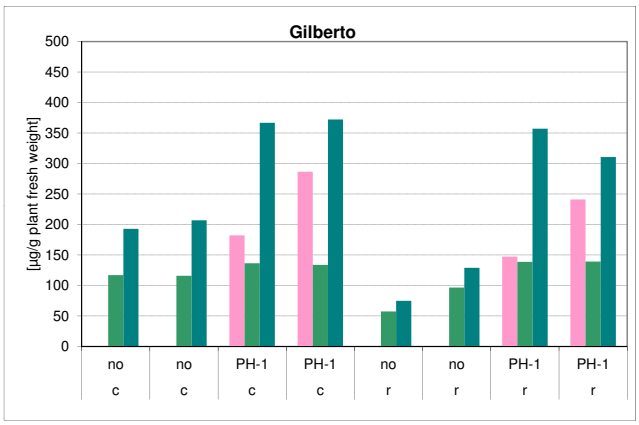
(a)



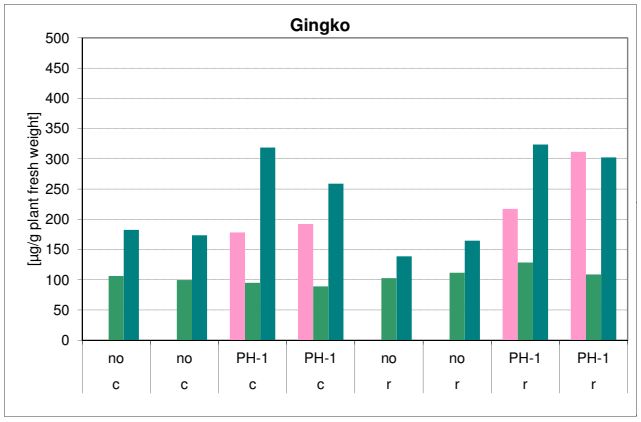
(b)



(c)



(d)



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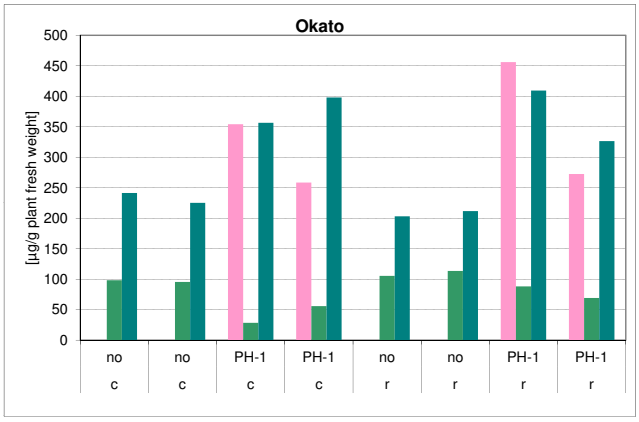
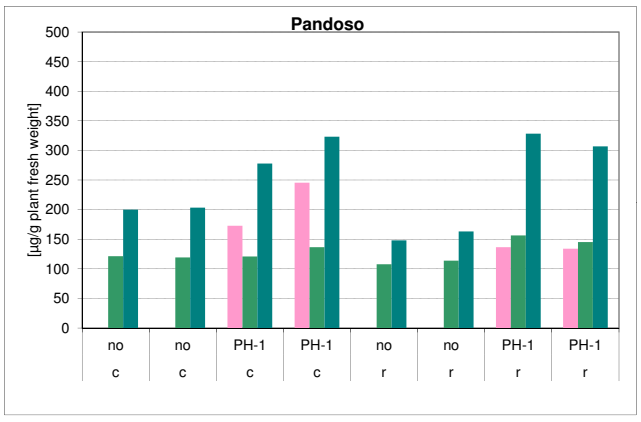


(f)



(g)

Figure 5.25: Ergosterol, sitosterol and stigmasterol concentrations [$\mu g g^{-1}$] which were normalized by fresh weight of the plant (n=1). c...cultivated in climate chamber PolEco; r...cultivated in climate room, no...not infected (control), PH-1...infected with *F. graminearum* PH-1. Concentrations were determined for the following maize cultivars: (a) 'Fortress', (b) 'Marcello', (c) 'Eduardo', (d) 'Gilberto', (e) 'Gingko', (f) 'Okato', (g) 'Pandoso'.

Table 5.12: Calibration lines for absolute quantification of ergosterol, sitosterol and stigmasterol (n=3).

target analyte	calibration line	R^2	calibration range [mg/L]
ergosterol (m/z 363)	y=408784·x-214508	0.9734	1-50
sitosterol (m/z 396)	y=159513·x+14059	0.9808	0.25-50
stigmasterol (m/z 394)	y=283079·x+23425	0.9721	0.25-50

Figure 5.25 shows the concentrations in μgg^{-1} fresh weight of ergosterol, sitosterol and stigmasterol. The concentrations of ergosterol differed significantly between cultivars and also between climate chamber and climate room. The most consistent concentrations were determined for the cultivars (d) 'Gilberto' and (e) 'Gingko'. For 'Marcello' (b) ergosterol was even determined in small amounts in the control samples. There was a contamination of fungal origin in these seedlings. Therefore, these seeds are not of further interest to the researchers. In (a) 'Fortress', the observed amount of ergosterol were below 0.05 mgg^{-1} fresh weight, which is lower than the detected amount in other cultivars (up to 0.45 mgg^{-1}). Seeds of 'Fortress' were used in previous experiments and therefore, this cultivar will be studied in future experiments for comparison.
The amount of ergosterol was between 130 - 280 μgg^{-1} in 'Eduardo', 'Gilberto', 'Gingko' and 'Pandoso' if cultivated in the climate chamber, but varied between 50-300 μgg^{-1} for these cultivars if cultivated in the climate room. 'Okato' samples showed the highest amount of ergosterol in the climate chamber (250-350 μgg^{-1}) and the climate room (260-450 μgg^{-1}). Since all of these values are results from single measurements only, it is difficult to evaluate which differences are significant and which are not. In a follow-up experiment, significant differences between the cultivars should be confirmed.

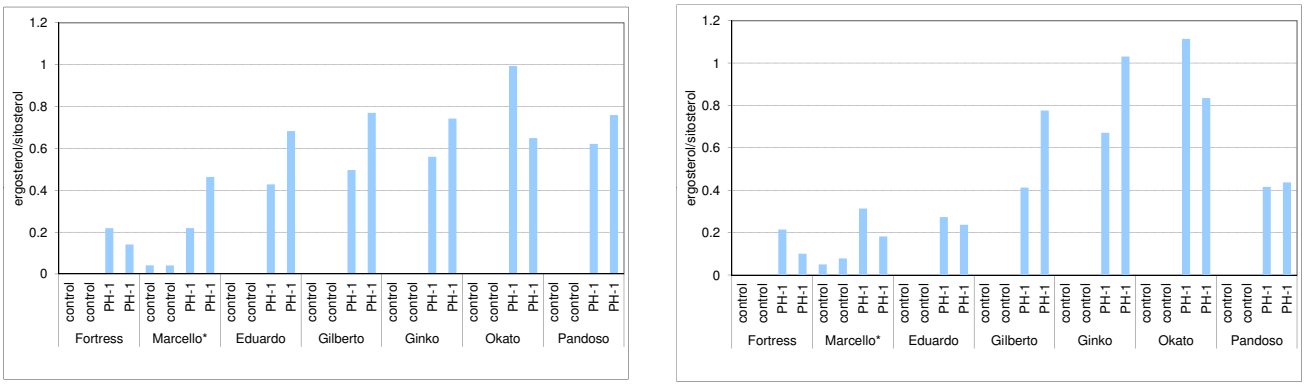
Table 5.13: Enhancement of sitosterol and stigmasterol content due to *Fusarium*-infection (n=2).

cultivar	climate chamber/room	enhancement factor sitosterol	enhancement factor stigmasterol
Fortress	chamber	1.47	1.07
	room	2.06	1.33
Marcello	chamber	1.39	1.07
	room	1.56	1.24
Eduardo	chamber	1.83	1.28
	room	2.50	1.61
Gilberto	chamber	1.85	1.16
	room	3.27	1.80
Gingko	chamber	1.62	0.89
	room	2.06	1.11
Okato	chamber	1.62	0.43
	room	1.77	0.72
Pandoso	chamber	1.49	1.07
	room	2.04	1.36

The amount of sitosterol was expected to be directly correlated with plant biomass (Griebel and Zeier, 2010), independent from the fungal infection, resulting in similar values for the sitosterol content per plant fresh weight. Only then it would be a reliable biomarker for plant biomass. However, it can be seen in figure 5.25 that the sitosterol content was rising with the amount of ergosterol detected. Especially in the cultivars (d) 'Gilberto', (e) 'Gingko' and (f) 'Okato', this enhancement in the sitosterol content can be seen. Comparing the concentrations for each cultivar and condition (climate chamber or room) individually, an increase of factor 1.5 to 3 was found for sitosterol during infection, see table 5.13. An average concentration of 250 μgL^{-1} ±28.54 % was determined for the

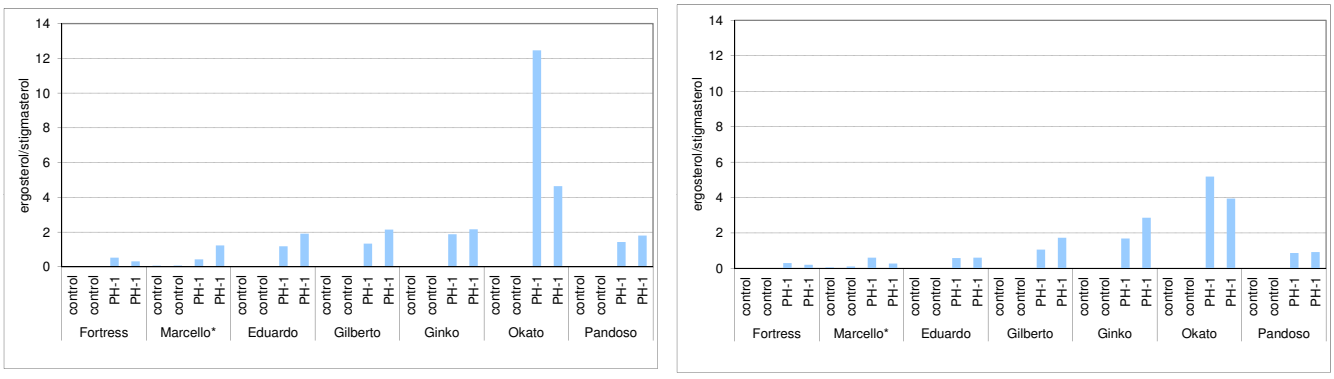
sitosterol content over all cultivars for the climate chamber, 110 mgL^{-1} ±41.24 % for the climate room. Whether the increase in the sitosterol concentration in infected plants is significant and reproducibly connected with *Fusarium* infection needs to be determined in replicate experiments, since this experiment revealed only two values per cultivar and condition.
The concentration of stigmasterol did not seem to be affected in the same intensity as the sitosterol concentration. The average concentration and relative standard deviation of samples in the climate chamber was 0.22 mgL^{-1} ±21.38 %, in the climate room 0.11 μgL^{-1} ±22.51 %. Interestingly, stigmasterol was described to be enhanced during pathogen attack in literature, whereas sitosterol content was not influenced (Griebel and Zeier, 2010). Table 5.13 shows that the enhancement of stigmasterol is different to the enhancement of sitosterol due to *Fusarium*-infection. It is not enhanced as much as the sitosterol content after *Fusarium* infection. Besides the cultivar 'Okato', where there stigmasterol content was lower in infected samples than in control samples, the enhancement of stigmasterol seemed to be not significant in the climate chamber, but in the climate room. If this data can be reproduced in a replicate experiment, stigmasterol could serve as a biomarker for plant biomass for maize cultivars (except 'Okato') cultivated in the climate chamber PolEco.

