Thesis of Dissertation

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Early detection of mushroom disease-related moulds using their volatile secondary metabolites

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I. Introduction

Production of bisporic bottom mushroom is a dynamically developing sector not only in Hungary, but all over the world. To achieve excellent quality, it is necessary to produce good quality raw material, the so-called mushroom compost. The compost consists of wheat straw, horse -and poultry manure, gypsum and water. During complex biological processes, this mixture is turned into compost. After heat treatment, the compost is inoculated with mushroom mycelium, which grow fleshy and edible fruit bodies. The last phase of mushroom cultivation is the harvesting. It is easy to see that not inappropriate quality of the compost will result yield loss and even the total loss of the crop.

Decreased quality can be caused by different infections presented on the compost, which should be identified as early as it possible. Besides maintaining the quality, it is also important to prevent or avoid these