If the increase in the sitosterol and stigmasterol concentrations due to *Fusarium* infection, are shown to be not significant in replicate experiments, the hypothesis is that both phytosterols can be used as biomarkers for the plant biomass. Subsequently, the detected concentrations (not normalized to the plant fresh weight) for ergosterol, sitosterol and stigmasterol were used to calculate ratios which should provide information about the infection degree. The ratio ergosterol/sitosterol, see figure 5.26, should be comparable to the ratio ergosterol/stigmasterol, see figure 5.27. The results confirmed this hypothesis (except for 'Okato' in the climate chamber), leading to the same conclusions regarding the infection degree: The cultivar 'Okato' showed the highest infection degree, followed by the cultivars 'Gilberto', 'Gingko' and 'Pandoso', and the lowest infectin degree was determined in the cultivars 'Eduardo' and 'Fortress' ('Marcello' seedlings were contaminated prior to infection). Except for the seedlings of cultivar 'Fortress', the infection degree was higher in seedlings cultivated in the climate chamber than in the climate room.



(a) (b)

Figure 5.26: Ergosterol/sitosterol ratio of seven different maize culitvars which were cultivated in (a) climate chamber PolEco or in (b) climate room. *contaminated



(a) (b)

Figure 5.27: Ergosterol/stigmasterol ratio of seven different maize cultivars which were cultivated in (a) climate chamber PolEco or in (b) climate room. *contaminated

5.4.2 Target analysis of ACC and 2-ketobutyric acid

In plants, ACC is the immediate precursor to ethylene, see 2.7, an important plant hormone that regulates fruit ripening, seed germination, leaf senescence and responses to biotic and abiotic stress. In higher plants, S-adenosylmethionine is used as a substrate for ACC synthesis by ACC synthases. In bacteria, ACC deaminases were described to be used for carbon and nitrogen generation (Thibodeaux and Liu, 2011). In fungi, the role of ACC is unclear. In *F. graminearum* three genes encoding for ACC synthases and two genes encoding for ACC deaminases were detected during genome sequencing (Cuomo et al., 2007): FGSG_05184 (named A1), FGSG_07606 (named A2), FGSG_13587 (named A3), FGSG_02678 (named D1), FGSG_12669 (named D2). For further details see the *Fusarium graminearum* DataBase (MIPS, 2003). Furthermore, 2-ketobutyric acid was described as degradation product of ACC from *Trichoderma* (Viterbo et al., 2010). We assumed that it is also the degradation product of *Fusarium* ACC deaminases.
To study the biosynthesis products of fungal ACC synthases and ACC deaminases and their relevance in plant::fungus interactions, a target method for ACC and its degradation product 2-ketobutyric acid was developed using GC-MS.

Development of target method for ACC and 2-ketobutyric acid and determination of matrix effects

For the development of a target method using GC-MS for ACC measurement, the online-derivatisation GC-MS method for metabolic profiling of non-volatile, polar metabolites was used as a start. Standard solutions of ACC and 2-ketobutyric acid were prepared, see 4.1.2 and measured using this method in a final concentration of 5 mgL^{-1} in solvent and in different cultivation media (diluted 1:10 with pure methanol), e.g. *Fusarium* minimal medium (FMM), yeast peptone dextrose medium (YPD), synthetic complete medium without isoleucine (SC-Ile), malt extract (ME) and lysogeny broth medium (LB). The media were selected because they can be used for the cultivation of fungi (FMM), yeasts (YPD, SC-Ile, ME) and bacteria (LB). These organisms can be used as host systems for the transformation of the *Fusarium* genes encoding ACC synthase or ACC deaminase.
Matrix effects affecting the signal intensity of ACC derivatives were observed for all cultivation media tested using the online-derivatisation GC-MS method for metabolic profiling. Therefore, derivatisation conditions had to be optimized: A derivatisation temperature of 90 °C for 30 min was required for the reproducible derivatisation of ACC to ACC 1TMS and ACC 2TMS from extracts of the cultivation media, see figure 5.28.
The methoximation step before silylation lead to a higher amount of incomplete deriva-

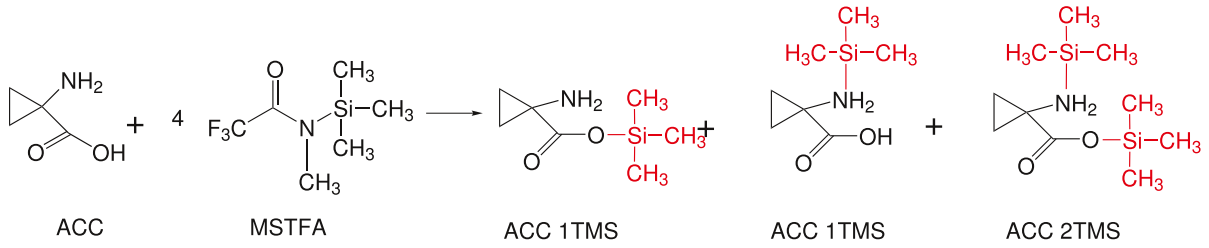


Figure 5.28: Silylation of ACC using MSTFA. Reaction products are ACC 1TMS and ACC 2TMS. There are two derivatives ACC 1TMS possible, but the first one might be the major product of them.

tives (ACC 1TMS) than complete derivatives (ACC 2TMS), artefacts and the chromatographic resolution was worse. The signal intensity of ACC 1TMS was twice as high if

methoximation was performed prior to silylation, whereas the signal intensity of ACC 2TMS dropped to less than half. There was no increase in signal intensity for ACC 2TMS whether 50 or 100 μL MSTFA were used. The RSD value for six replicates was 14.5 % for ACC 1TMS and 9 % for ACC 2TMS. Therefore, from the viewpoint of ACC, 50 μL MSTFA and 50 μL pryidine were enough for successful, reproducible derivatisation and saved some cost-intensive reagent.
In addition, MTBSTFA was considered for derivatisation of ACC because a method for profiling of phytohormones was published using MTBSTFA (Birkemeyer et al., 2003). Using MTBSTFA, the signal intensities of ACC derivatives was less intense than for the TMS derivatives using MSTFA.

2-Ketobutyric acid has a reactive ketogroup, which could be methoximated, and a carboxylic group which can be attacked by MSTFA. 2-Ketobutyric acid was successfully methoximated and then silylated to 2-methoxime-butanoic acid 1TMS using the two-step derivatisation of the online-derivatisation GC-MS method, see reaction 5.29. Since ACC was shown to require a higher derivatisation temperature than used for metabolic profiling of non-volatile, polar metabolites, and methoximation was shown to disturb the full derivatisation of ACC, the methoximation step was omitted and silylation was performed at 90 °C using 50 μL MSTFA and 50 μL pyridine.
If the carbonylgroup of 2-ketobutyric acid was not stabilized as methoxime group using

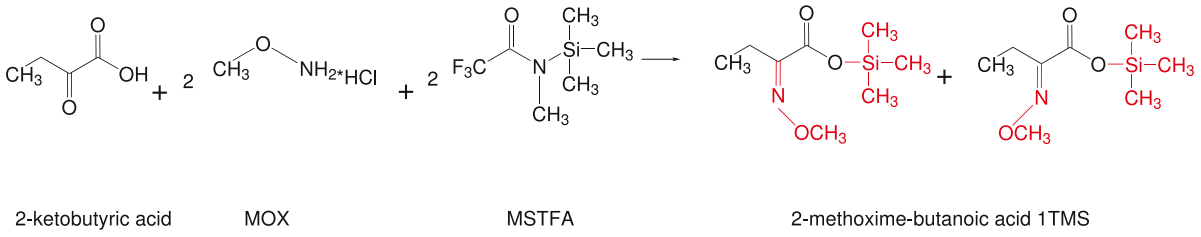


Figure 5.29: methoximation and silylation of 2-ketobutyric acid using MSTFA.

MOX prior to silylation, the keto-enol tautomerie product 2-butenoic acid 2TMS was detected, see reaction 5.30. This rearrangement was described by Little (1999) before as a derivatisation artefact. We tested whether the same rearrangement occurred in matrix containing extracts and found that it was reproducible. The RSD of 2-butenoic acid 2TMS was found 23 % for six replicate measurements.

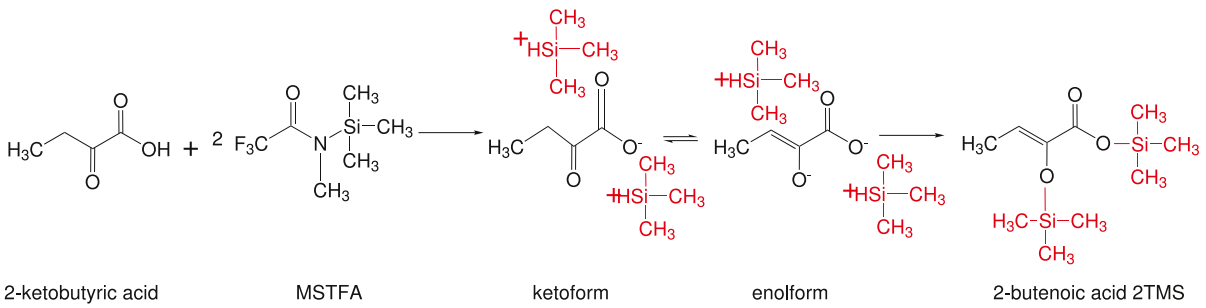


Figure 5.30: Silylation of 2-ketobutyric acid using MSTFA.

m/z 55 and 130 for ACC 1TMS, m/z 147 and 202 for ACC 2TMS and m/z 147 and 231 for 2-butenoic acid 2TMS were chosen for the measurement of ACC and 2-ketobutyric acid

derivatives using MSTFA in SIM mode. The SIM chromatogram of a standard solution containing ACC and 2-ketobutyric acid is shown in figure 5.31.

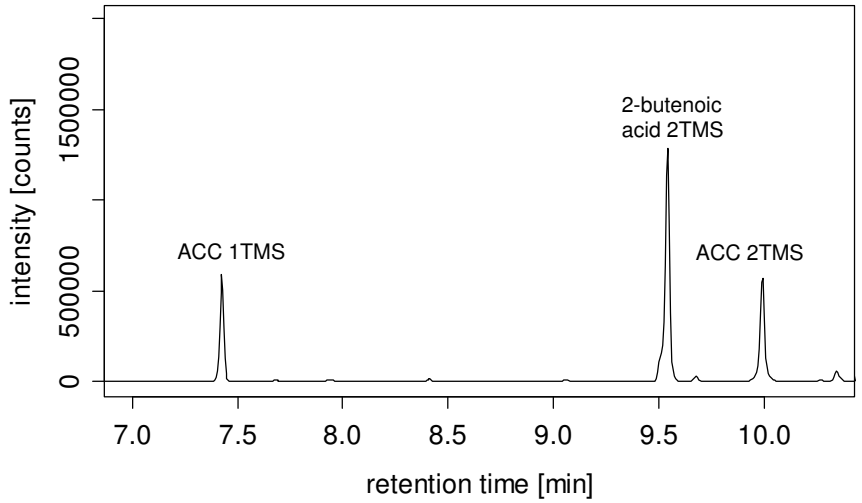


Figure 5.31: SIM chromatogram of a 10 mgL^{-1} standard solution containing ACC and 2-ketobutyric acid. Selected ions, which were monitored, were m/z 55 and 130 for ACC 1TMS, m/z 147 and 202 for ACC 2TMS and m/z 147 and 231 for 2-butenoic acid 2TMS.

In the following, cell extracts containing bacterial or yeast cells were tested for matrix effects. In 90% aqueous methanol extracts of *E. coli*, 92 % of spiked ACC were detected, whereas only 37 % of ACC were recovered from extracts containing cells of *S. cerevisiae* suggesting that ACC was adsorbed to yeast cells. Therefore, transformations of fungal genes encoding for ACC synthases or deaminases were carried out in *E. coli*. LB medium was used for cultivation of bacterial cells. Therefore, LB medium was tested with different amounts of NaCl using the optimized derivatisation conditions. 80 % of spiked ACC were found in LB medium containing 10 gL^{-1} NaCl, whereas 90 % of ACC were found in LB medium with 5 gL^{-1} NaCl or no NaCl (results from two replicate experiments). For future experiments, methionine and IPTG were supplemented in the medium. The addition of methionine to LB medium lead to 101 % recovery of spiked ACC. IPTG did not influence ACC derivatisation. Therefore, LB medium without NaCl was used for the cultivation of bacterial transformants, IPTG was used induction of bacterial cells and methionine was used as a substrate for ACC synthase induction.

Study of fungal ACC synthase and ACC deaminase biosynthesis products in bacterial cells

Having optimized the derivatisation conditions for the target analysis of ACC and 2-ketobutyric acid, a pilot experiment with transformants of bacterial cells was started. Three bacterial cultures were transformed with genes encoding for ACC synthases (one gene per tranformant), named A1 (FGSG_05184), A2 (FGSG_07606), A3 (FGSG_13587) and two cultures were transformed with single genes encoding for ACC deaminase, named D1 (FGSG_02678), D2 (FGSG_12669).

In this pilot experiment, none of the transformants including a gene for ACC synthase produced ACC in significant, reproducible amounts. On a genomic level, the expression of active genes encoding for ACC synthases could be tested or in a protein assay it could be checked whether ACC synthases were successfully transcribed from the genes.
Fungal ACC deaminase uses ACC as a substrate and degrades it to 2-ketobutyric acid (Thibodeaux and Liu, 2011; Viterbo et al., 2010). One of the transformants including a gene for fungal ACC deaminase, D2 (FGSG_12669), was found to degrade ACC after 3 h in small amounts and significantly after 24 h to a metabolite which could be annotated as 2-aminobutyric acid 2TMS. 2-Aminobutyric acid was assumed to be a transamination product of 2-ketobutyric acid prior to derivatisation, see reaction 5.32. This transamina-

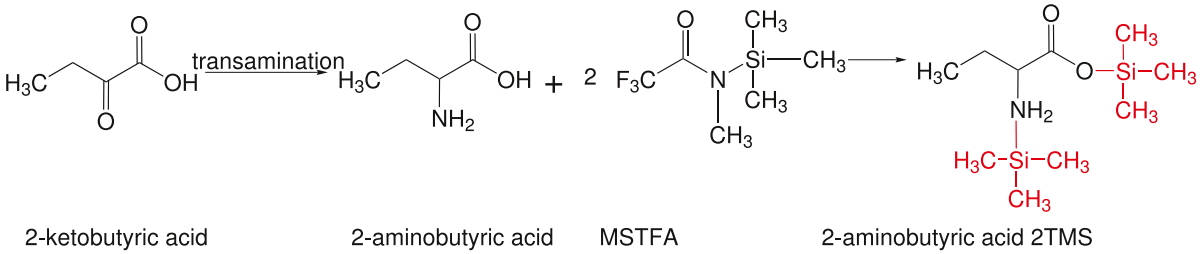


Figure 5.32

tion could be achieved by bacterial transaminases. Protein extracts which were induced with 2-ketobutyric acid did not reveal this metabolite supporting the hypothesis that bacterial enzymes convert the ACC degradation product 2-ketobutyric acid to 2-aminobutyric acid. However, in protein extracts no 2-butenoic acid 2TMS was found either, but after 24 h ACC was degraded.
In the following repetitive experiment, transformants with deaminases were induced with 2-ketobutyric acid as well as ACC. After 3 h and 24 h, 2-aminobutyric acid 2TMS was detected in all samples which were induced with 2-ketobutyric acid. Only D2 synthesized 2-aminobutyric acid 2TMS in significant amounts at 3 h and 24 h after ACC induction, supporting the results of the first experiment.
For the future, matrix effects from *Fusarium* cultures should be studied, including different cultivation medium and supplements. If there is no adsorption to fungal cells and growth medium, ACC synthases and deaminases should be studied directly from fungal cells. This might increase the efficiency of ACC synthesis and degradation and might avoid the transamination of 2-ketobutyric acid. Then calibration should be achieved for the new system under investigation to be able to quantify the target metabolites in absolute concentrations.
The physiological significance of ACC and its degradation product for fungi is yet unclear but it could be important for the interaction of plant::fungus during infection processes: ACC deaminases are useful to the fungus to degrade ACC, the precursor of ethylene, to reduce the amount of ethylene produced by the plant as a defense response. However, ethylene was also described to reduce resistance in mono- and dicotyledonous plants (Chen et al., 2009). So, ACC synthesis by the fungus could enhance to amount of ethylene produced by the plant or even promote ethylene production by the fungus to enhance susceptibility. Ethylene itself was determined only in very low amounts in *F. graminearum* PH-1 cultures which were grown on methionine supplemented medium in a small study. This experiment needs to be repeated with S-adenosylmethionine supplemented medium to show whether ethylene production was significant.

Chapter 6

Conclusion

In the course of this thesis I developed and evaluated two main GC-MS methods, a HS SPME GC-MS and a two-step, online-derivatisation GC-MS method, for different applications regarding the metabolic studies of *Fusarium* and wheat as well as target analysis of selected metabolites. I have studied volatile metabolic profiles of fungal cultures of *F. graminearum* PH-1, of *F. graminearum* strains of the Northland population and of Δ*tri5* mutant, and of wheat ears dependent on their developmental stage and changes due to *Fusarium*-infection. Furthermore, a method for the metabolic profiling of non-volatile, polar metabolites was established and adapted for the target analysis of selected sterols (ergosterol, sitosterol and stigmasterol) to serve as fungal and plant biomarkers and of the phytohormone ACC and its degradation product 2-ketobutyric acid.
The thesis was carried out in the project SFB *Fusarium* which was funded by the Austrian Science Fund FWF. This project was undertaken to study Fusarium Head Blight disease regarding the interaction of *Fusarium* and its host plants from various aspects including a metabolomics approach. Therefore, a metabolomics platform was established at the Center for Analytical Chemistry. The presented thesis, which was part of the establishment for the metabolomics platform, focused on the development and application of GC-MS methods for metabolic profiling of volatiles and non-volatile, polar metabolites as well as target analysis of selected metabolites as a service to collaborators within the SFB project.
As a first step, reference mass spectra and RIs were collected for the in-house libraries for volatiles and for non-volatile, polar metabolites. For both libraries, measurements of mass spectra and RI from authentic standards were done. The in-house library for non-volatile polar metabolites contains 147 spectra and RIs from measurements of authentic standards. Regarding the library for volatiles, mass spectra from the greatest commercially available database NIST and RIs from NCWB were appended for metabolites, which were annotated in fungal cultures and wheat ears repetitively. Also metabolites, which could not be annotated but were found in several experiments of fungal cultures, were added to the in-house library for volatiles. In total, the in-house library for volatiles contains 296 spectra, namely 139 spectra and RIs from measurements of authentic standards, 116 NIST mass spectra and RIs from NCWB and 41 metabolites, which could not be annotated.
As a part of the method evaluation, the accuracy of measured LTPRI values, in comparison to the LTPRI values listed in NCWB, and the precision over long-time sequences using HS SPME was determined for selected standard substances. Furthermore, the volatile metabolic profiles of fungal cultures of *F. graminearum* PH-1 were determined for flushed and non-flushed cultures (regarding flushing with synthetic air prior to extraction). Both

types of cultures were found to reveal a similar volatile metabolic pattern. The flushed cultures were studied in HS-vials over one week using HS SPME GC-MS. 32 metabolites were annotated and 19 were identified. Another 25 metabolites, mainly sesquiterpenes, could not be annotated according to the criteria set. The presence of mycotoxins synthesized by the fungal cultures in the HS-vials was determined after one week. Interestingly, a high number of mycotoxins could be revealed, including DON and its derivatives 3-acetyl-DON and 15-acetyl-DON. Especially the presence of high amounts of culmorin was surprising since longiborneol, the volatile precursor could not be determined in the HS profile. α-Longifolene was detected in the volatile metabolic profile, which was mentioned to be a precursor for longiborneol in plant (McCormick et al., 2010). The relevance of α-longifolene and longiborneol for culmorin biosynthesis in *F. graminearum* PH-1 should be further determined.

Based on the results of this study, matrix effects were carried out including the cultivation medium, volatiles themselves and the fungal mycelium. The SPME efficiency of minor constituents in the HS profile like alcohols, monoterpenes or C8-compounds was influenced mainly by the cultivation medium. In addition, the signal intensities of the monoterpenes was affected by the presence of sesquiterpenes and the intensities of C8-compounds by the presence of fungal mycelium. In contrast, the signal intensities of the sesquiterpenes remained unaffected by the matrices tested. For commercially available sesquiterpenes, calibration lines were made and evaluated. Since reproducibility was too low for absolute quantification, only relative quantification was considered for the comparison of signal intensities of the sesquiterpenes. For volatiles of other chemical classes not even a comparison of their intensity was feasible because the signal intensities were influenced differently at different time points of cultivation due to changing concentrations of the sesquiterpenes and increasing amounts of fungal mycelium. Having studied possible calibration techniques from literature, no suitable technique was found for living fungal cultures which could overcome these limitations. Regarding *Fusarium*-infected wheat ears, the HS SPME method differs regarding the extraction temperature and the matrices. Matrix effects need to be determined first, but multiple HS extraction could be an option for a standard-free calibration.

Anyway, the HS SPME method was used to study metabolic differences on a qualitative level between cultures of *F. graminearum* PH-1 and four strains of *F. graminearum* of the Northland population and between *F. graminearum* PH-1 (wild-type) and Δ*tri5* (mutant). The most significant outcome of these studies was the comparison of fungal cultures of the wild-type and the mutant revealing that not only trichodiene synthesis, but most sesquiterpenes were knocked-out due to the deletion of the *tri5* gene. It would be interesting whether the volatile metabolic pattern of wheat ears infected with the Δ*tri5* mutant would also reveal a lower number of sesquiterpenes.

The HS SPME method for the determination of volatile metabolic profiles of wheat ears differed in extraction time and temperature. For the study of wheat ears a higher extraction is necessary to successfully extract volatiles from ground wheat powder. The volatile profiles of wheat ears from different developmental stages and after *Fusarium*-infected were determined in the cultivar 'Capo' as a first study of plant volatiles. The effects of *Fusarium* infection on the volatile metabolic profile were studied in the resistent cultivar 'CM' and the susceptible cultivar 'Remus'. 96 h after *Fusarium*-infection a number of sesquiterpenes, longiborneol and p-xylene were detected, which could not be found at earlier time points or in healthy wheat ears. Whether these metabolites were synthesized by the fungus or the plant could not be determined with this setup in all cases, but they seem to play a crucial role in FHB disease.

The HS SPME method used for volatile metabolic profiling of wheat ears in this thesis was not evaluated for matrix effects or other extraction temperatures. The method should be optimized in a way that labeling experiments are possible. The interaction of plant::fungus is best studied if e.g. the plant material was isotopically labeled, to see which metabolites originate from which organisms. It could also be interesting to separate the roots in space from the residual plant, infect the roots of a plant culture with *Fusarium* and monitor the volatile profiles of the plant. There are two limitations for this idea: First, *Fusarium* usually infects wheat ears during anthesis and we do not know if infection of the roots results in similar metabolic changes. Second, for the determination of volatiles from living plant cultures a different extraction technique than HS SPME must be used because HS SPME was not found sensitive enough to measure volatiles from living plant cultures in plant tissue culture boxes.
Regarding non-volatile, polar metabolites, the extraction conditions chosen were based on protocols published. Precision of the method was determined for a number of metabolites extracted from wheat ears in preliminary experiments. The derivatisation procol was adapted for the target analysis of selected metabolites. Ergosterol, sitosterol and stigmasterol were considered as biomarkers for fungal and plant biomass. The infection degree corresponding to the ratio of ergosterol/sitosterol was compared for seven maize cultivars. 'Okato' showed the highest infection degree, followed by 'Gilberto', 'Gingko' and 'Pandoso', then 'Eduardo' and 'Fortress'. However, sitosterol, which was reported in literature to be independent from metabolic changes to infection (Griebel and Zeier, 2010), was found to be influenced by *Fusarium*-infection. These data need confirmation in a repetitive experiment with higher number of biological replicates, but the suitability of sitosterol as a biomarker for plant biomass should be discussed.
A second targeted method was established for the measurement of ACC and 2-ketobutyric acid. ACC synthases and deaminases from *F. graminearum* PH-1 were studied in the expression system *E. coli*. After the addition of substrates (methionine for ACC synthesis and ACC for 2-ketobutyric acid synthesis), ACC and its degradation product 2-ketobutyric acid were monitored. In none of the transformants including ACC synthases ACC was detected. One of two transformants including a ACC deaminase successfully degraded ACC to 2-aminobutyric acid. This was possibly the transamination product (by bacterial transaminases) of 2-ketobutyric acid. The annotation of 2-aminobutyric acid needs to be confirmed with an authentic standard. Moreover, the expression system should be changed to *Fusarium* after matrix effects have been determined properly for an appropiate cultivation medium and the fungal mycelium. This would avoid the bacterial transamination step and could lead to higher expression of the other ACC deaminase and ACC synthases. The addition of S-adenosylmethionine instead of methionine could further enhance the synthesis of ACC by ACC synthases.
This research has focused on defined parts of the metabolome. Regarding further research, mass spectral data of different instruments (GC-MS data from volatiles and non-volatile, polar metabolites, HR-LC-MS data from secondary and larger mid- to nonpolar metabolites) need to be linked and used together for statistical evaluation. This enhances the scientific output from metabolic (pathway) analysis, which needs to be established in the metabolomics platform. Additionally, new standard substances available should be continuously included into the in-house libraries to enlarge the focus of the individual studies.
The information gained from metabolic changes in wheat ears due to *Fusarium* infection should be connected with results from transcriptomics data to confirm the findings from metabolomics experiments on a genomic level. Future research should therefore concentrate on linking the genomic expression data with metabolomics data to unravel new

pathways involved in the plant's susceptibility or resistance and to improve knowledge about existing pathways and their role in FHB disease.

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Appendix A

Additional tables

Table A.1: Qualitative analysis of fungal cultures using HS SPME. Retention indices (RI) listed from HP5ms or equivalent column type.

chemical group	metabolite	RI	organism	literature
alcohols	3-methyl-1-butanol		*Fusarium*, *Aspergillus*, *Penicillium*	Eifler et al. (2011); Jelen et al. (1997); Jelen (2003); Borjesson et al. (1989)
	2-methyl-1-butanol		*Fusarium*, *Trichoderma*	Eifler et al. (2011); Jelen et al. (1997); Nemcovic et al. (2008)
	2-methyl-1-propanol		*Fusarium*, *Aspergillus*, *Penicillium*, *Trichoderma*	Eifler et al. (2011); Borjesson et al. (1989); Nemcovic et al. (2008)
	2-butanol		*Penicillium*	Nilsson et al. (1996)
	isopentyl alcohol		*Penicillium*	Nilsson et al. (1996)
	isobutyl alcohol		*Penicillium*	Nilsson et al. (1996)
	3-methyl-3-buten-1-ol		*Trichoderma*	Nemcovic et al. (2008)
	2-(2-ethoxy)-ethoxy-ethanol		*Trichoderma*	Nemcovic et al. (2008)
	phenylethylalcohol	1116	*Trichoderma*	Stoppacher et al. (2010)
	ethanol		*Fusarium*	Eifler et al. (2011)
aldehydes	3-methyl-butanal		*Fusarium*	Eifler et al. (2011)
	2-methylpropanal		*Fusarium*	Eifler et al. (2011)
	hexanal		*Fusarium*	Eifler et al. (2011)
	nonanal		*Penicillium*	Chitarra et al. (2004)
	decanal		*Penicillium*	Chitarra et al. (2004)
	trans-cinnamaldehyde	1266	*Penicillium*	Demyttenaere et al. (2003)
	benzacetaldehyde		*Fusarium*	Eifler et al. (2011)
ketones	2-butanone		*Trichoderma*	Nemcovic et al. (2008)
	2-pentanone		*Fusarium*, *Aspergillus*, *Penicillium*	Nilsson et al. (1996); Borjesson et al. (1989)
	2-heptanone	893	*Trichoderma*	Stoppacher et al. (2010)
	6-methyl-2-heptanone		*Trichoderma*	Nemcovic et al. (2008)
	2-nonanone	1093	*Trichoderma*	Stoppacher et al. (2010)
	2-nonadecanone		*Trichoderma*	Stoppacher et al. (2010)
	2-decanone		*Trichoderma*	Nemcovic et al. (2008)
	2-undecanone	1292	*Trichoderma*	Stoppacher et al. (2010)
	acetone		*Penicillium*, *Trichoderma*	Nilsson et al. (1996); Nemcovic et al. (2008)
lactones	6-pentyl-α-pyrone	1465	*Trichoderma*	Stoppacher et al. (2010)

Table A.1: (continued)

chemical group	metabolite	RI	organism	literature
C8-compounds	1-octen-3-ol	978, 984	*Fusarium*, *Aspergillus*, *Penicillium*, *Trichoderma*	Eifler et al. (2011); Jelen et al. (1997); Borjesson et al. (1989); Stoppacher et al. (2010); Nemcovic et al. (2008); Combet et al. (2006); Nilsson et al. (1996); Chitarra et al. (2004); Demyttenaere et al. (2003); Jelen (2003)
	1-octen-3-ol acetate		*Penicillium*	Nilsson et al. (1996)
	3-octanone	987	*Fusarium*, *Trichoderma*, *Penicillium*	Eifler et al. (2011); Jelen et al. (1997); Stoppacher et al. (2010); Nemcovic et al. (2008); Combet et al. (2006); Nilsson et al. (1996); Chitarra et al. (2004); Jelen (2003)
	3-octanol	997	*Trichoderma*, *Penicillium*	Stoppacher et al. (2010); Combet et al. (2006); Nilsson et al. (1996); Chitarra et al. (2004); Jelen (2003)
	3-octanol acetate		*Penicillium*	Nilsson et al. (1996)
	1,3-octadiene (*cis* and *trans*)		*Penicillium*	Chitarra et al. (2004); Nilsson et al. (1996)
	1,3-*trans*-5-*cis*-octatriene		*Penicillium*	Nilsson et al. (1996)
	1,5-octadien-3-ol		*Penicillium*	Nemcovic et al. (2008); Combet et al. (2006); Chitarra et al. (2004)
	1-octen-3-one		*Penicillium*	Nilsson et al. (1996); Combet et al. (2006)
	5-octen-3-one		*Penicillium*	Chitarra et al. (2004)
	3-octen-2-ol		*Aspergillus*, *Penicillium*	Borjesson et al. (1989)
	butyrolactone		*Fusarium*	Eifler et al. (2011)
acids and esters	ethyl acetate		*Fusarium*	Borjesson et al. (1989)
	2-methylpropanoic acid		*Penicillium*	Jelen (2003)
	3-methyl-1-butanol acetate		*Fusarium*, *Penicillium*	Jelen et al. (1997); Demyttenaere et al. (2003); Jelen (2003)
	isobutyl acetate		*Fusarium*, *Penicillium*	Jelen et al. (1997); Nilsson et al. (1996)
	hexyl acetate		*Fusarium*	Jelen et al. (1997)
	acetic acid 2-methylpropylester		*Penicillium*	Jelen (2003)
	2-methyl-propanic acid 2-methylpropylester		*Penicillium*	Jelen (2003)
	2-methyl-propanic acid 3-methylbutylester		*Penicillium*	Jelen (2003)
	2-methyl-butanoic acid 2-methylpropylester		*Penicillium*	Jelen (2003)
	butanoic acid 3-methylbutylester		*Penicillium*	Jelen (2003)
	2,2-dimethyl-1-propanol benzoate		*Fusarium*	Eifler et al. (2011)
alkanes	heptane		*Penicillium*	Jelen (2003)
	undecane		*Penicillium*	Jelen (2003)
	hexadecane		*Fusarium*	Eifler et al. (2011)
	2,4-dimethylhexan		*Fusarium*	Borjesson et al. (1989)
	2,3,5-trimethylhexane		*Fusarium*	Borjesson et al. (1989)
	3,3,4-trimethylhexane		*Fusarium*	Eifler et al. (2011)
	3,7-dimethyldecane		*Fusarium*	Eifler et al. (2011)
alkenes	1,3-octadiene		*Penicillium*	Jelen (2003)
furans	2-methyl-furan		*Trichoderma*	Nemcovic et al. (2008)

Table A.1: (continued)

chemical group	metabolite	RI	organism	literature
	5-methyl-2-ethyl-furan		*Trichoderma*	Nemcovic et al. (2008)
	2-pentyl-furan	992	*Aspergillus*, *Fusarium*, *Trichoderma*	Syhre et al. (2008); Stoppacher et al. (2010); Nemcovic et al. (2008)
	2-n-heptylfuran	1191	*Trichoderma*	Stoppacher et al. (2010)
	5-ethylene-dihydro-5-methyl-2-furanone		*Trichoderma*	Nemcovic et al. (2008)
sulfuric compounds	dimethyldisulfide		*Aspergillus*, *Penicillium*, *Cladosporium*, *Paecilomyces*, *Phialophora*, lactic acid bacteria	Sunesson et al. (1995); Yvon and Rijnen (2001)
cyclic and aromatic compounds	C3-benzene		*Trichoderma*	Nemcovic et al. (2008)
	ethylbenzene		*Fusarium*	Jelen et al. (1997)
	dimethylbenzene		*Fusarium*	Jelen et al. (1997)
	1-methyl-4-(1-methyl-ethyl)-benzene		*Penicillium*	Jelen (2003)
	1-methoxy-3-methylbenzene		*Penicillium*	Nilsson et al. (1996)
	tert-p-methoxybenzene		*Trichoderma*	Nemcovic et al. (2008)
	benzothiazole		*Trichoderma*	Nemcovic et al. (2008)
	styrene		*Penicillium*	Jelen (2003)
	toluene		*Trichoderma*, *Penicillium*	Nemcovic et al. (2008); Jelen (2003)
	p-xylene		*Fusarium*, *Penicillium*	Eifler et al. (2011); Jelen (2003)
	1,1-dimethylethyl-2-methylphenol		*Penicillium*	Jelen (2003)
	4-ethyl-2-methoxyphenol		*Fusarium*	Jelen et al. (1997)
chlorogenic hydrocarbons	chloroform		*Trichoderma*	Nemcovic et al. (2008)
	1,2-dichloroethane		*Trichoderma*	Nemcovic et al. (2008)
monoterpenes	bornylene		*Penicillium*	Nilsson et al. (1996)
	camphene		*Penicillium*	Nilsson et al. (1996)
	(+)-2-carene		*Penicillium*	Jelen (2003)
	3-carene		*Fusarium*, *Penicillium*	Jelen et al. (1997); Jelen (2003)
	eucalyptol		*Penicillium*	Nilsson et al. (1996)
	limonene	1031	*Fusarium*, *Penicillium*, *Trichoderma*	Jelen et al. (1997); Nilsson et al. (1996); Demyttenaere et al. (2003); Jelen (2003); Nemcovic et al. (2008)
	linalool		*Penicillium*	Nilsson et al. (1996)
	myrcene	989	*Penicillium*	Nilsson et al. (1996); Demyttenaere et al. (2003); Jelen (2003)
	cis-nerolidol		*Penicillium*	Nilsson et al. (1996)
	(E)-β-ocimene	1053	*Penicillium*	Demyttenaere et al. (2003)
	α-pinene		*Fusarium*, *Penicillium*	Jelen et al. (1997); Nilsson et al. (1996)
	β-pinene		*Penicillium*	Nilsson et al. (1996)
	α-phellandrene	1005	*Trichoderma*, *Penicillium*	Stoppacher et al. (2010); Jelen (2003)
	β-phellandrene	1030	*Trichoderma*	Stoppacher et al. (2010)
	sabinene		*Penicillium*	Nilsson et al. (1996)
	α-terpinene	1018, 1017	*Trichoderma*, *Penicillium*	Stoppacher et al. (2010); Demyttenaere et al. (2003)

Table A.1: (continued)

chemical group	metabolite	RI	organism	literature
	γ-terpinene	1061,1062	*Trichoderma*, *Penicillium*	Stoppacher et al. (2010); Demyttenaere et al. (2003); Nilsson et al. (1996)
	terpinolene	1090, 1091	*Trichoderma*, *Penicillium*	Stoppacher et al. (2010); Demyttenaere et al. (2003); Nilsson et al. (1996)
monoterpenoids	p-menth-2-en-7-ol	1259	*Trichoderma*	Stoppacher et al. (2010)
	camphor		*Penicillium*	Nilsson et al. (1996)
	borneol		*Penicillium*	Nilsson et al. (1996)
sesquiterpenes	acoradiene	1483,1476	*Fusarium*. *Penicillium*	Demyttenaere et al. (2004); Jelen et al. (1995, 1997); Nilsson et al. (1996)
	10-epi-β-acoradiene	1473 (KI)	*Fusarium*	Girotti et al. (2010)
	(+)-aristolochene	1482	*Penicillium*	Demyttenaere et al. (2003); Jelen (2003)
	barbatene	1420 (KI)	*Fusarium*	Girotti et al. (2010)
	α-bergamotene	1394, 1440	*Fusarium*, *Penicillium*, *Trichoderma*	Jelen et al. (1995); Nilsson et al. (1996); Stoppacher et al. (2010)
	β-bisabolene	1517,1516, 1512, 1513 (KI)	*Fusarium*, *Penicillium*, *Trichoderma*	Demyttenaere et al. (2004); Jelen et al. (1995, 1997); Girotti et al. (2010); Jelen (2003); Nilsson et al. (1996); Stoppacher et al. (2010)
	γ-cadinene		*Fusarium*	Jelen et al. (1997)
	trans-β-caryophyllene	1417	*Penicillium*	Nilsson et al. (1996); Demyttenaere et al. (2003)
	9-epi-*trans*-caryophyllene	1465	*Penicillium*	Demyttenaere et al. (2003); Nilsson et al. (1996)
	α-cedrene	1521	*Fusarium*	Demyttenaere et al. (2004)
	diepi-α-cedrene	1487	*Fusarium*, *Penicillium*	Jelen et al. (1995); Jelen (2003)
	α-chamigrene	1428	*Fusarium*, *Penicillium*	Demyttenaere et al. (2004); Jelen (2003); Nilsson et al. (1996)
	β-chamigrene	1490,1481, 1473, 1480 (KI)	*Fusarium* , *Penicillium*	Demyttenaere et al. (2004); Jelen et al. (1995); Demyttenaere et al. (2003); Girotti et al. (2010); Nilsson et al. (1996)
	α-copaene		*Penicillium*	Nilsson et al. (1996)
	cuparene	1523	*Fusarium*	Demyttenaere et al. (2004)
	α-cubebene		*Penicillium*	Nilsson et al. (1996)
	aryl-curcumene	1492,1490, 1484 (KI)	*Fusarium*	Demyttenaere et al. (2004); Jelen et al. (1995); Girotti et al. (2010)
	α-curcumene	1486	*Trichoderma*	Stoppacher et al. (2010)
	γ-curcumene	1483	*Trichoderma*	Stoppacher et al. (2010)
	β-elemene	1390	*Penicillium*	Demyttenaere et al. (2003); Jelen (2003)
	β-elemene enantiomer	1383	*Penicillium*	Demyttenaere et al. (2003)
	elixene	1445	*Fusarium*	Jelen et al. (1995)
	α-farnesene	1538, 1508	*Fusarium*, *Trichoderma*	Jelen et al. (1995, 1997); Stoppacher et al. (2010)

Table A.1: (continued)

chemical group	metabolite	RI	organism	literature
	trans-β-farnesene	1461,1462, 1453 (KI), 1457	*Fusarium*, *Trichoderma*	Demyttenaere et al. (2004); Jelen et al. (1995, 1997); Girotti et al. (2010); Stoppacher et al. (2010)
	germacrene A	1502	*Penicillium*	Demyttenaere et al. (2003)
	germacrene D		*Fusarium*	Jelen et al. (1997)
	geosmin		*Penicillium*	Nilsson et al. (1996)
	β-gurjunene		*Penicillium*	Jelen (2003)
	α-himachalene	1510	*Fusarium*	Demyttenaere et al. (2004)
	β-himachalene	1510,1527, 1509 (KI)	*Fusarium*, *Penicillium*	Jelen et al. (1995); Demyttenaere et al. (2004); Girotti et al. (2010); Jelen (2003); Nilsson et al. (1996)
	isobazzanene	1444 (KI)	*Fusarium*	Girotti et al. (2010)
	2-methylisoborneol		*Penicillium*	Nilsson et al. (1996)
	italicene		*Penicillium*	Nilsson et al. (1996)
	longifolene	1415 (KI)	*Fusarium*, *Penicillium*	Girotti et al. (2010); Jelen et al. (1995); Nilsson et al. (1996)
	β-maaliene		*Penicillium*	Nilsson et al. (1996)
	α-panasinsene		*Penicillium*	Jelen (2003)
	γ-patchoulene		*Penicillium*	Jelen (2003)
	β-santalene	1474,1468, 1463 (KI)	*Fusarium*	Demyttenaere et al. (2004); Jelen et al. (1995); Girotti et al. (2010)
	α-selinene		*Penicillium*	Jelen (2003)
	β-selinene	1550	*Fusarium*	Jelen et al. (1995)
	β-sesquiphellandrene	1535, 1529	*Fusarium*, *Trichoderma*	Demyttenaere et al. (2004); Stoppacher et al. (2010)
	thujopsene	1450, 1439 (KI)	*Fusarium*, *Penicillium*	Demyttenaere et al. (2004); Girotti et al. (2010); Nilsson et al. (1996)
	trichodiene	1533,1541, 1533 (KI)	*Fusarium*	Jelen et al. (1995, 1997); Jelen (2003); Demyttenaere et al. (2004); Girotti et al. (2010)
	valencene	1490	*Penicillium*	Demyttenaere et al. (2003); Jelen (2003)
	widdrol		*Penicillium*	Nilsson et al. (1996)
	α-zingiberene	1498	*Trichoderma*	Stoppacher et al. (2010)
	unidentified sesquiterpenes			
sesquiterpenoids	longiborneol	1592	*Fusarium*	McCormick et al. (2010)
	nerolidol	1566	*Trichoderma*	Stoppacher et al. (2010)
diterpenes	unidentified M=272		*Trichoderma*	Nemcovic et al. (2008)

Table A.2: Qualitative analysis of volatiles detected in infected wheat samples. Retention indices (RI) listed from HP5ms or equivalent column type.

chemical group	metabolite	RI	organism	literature
alcohols	ethanol		*Fusariumpoae* on wheat	Presicce et al. (2006)
	2-phenoxyethanol	1221	natural infection of wheat by fungi	Busko et al. (2010)
	propanol		*Fusariumpoae* on wheat	Presicce et al. (2006)
	2-methylpropanol		*Fusariumpoae* on wheat	Presicce et al. (2006)
	butanol		*Fusariumpoae* on wheat	Presicce et al. (2006)

Table A.2: (continued)

chemical group	metabolite	RI	organism	literature
	3-methyl-butanol		*Fusarium*poae on wheat	Presicce et al. (2006)
	1-pentanol		*Fusarium*poae on wheat	Presicce et al. (2006)
	2-pentanol		*Fusarium*poae on wheat	Presicce et al. (2006)
	hexanol	873	*Fusarium*poae on wheat, natural infection of wheat by fungi	Presicce et al. (2006); Busko et al. (2010)
	2-ethyl-1-hexanol	1029	natural infection of wheat by fungi	Busko et al. (2010)
	1-heptanol	969	natural infection of wheat by fungi	Busko et al. (2010)
	1-decanol	1276	natural infection of wheat by fungi	Busko et al. (2010)
acids and esters	ethyl acetate		*Fusarium*poae on wheat	Presicce et al. (2006)
	acetic acid		*Fusarium*poae on wheat	Presicce et al. (2006)
	benzyl acetate		*Fusarium*spp. on wheat	Wenda-Piesik et al. (2010)
	2-phenylethyl acetate		*Fusarium*poae on wheat	Presicce et al. (2006)
	ethyldecanoate		*Fusarium*poae on wheat	Presicce et al. (2006)
	ethyl-9-decenoate		*Fusarium*poae on wheat	Presicce et al. (2006)
	octanoic acid	1192	natural infection of wheat by fungi	Busko et al. (2010)
	nonanoic acid	1283	natural infection of wheat by fungi	Busko et al. (2010)
aldehydes	3-methylbutanal		*Fusarium*poae on wheat	Presicce et al. (2006)
	hexanal		*Fusarium*poae on wheat	Presicce et al. (2006)
	heptanal	901	natural infection of wheat by fungi	Busko et al. (2010)
	nonanal	1105	natural infection of wheat by fungi	Busko et al. (2010)
	decanal	1206	natural infection of wheat by fungi	Busko et al. (2010)
ketones	2-butanone		*Fusarium*poae on wheat	Presicce et al. (2006)
	3-hydroxy-2-butanone		*Fusarium*poae on wheat	Presicce et al. (2006)
	2-pentanone/3-methylbutanone		*Fusarium*poae on wheat	Presicce et al. (2006)
	2-heptanone		*Fusarium*poae on wheat	Presicce et al. (2006)
	6-methyl-5-hepten-2-one	985	natural infection of wheat by fungi	Busko et al. (2010)
	nona-2-none	1092	natural infection of wheat by fungi	Busko et al. (2010)
	3,5,5-trimethylcyclohexen-2-one	1192	natural infection of wheat by fungi	Busko et al. (2010)
furans	2-methylfuran		*Fusarium*poae on wheat	Presicce et al. (2006)
	2-ethylfuran		*Fusarium*poae on wheat	Presicce et al. (2006)
	2-pentylfuran		*Fusarium*poae on wheat	Presicce et al. (2006)
	5-butyldihydro-2(3H)-furanone	1261	natural infection of wheat by fungi	Busko et al. (2010)
alkanes and alkenes	2,4-dimethylheptene		*Fusarium*poae on wheat	Presicce et al. (2006)
	indane	1030	natural infection of wheat by fungi	Busko et al. (2010)

Table A.2: (continued)

chemical group	metabolite	RI	organism	literature
	undecane	1099	natural infection of wheat by fungi	Busko et al. (2010)
	dodecane	1199	natural infection of wheat by fungi	Busko et al. (2010)
	tetradecane	1400	natural infection of wheat by fungi	Busko et al. (2010)
	pentadecane	1502	natural infection of wheat by fungi	Busko et al. (2010)
	hexadecane	1599	natural infection of wheat by fungi	Busko et al. (2010)
	eicosane	1999	natural infection of wheat by fungi	Busko et al. (2010)
	triacontane	3007	natural infection of wheat by fungi	Busko et al. (2010)
cyclic and aromatic compounds	propylbenzene	948	natural infection of wheat by fungi	Busko et al. (2010)
	1-ethyl-2-methylbenzene	957	natural infection of wheat by fungi	Busko et al. (2010)
	1-ethyl-3-methylbenzene	974	natural infection of wheat by fungi	Busko et al. (2010)
	1,3,5-trimethylbenzene	963	natural infection of wheat by fungi	Jelen and Wasowicz (1998); Olsson et al. (2000); Busko et al. (2010)
	1,2,4-trimethylbenzene	988	natural infection of wheat by fungi	Jelen and Wasowicz (1998); Olsson et al. (2000); Busko et al. (2010)
	p-xylene/ethylbenzene		*Fusarium poae* on wheat	Presicce et al. (2006)
	p-xylene	870	natural infection of wheat by fungi	Busko et al. (2010)
	styrene	889	natural infection of wheat by fungi	Busko et al. (2010)
	4-ethyl-2-methoxyphenol		*Fusarium poae* on wheat	Presicce et al. (2006)
	4-ethylphenol		*Fusarium poae* on wheat	Presicce et al. (2006)
	indole		*Fusarium* spp. on wheat	Wenda-Piesik et al. (2010)
	butyrolactone	916	natural infection of wheat by fungi	Busko et al. (2010)
	naphtalene	1178	natural infection of wheat by fungi	Busko et al. (2010)
	1-methylnaphtalene	1308	natural infection of wheat by fungi	Busko et al. (2010)
	2-methylnaphtalene	1291	natural infection of wheat by fungi	Busko et al. (2010)
	diethylphthalate	1597	natural infection of wheat by fungi	Busko et al. (2010)
C6 GLVs	*cis*-3-hexenal		*Fusarium* spp. on wheat, oat, barley	Piesik et al. (2011b)
	trans-2-hexenal		*Fusarium* spp. on wheat, oat, barley	Piesik et al. (2011b)
	trans-2-hexenol		*Fusarium* spp. on wheat, oat, barley	Piesik et al. (2011b)
	cis-3-hexen-1-ol		*Fusarium* on wheat	Wenda-Piesik (2011)
	cis-3-hexenyl acetate		*Fusarium* spp. on wheat, oat, barley	Piesik et al. (2011b)
	1-hexyl acetate		*Fusarium* spp. on wheat, oat, barley	Piesik et al. (2011b)

Table A.2: (continued)

chemical group	metabolite	RI	organism	literature
	cis-3-hexen-1-yl acetate		*Fusarium*spp. on wheat, oat, barley	Piesik et al. (2011b)
C8-compounds	1-octen-3-ol	978	*Fusariumpoae* on wheat, natural infection of wheat by fungi	Presicce et al. (2006); Busko et al. (2010); Eifler et al. (2011)
	2-octanone	989	natural infection of wheat by fungi	Busko et al. (2010)
	3-octen-2-one	1038	natural infection of wheat by fungi	Busko et al. (2010)
	(*trans,trans*)-3,5-octadiene-2-one	1094	natural infection of wheat by fungi	Busko et al. (2010)
monoterpenoids	3-carene	1005	natural infection of wheat by fungi	Busko et al. (2010)
	cymene	1020	natural infection of wheat by fungi	Busko et al. (2010)
	D-limonene	1024	*Fusariumpoae* on wheat, natural infection of wheat by fungi	Presicce et al. (2006); Busko et al. (2010)
	β-linalool		*Fusarium*spp. on wheat, oat, barley	Piesik et al. (2011b)
	linalool oxide		*Fusarium*spp. on wheat	Wenda-Piesik et al. (2010)
	cis-ocimene		*Fusarium*spp. on wheat	Wenda-Piesik et al. (2010)
	α-pinene	929	natural infection of fungi on wheat	Busko et al. (2010)
sesquiterpenoids	β-caryophyllene		*Fusarium*spp. on wheat, oat, barley	Piesik et al. (2011b)
	β-farnesene		*Fusarium*spp. on wheat	Wenda-Piesik (2011)
	lilial	1529	natural infection of wheat by fungi	Busko et al. (2010)
	trichodiene	1520	*Fusarium*spp. on wheat, natural infection of wheat by fungi	Perkowski et al. (2008); Busko et al. (2010)

Table A.3: Reference substances, whose mass spectra and RIs (on HP5-ms column) are collected in the in-house library of volatiles.

chemical class	substance	origin	organism
acid	4-methylvaleric acid	standard	plant
acid	acetic acid	NIST	plant
acid	hexadecanoic acid	NIST	plant
acid	hexanoic acid	NIST	plant
acid	octanoic acid	NIST	plant
acid	pentadecanoic acid	NIST	plant
acid	tetradecanoic-acid	NIST	plant
alcohol	(E)-3-hexen-1-ol	standard	plant
alcohol	(E,E)-2,4-hexadienol	standard	plant
alcohol	(Z)-5-octen-1-ol	standard	plant
alcohol	1,3-propanediol	standard	plant
alcohol	1-decanol	standard	plant
alcohol	1-heptanol	NIST	plant
alcohol	1-hexanol	standard	plant
alcohol	1-hexanol-2-ethyl-	NIST	plant
alcohol	1-nonanol	NIST	plant
alcohol	1-octanol	standard	fungal, plant
alcohol	1-octen-3-ol	standard	fungal, plant
alcohol	1-pentanol	standard	fungal,plant
alcohol	1-tetradecanol	NIST	plant
alcohol	1-tridecanol	standard	plant
alcohol	2-heptanol	standard	plant

Table A.3: (continued)

chemical class	substance	origin	organism
alcohol	2-methyl-1-butanol	standard	fungal,plant
alcohol	2-methyl-1-pentanol	standard	plant
alcohol	2-methyl-1-propanol	standard	fungal,plant
alcohol	2-methyl-2-pentanol	standard	plant
alcohol	2-octen-1-ol	NIST	plant
alcohol	2-phenoxyethanol	standard	plant
alcohol	2-propyl-1-pentanol	standard	plant
alcohol	2-tridecanol	NIST	plant
alcohol	3-ethoxy-1-propanol	standard	plant
alcohol	3-methyl-1-butanol	standard	fungal,plant
alcohol	3-octanol	standard	fungal,plant
alcohol	3-octen-1-ol	NIST	fungal
alcohol	3-pentanol	standard	plant
alcohol	4-penten-2-ol	NIST	fungal
alcohol	5-hexen-1-ol	standard	plant
alcohol	6-methyl-5-heptene-2-ol	standard	plant
alcohol	benzyl alcohol	standard	plant
alcohol	phenylethylalcohol	standard	plant
alcohol	R(-)2-methyl-1,4-pentanediol	standard	plant
aldehyde	(E)-2-methyl-2-butenal	standard	plant
aldehyde	(E)-2-pentenal	standard	plant
aldehyde	1H-pyrrole-2-carboxaldehyde	NIST	plant
aldehyde	2-(E)-hexenal	standard	plant
aldehyde	2,4-decadienal(E,E)- 1	NIST	plant
aldehyde	2,4-heptadienal(E,E)-	NIST	plant
aldehyde	2,4-nonadienal(E,E)-	NIST	plant
aldehyde	2,6-nonadienal(E,Z)-	NIST	plant
aldehyde	2-heptenal-(Z)-	NIST	plant
aldehyde	2-nonenal-(E)-	NIST	plant
aldehyde	6-nonenal-(Z)-	NIST	plant
aldehyde	9,17-octadecadienal-(Z)-	NIST	plant
aldehyde	acetaldehyde	NIST	plant
aldehyde	benzaldehyde	standard	plant
aldehyde	benzaldehyde-2-4-dimethyl-	NIST	plant
aldehyde	benzaldehyde-4-hydroxy-	NIST	plant
aldehyde	butanal	NIST	plant
aldehyde	butanal-2-methyl-	NIST	plant
aldehyde	butanal-3-methyl-	NIST	plant
aldehyde	decanal	NIST	plant
aldehyde	dodecanal	NIST	plant
aldehyde	heptanal	NIST	plant
aldehyde	hexanal	NIST	plant
aldehyde	nonanal	NIST	plant
aldehyde	octanal	NIST	plant
aldehyde	pentanal	NIST	plant
aldehyde	propanal-2-methyl-	NIST	plant
aldehyde	tetradecanal	NIST	plant
aldehyde	tridecanal	NIST	plant
alkane	butane-2-2-3-3-tetramethyl-	NIST	plant
alkane	decane	standard	plant
alkane	docosane	standard	plant
alkane	dodecane	standard	plant
alkane	eicosane	standard	plant
alkane	heneicosane	standard	plant
alkane	heptacosane	standard	plant
alkane	heptadecane	standard	plant
alkane	heptane	standard	plant
alkane	hexacosane	standard	plant
alkane	hexadecane	standard	plant
alkane	hexane	standard	plant
alkane	hexane-2-2-dimethyl-	NIST	plant
alkane	isooctan	NIST	plant
alkane	nonacosane	standard	plant
alkane	nonadecane	standard	plant

Table A.3: (continued)

chemical class	substance	origin	organism
alkane	nonane	standard	plant
alkane	octacosane	standard	plant
alkane	octadecane	standard	plant
alkane	octane	standard	plant
alkane	pentacosane	NIST	plant
alkane	pentacosane	standard	plant
alkane	pentadecane	standard	plant
alkane	pentane	standard	plant
alkane	pentane-2-2-4-4-tetramethyl-	NIST	plant
alkane	tetracosane	standard	plant
alkane	tetradecane	standard	plant
alkane	triacontane	standard	plant
alkane	tricosane	standard	plant
alkane	tridecane	standard	plant
alkane	undecane	standard	plant
alkene	9-tricosene-(Z)-	NIST	plant
cyclic compound	1,3,5-cycloheptatriene	standard	fungal, plant
cyclic compound	2(4H)-benzofuranone, 5,6,7,7a-tetrahydro-4,4,7a-trimethyl-(R)-	NIST	plant
cyclic compound	2,3-dimethylanisole	NIST	plant
cyclic compound	2-methoxy-4-vinylphenol	NIST	plant
cyclic compound	2-pentyl-furan	standard	plant
cyclic compound	3-furaldehyde	NIST	plant
cyclic compound	5-methyl-furfural	standard	plant
cyclic compound	6-pentyl-2-pyron	standard	plant
cyclic compound	anisole	standard	plant
cyclic compound	benzene 1-2-3-trimethyl-	NIST	fungal
cyclic compound	benzene 1-ethyl-3-methyl-	NIST	fungal
cyclic compound	benzene-1-2-4-trimethyl-	NIST	fungal
cyclic compound	benzene-1-3-5-trimethyl-	NIST	fungal
cyclic compound	dimethylfulvene	NIST	fungal
cyclic compound	ethylbenzene	NIST	fungal
cyclic compound	furan-2-ethyl	standard	plant
cyclic compound	furan-2-ethyl-5-methyl-	NIST	plant
cyclic compound	furan-2-methyl-	NIST	plant
cyclic compound	furan-3-methyl-	NIST	plant
cyclic compound	furfural	NIST	plant
cyclic compound	γ-octalactone-2(3H)-furanone, 5-butyldihydro-	standard	plant
cyclic compound	indole	NIST	plant
cyclic compound	m-xylene	standard	fungal, plant
cyclic compound	oxirane	NIST	plant
cyclic compound	o-xylene	standard	fungal, plant
cyclic compound	p-xylene	standard	fungal, plant
cyclic compound	pyrazine-2-6-dimethyl-	NIST	plant
cyclic compound	pyridine-3-phenyl-	NIST	plant
cyclic compound	toluene	NIST	plant
cyclic compound	vanillin	NIST	plant
ester	(E)-2-hexenylacetate	standard	plant
ester	1-butanol-3-methyl-acetate	NIST	fungal, plant
ester	2-methyl-1-butanol acetate	standard	fungal, plant
ester	2-methyl-butyric-acid-ethylester	standard	plant
ester	acetic acid n-butyl ester	standard	plant
ester	acetic acid nonylester	standard	plant
ester	allylisovalerate	standard	plant
ester	allylpropionate	standard	plant
ester	amyl propionate	standard	plant
ester	benzyl acetate	standard	plant
ester	benzyl benzoate	standard	plant
ester	butanoic-acid-3-methyl-ethylester	NIST	plant
ester	butylbutyrate	standard	plant
ester	citronellyl acetate	standard	plant
ester	ethylacetate	standard	fungal,plant
ester	ethylbutyrate	standard	plant

Table A.3: (continued)

chemical class	substance	origin	organism
ester	ethyldecanoate	standard	plant
ester	ethyldodecanoate	standard	plant
ester	ethylhexanoate	standard	plant
ester	ethylisovalerate	standard	plant
ester	geranylacetate	standard	plant
ester	heptylformiate	standard	plant
ester	hexadecanoic-acid-methylester	NIST	plant
ester	hexanoic-acid-2-ethyl-methylester	NIST	plant
ester	hexanoic-acid-ethylester	standard	plant
ester	isobutylacetate	standard	plant
ester	isopropylacetate	standard	plant
ester	linalylacetate	standard	plant
ester	methylhexanoate	standard	plant
ester	methylsalicylate	standard	plant
ester	nerylacetate	standard	plant
ester	n-pentylacetate	standard	plant
ester	pentylbutyrate	standard	plant
ester	propylbutyrate	standard	plant
ester	propylpropionate	standard	plant
ketone	2-butanone	NIST	plant
ketone	2-butanone-3-hydroxy-	NIST	plant
ketone	2-heptadecanone	NIST	plant
ketone	2-heptanone	standard	fungal, plant
ketone	2-hexanone	standard	plant
ketone	2-octanone	standard	fungal, plant
ketone	2-pentadecanone	NIST	plant
ketone	2-propanone, 1-hydroxy-	NIST	plant
ketone	2-tridecanone	NIST	plant
ketone	2-undecanone	NIST	plant
ketone	3,5-octadien-2-one (EE)-	NIST	plant
ketone	3-heptanone-5-methyl-	NIST	plant
ketone	3-octanone	standard	fungal, plant
ketone	3-pentanone	standard	plant
ketone	4-hexen-3-one	standard	plant
ketone	4-octanone	NIST	plant
ketone	acetone	NIST	plant
ketone	heptan-4-one	standard	plant
monoterpene	(+)-4-carene	NIST	plant
monoterpene	3-carene	standard	fungal, plant
monoterpene	β-myrcene	standard	fungal, plant
monoterpene	cis-β-ocimene 1	NIST	plant
monoterpene	γ-terpinene	standard	fungal, plant
monoterpene	limonene	standard	fungal, plant
monoterpene	m-cymol	standard	fungal, plant
monoterpene	m-sylvestrene	NIST	fungal, plant
monoterpene	o-cymol	standard	fungal, plant
monoterpene	p-cymol	standard	fungal, plant
monoterpene	R(+)-limonene	standard	fungal, plant
monoterpene	terpinolene	standard	fungal, plant
monoterpene	trans-β-ocimene	NIST	plant
sesquiterpene	(-)-alloaromadendrene	NIST	fungal, plant
sesquiterpene	(-)epiglobulol	standard	fungal, plant
sesquiterpene	(+)-β-gurjunene (calarene)	NIST	fungal, plant
sesquiterpene	(+)-epi-bicyclosesquiphellandrene	NIST	fungal, plant
sesquiterpene	(E)-β-farnesene	standard	fungal, plant
sesquiterpene	9,10 dehydro isolongifolene	standard	fungal, plant
sesquiterpene	acoradiene	NIST	fungal, plant
sesquiterpene	α-(Z)-bisabolene	NIST	fungal, plant
sesquiterpene	α-bergamotene	NIST	fungal, plant
sesquiterpene	α-caryophyllene	standard	fungal, plant
sesquiterpene	α-cedrene	standard	fungal, plant
sesquiterpene	α-chamigrene	NIST	fungal, plant
sesquiterpene	α-farnesene	NIST	fungal, plant
sesquiterpene	α-himachalene	NIST	fungal, plant
sesquiterpene	α-humulene	standard	fungal, plant

Table A.3: (continued)

chemical class	substance	origin	organism
sesquiterpene	α-longifolene	NIST	fungal, plant
sesquiterpene	α-longipinene	standard	fungal, plant
sesquiterpene	α-phellandrene	NIST	fungal, plant
sesquiterpene	α-pinene	standard	fungal, plant
sesquiterpene	α-selinene	NIST	fungal, plant
sesquiterpene	α-terpinene	standard	fungal, plant
sesquiterpene	ar-curcumene	NIST	fungal, plant
sesquiterpene	aromadendrene	NIST	fungal, plant
sesquiterpene	β-bisabolene	NIST	fungal, plant
sesquiterpene	β-caryophyllene	standard	fungal, plant
sesquiterpene	β-chamigrene	NIST	fungal, plant
sesquiterpene	β-cubebene	NIST	fungal, plant
sesquiterpene	β-cyclocitral	NIST	fungal, plant
sesquiterpene	β-himachalene	NIST	fungal, plant
sesquiterpene	β-humulene	standard	fungal, plant
sesquiterpene	butylisobutyrate	standard	plant
sesquiterpene	butylpropionate	standard	plant
sesquiterpene	calamenene	NIST	fungal, plant
sesquiterpene	cycloisolongifolene,-8,9-dehydro	standard	plant
sesquiterpene	ethylnonanoate	standard	plant
sesquiterpene	ethyloctanoate	standard	plant
sesquiterpene	ethylphenylacetate	standard	plant
sesquiterpene	ethylsalicylate	standard	plant
sesquiterpene	ethylsorbate	standard	plant
sesquiterpene	ethylvalerate	standard	plant
sesquiterpene	γ-(E)-bisabolene	NIST	fungal, plant
sesquiterpene	γ-gurjunene	NIST	fungal, plant
sesquiterpene	γ-humulene	standard	fungal, plant
sesquiterpene	γ-patchoulene	NIST	fungal, plant
sesquiterpene	isolongifolene	standard	fungal, plant
sesquiterpene	longiborneol	NIST	fungal, plant
sesquiterpene	thujopsene	standard	fungal, plant
sesquiterpene	trichodiene	NIST	fungal
sesquiterpene	Fg1 RI727		fungal
sesquiterpene	Fg1		fungal
sesquiterpene	Fg2 RI1411		fungal
sesquiterpene	Fg3 RI1467		fungal
sesquiterpene	Fgstrain2		fungal
sesquiterpene	Fgstrain3		fungal
sesquiterpene	monoterpene1		fungal
sesquiterpene	sesquiterpene1		fungal
sesquiterpene	sesquiterpene2		fungal
sesquiterpene	sesquiterpene3		fungal
sesquiterpene	sesquiterpene4		fungal
sesquiterpene	sesquiterpene6		fungal
sesquiterpene	sesquiterpene7		fungal
sesquiterpene	sesquiterpene8		fungal
sesquiterpene	sesquiterpene9		fungal
sesquiterpene	sesquiterpene10 RI1510		fungal
sesquiterpene	sesquiterpene10		fungal
sesquiterpene	sesquiterpene11		fungal
sesquiterpene	sesquiterpene12		fungal
sesquiterpene	sesquiterpene13		fungal
sesquiterpene	sesquiterpene14		fungal
sesquiterpene	sesquiterpene15		fungal
sesquiterpene	sesquiterpene16		fungal
sesquiterpene	sesquiterpene17 RI1415		fungal
sesquiterpene	sesquiterpene18 RI1436		fungal
sesquiterpene	sesquiterpene19 RI1475		fungal
sesquiterpene	sesquiterpene20 RI1509		fungal
sesquiterpene	sesquiterpene21 RI1513		fungal
sesquiterpene	sesquiterpene22 RI1515		fungal
sesquiterpene	sesquiterpene23 RI1527		fungal
sesquiterpene	sesquiterpene24 RI1538		fungal
sesquiterpene	sesquiterpene25 RI1384		fungal
sesquiterpene	sesquiterpene26 RI1388		fungal
sesquiterpene	sesquiterpene27 RI1424		fungal

Table A.3: (continued)

chemical class	substance	origin	organism
sesquiterpene	sesquiterpene28 RI1427		fungal
sesquiterpene	sesquiterpene29 RI1445		fungal
sesquiterpene	sesquiterpene30 RI1453		fungal
sesquiterpene	sesquiterpene31 RI1469		fungal
sesquiterpene	sesquiterpene32 RI1522		fungal
sesquiterpene	sesquiterpene33 RI1539		fungal
sesquiterpene	similaralkane RI713		fungal
sesquiterpene	ylangene	NIST	fungal, plant
sterol	phytol	standard	plant
sterol	phytone	NIST	plant
sulphuric compound	1,3-dimethylsulfane	NIST	fungal,plant
sulphuric compound	dimethyl sulfide	NIST	fungal, plant
sulphuric compound	disulfide dimethyl	NIST	fungal, plant

Table A.4: Standard substances measured on HP5-ms column using derivatisation GC-MS with the detected derivatives. BP=bypeak, MP=mainpeak,MOX=methoxime group, TMS=trimethylsilyl group.

chemical class	substance	derivatives detected
acid	2-aminoadipic acid	2TMS
acid	2-Hydroxyglutaric acid	3TMS
acid	2-ketocaproic acid	1MOX 1TMS BP, 1MOX 1TMSMP, 1MOX 2TMS BP, 1MOX 2TMS MP
acid	2-methylcitric acid	4TMS BP, 4TMS MP
acid	3-methyl-2-ketobutanoic acid	1 MOX 1TMS
acid	azelaic acid	2TMS
acid	Citric acid	4TMS
acid	Fumaric acid	2 TMS
acid	Gluconic acid	6TMS
acid	Gluconic acid-6-P	7TMS
acid	Glyceric acid	3TMS
acid	Glyceric acid-2-P	4TMS
acid	Glyceric acid-3-P	4TMS
acid	Isocitric acid	4TMS
acid	Lactic acid	2TMS
acid	Maleic acid	2TMS
acid	Malic acid	3TMS
acid	Pantothenic acid	3TMS
acid	Phosphoenolpyruvic acid	3TMS
acid	Phosphoric acid	3TMS
acid	Pyruvic acid	1MOX 1TMS
acid	Succinic acid	2TMS
acid	Glucuronic acid	1MOX 5TMS BP, 1MOX 5TMS MP
acid	Glycerol-2-P	4TMS
alkane	nonane	
alkane	decane	
alkane	undecane	
alkane	dodecane	
alkane	tridecane	
alkane	tetradecane	
alkane	pentadecane	
alkane	hexadecane	
alkane	heptadecane	
alkane	octadecane	
alkane	nonadecane	
alkane	eicose	
alkane	heneicosane	
alkane	docosane	
alkane	tricosane	
alkane	tetracosane	
alkane	pentacosane	
alkane	hexacosane	
alkane	heptacosane	
alkane	octacosane	
alkane	nonacosane	
alkane	triacontane	
alkane	hentriacontane	
alkane	dotriacontane	

Table A.4: (continued)

chemical class	substance	derivatives detected
alkane	tritriacontane	
alkane	tetratriacontane	
alkane	pentatriacontane	
alkane	hexatriacontane	
alkane	heptatriacontane	
alkane	octatriacontane	
alkane	nonatriacontane	
amine	Agmatine_Putrescine	3TMS, 4TMS
amine	Agmatine-NH3	3TMS
amine	Putrescine_Agmatine	3TMS, 4TMS
amine	Serotonine	3TMS, 4TMS
amine	Spermidin	4TMS, 5TMS 1, 5TMS 2, 6TMS
amine	Tryptamine	2TMS, 3TMS
amine	Tyramine	3TMS
amino acid	Alanin	1TMS, 2TMS, 3TMS
amino acid	Arginine_Citrulline_Ornithine	3TMS, 4TMS 1, 4TMS2
amino acid	Asparagin	4TMS
amino acid	Aspartic acid	2TMS, 3TMS
amino acid	Cystein	3TMS, 4TMS
amino acid	Glutamic	acid 3TMS
amino acid	Glutamin	3TMS
amino acid	Glycin	2TMS, 3TMS
amino acid	Histidin	3TMS
amino acid	Isoleucin	1TMS, 2TMS
amino acid	Leucin	1TMS, 2TMS
amino acid	Lysin	4TMS
amino acid	Methionin	1TMS, 2TMS
amino acid	Phenylalanin	1TMS, 2TMS
amino acid	Prolin	1TMS, 2TMS
amino acid	Serin	2TMS, 3TMS
amino acid	Threonin	2 TMS, 3TMS
amino acid	Tryptophan	1TMS, 2TMS 1, 2TMS 2, 3TMS
amino acid	Tyrosin	2TMS, 3TMS
amino acid	Valin	1TMS, 2TMS
amino acid	Citrulline_Ornithin_Arginine	3TMS, 4TMS 1, 4TMS 2
amino acid	Norleucin	1TMS, 2TMS
amino acid	Norvalin	2TMS
amino acid	Ornithin_Arginine_Citrulline	3TMS, 4TMS 1, 4TMS 2
amino acid derivative	4-aminobutyric acid (GABA)	3TMS
amino acid derivative	β-Alanin	2TMS, 3TMS
amino acid derivative	Homoserin	2TMS, 3TMS
amino acid derivative	Hydroxyprolin	3TMS
amino acid derivative	Pyroglutamic acid	2TMS
amino acid derivative	Sarcosin	2TMS
amino acid derivative	S-methyl-cysteine	2TMS
base	Adenine	2 TMS
base	Thymin	2TMS
base	Uracil	2TMS
IS	13C6glucose	1MOX 5TMS
IS	nonadecanoic acid methyl ester	
IS	Succinic-d4-acid	2TMS
IS	valine-d8	1 TMS, 2TMS
lactone	Gluconic acid 1,4-lactone	4TMS
lactone	Gluconic acid 1,5-lactone	4TMS
nucleoside	Adenosin	3TMS, 4TMS
nucleoside	Adenosin 3,5-cyclic monoP	3TMS
nucleoside-mono-phosphate	Adenosin-5-P	5TMS
phenylpropanoid	caffeic acid	3TMS
phenylpropanoid	chlorogenic acid	6TMS
phenylpropanoid	ferulic acid	2TMS
phenylpropanoid	gallic acid	4TMS
phenylpropanoid	OH-trans-cinnamic acid	2 TMS
phenylpropanoid	p-coumaric acid	2 TMS
phenylpropanoid	sinapic acid	2TMS
phenylpropanoid	Syringic acid	2TMS
phenylpropanoid	trans-cinammic acid	1TMS
phenylpropanoid	vanillic acid	2TMS

Table A.4: (continued)

chemical class	substance	derivatives detected
phytohormone	1-Aminocyclopropane-1-carboxylic acid	1TMS, 2TMS
phytohormone	Indol-3-acetic acid (auxin)	1TMS, 2TMS
phytohormone	Jasmonic acid	1 TMS, 1MOX 1TMS (5 peaks)
phytohormone	Shikimic acid	4TMS
sterol	Ergosterol	1TMS
sterol	Sitosterol	1TMS
sterol	Stigmasterol	1TMS
sugar	Cellobiose	1MOX 8TMS BP, 1MOX 8TMS MP
sugar	D-Arabinose	1MOX 4 TMS
sugar	Fructose	1MOX 5TMS BP, 1MOX 5TMS MP
sugar	Galactose	1MOX 5TMS BP, 1MOX 5TMS MP
sugar	Glucose	1MOX 5TMS BP, 1MOX 5TMS MP
sugar	Maltose	1MOX 8TMS BP, 1MOX 8TMS MP
sugar	Melibiose	1MOX 8TMS (3 peaks)
sugar	Rhamnose	1MOX 4TMS BP, 1MOX 4TMS MP
sugar	Ribose	1MOX 4TMS
sugar	Saccharose (sucrose)	8TMS
sugar	Trehalose	8TMS
sugar	Xylose	1MOX 4TMS BP, 1MOX 4TMS MP
sugar alcohol	Adonitol (Ribitol)	5 TMS
sugar alcohol	D-(+)-Arabitol	5 TMS
sugar alcohol	Mannitol	6TMS
sugar alcohol	Myoinositol	5TMS, 6TMS
sugar alcohol	Pinitol	5TMS
sugar alcohol	Xylitol	5TMS
sugar phosphate	Myoinositol-P	7TMS
sugar phosphate	Erythrose-4-P	1MOX 4TMS BP, 1MOX 4TMS MP
sugar phosphate	Fructose-6-P	1MOX 6TMS
sugar phosphate	Glucose-6-P	6TMS
sugar phosphate	Ribose-5-P	1MOX 5TMS BP, 1MOX 5TMS MP
sugar phosphate	Ribulose-5-P	1MOX 5TMS BP, 1MOX 5TMS MP
triosephosphate	Dihydroxyacetonphosphate	1MOX 3TMS BP, 1MOX 3TMS MP

Appendix B

Glossary

ACC	1-aminocyclopropane-1-carboxylic acid
AMDIS	Automated Mass Spectral Deconvolution and Identification System
CE	capillary electrophoresis
DIOS	direct ionisation on silicon
DIMS	direct injection MS
DON	deoxynivalenol
FHB	*Fusarium* Head Blight
FT-IF	Fourier transform infrared
GC	gas chromatography
HCA	hierarchical cluster analysis
HS	headspace
IPTG	isopropyl-β-D-thiogalactopyranosid
KI	Kovats retention index
LB medium	lysogeny broth for cultivation of bacteria
LC	liquid chromatography
LDI	laser desorption ionisation
LTPRI	linear temperature-programmed retention index
MALDI	matrix-assisted laser desorption ionisation
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MOX	methoxyamine hydrochloride
MSTFA	N-methyl-N-trimethylsilyltrifluoroacetamide
NCWB	NIST Chemistry WebBook
NIL	near isogenic line
NMR	nuclear magnetic resonance
OD	optical density
PC	principal component
PCA	principal component analysis
QTL	quantitative trait locus
RI	retention index
RSD	relative standard deviation
SIM	single ion monitoring
SPME	solid phase microextraction
TIC	total ion current
TMS	trimethylsilyl group (attached during derivatisation)
XIC	selected ion current

Curriculum vitae

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Education

05/2009-2012 (expected):	University of Natural Resources and Life Sciences Vienna **PhD: Food Science and Biotechnology** PhD thesis: "Towards Gas Chromatography - Mass Spectrometry Based Metabolomics for The Study of *Fusarium* and Wheat"
10/2007-04/2009:	University of Natural Resources and Life Sciences Vienna **Master studies: Biotechnology** Master thesis: "Performance characteristics of different antibodies for the detection of food allergens" finished with distinction, original paper published in Analytical Bioanalytical Chemistry
10/2004-10/2007:	University of Natural Resources and Life Sciences Vienna **Bachelor studies: Food Science and Biotechnology** Bachelor thesis: "ELISA test kits for rapid analysis of mycotoxins" finished with distinction
10/2002-06/2005:	University of Music and Performing Arts Vienna **Preparatory course for guitar**

Work Experience in Austria

05/2009-11/2012:	PhD at the Center for Analytical Chemistry, IFA Tulln, part of FWF-project "SFB *Fusarium*" (metabolomics group)
07/2008-02/2009:	Master thesis at the Center for Analytical Chemistry, IFA Tulln, part of "Christian Doppler Laboratory for Rapid Test Systems for Allergenic Food Contaminants" of Ao.Univ.Prof. DI Dr.rer.nat. Sabine Baumgartner
2008:	student job at "Quantas Analytics" (since 2010 Romer Labs) in Tulln,; analytics of mycotoxins
08-09/2007:	trainee at "Quantas Analytics" in Tulln; analytics of mycotoxins
12/2006-06/2008; 07/2009:	students job at Café "Winzig", Tulln
09/2005:	trainee at the Center for Analytical Chemistry, IFA-Tulln, in the "Christian Doppler Laboratory for Mycotoxin Research Mod. Romer" of Univ. Prof. Dipl.-Ing. Dr. techn. Rudolf Krska
09/2004-09/2007:	private teacher for guitar, mathematics, latin, english, german (school level)

International Work Experience

07/2006-09/2006:	trainee in Singapore at "Romer Labs Singapore", development of rapid test system for mycotoxin analysis

Personal Skills and Competences

German:	mother tongue
English:	fluent (spoken and written)
French:	basic knowledge
EDV:	MS Office (Word, Excel, Powerpoint) Agilent Chemstation, MassHunter, AMDIS, MetaboliteDetector R (including Bioconductor for XCMS), SPSS, Matlab
Driving licence:	Austrian driving licence B

Activities and Interests

Music (guitar, piano, singing), volleyball.

Publications in journals (peer-reviewed)

2009:	Huber D. et al (2009) Anal Bioanal Chem 394 (2) 539-548 "Effectiveness of natural and synthetic blocking reagents and their application for detecting food allergens in enzyme-linked immunosorbent assays"

List of publications during the PhD

Publications

Schöfbeck D., Wiesenberger G., Fiebrich A., Kluger B., Weingart G., Adam G., Krska R., Schuhmacher R. HS SPME GC-MS Method for the Characterisation of the Volatile Metabolic Profile of *Fusarium graminearum* And Critical Assessment of Matrix Effects. (in preparation)

Book chapters

Kluger B., Zeilinger S., Wiesenberger G., Schöfbeck D., Schuhmacher R. (2012) Detection and identification of fungal microbial volatile organic compounds by HS-SPME-GC-MS. In Gupta V.K., Tuohy M., Ayychamy M., Turner K.M., O. Donovan A. (Eds): Laboratory Protocols in Fungal Biology: Current Methods in Fungal Biology, Springer ISBN: 978-1-4614-2355-3. In press.

Conference contributions

2012

Schöfbeck D., Neumann N., Kluger B., Lemmens M., Adam G., Wiesenberger G., Krska R., Schuhmacher R. (2012) GC-MS Based Metabolomics To Study *Fusarium* Head Blight. In: ASAC, 8. ASAC JunganalytikerInnen Forum 2012, O26, p.34 [Presentation]

2011

Schöfbeck D., Fiebrich A., Wiesenberger G., Adam G., Krska R., Schuhmacher R. (2011) Towards the Quantification of the Volatile Metabolome of *Fusarium graminearum* PH-1 using HS SPME GC-MS. In: Metabolic Profiling Forum and VTT Technical Research Center of Finland, Metabomeeting 2011, SEP 25-28, 2011, Helsinki, FINLAND [Poster]

2010

Schöfbeck D., Wiesenberger G., Adam G., Krska R., Schuhmacher R. (2010) Metabolic Profiling of Volatiles of *Fusarium graminearum* by GC-MS. In: ALVA Wien Dr. Andreas Adler (Hrsg.), ALVA-Mitteilungen Heft 8/2010; ISSN 1811-7317 [Poster]

Schöfbeck D., Adam G., Wiesenberger G., Kluger B., Krska R., Schuhmacher R. (2010) Metabolic Profiling of Volatiles of *Fusarium graminearum* PH1 wild-type and $\Delta tri5$ Mutant. In: University of Technology Vienna, Austria, 4th Central and Eastern European Proteomics Conference meets International Metabolomics Austria, page 84 [Presentation]

Schöfbeck D., Weingart G., Lemmens M., Krska R., Schuhmacher R. (2010) Profiling of Volatiles of Wheat Ears Using Headspace SPME-GC-MS. In: The Munich Functional Metabolomics Initiative ZIEL Research Center for Nutrition and Food Sciences Helmholtz Zentrum München, Metabolomics and more: The Impact of Metabolomics on the Life Sciences, P140 [Poster]

Students

Fiebrich Alexandra, master student at FH Tulln, co-supervised during project and master thesis from 10/2010-05/2011

Köstlbauer Katharina, diploma student at TU Wien, co-supervised during diploma thesis 10/2009-06/2010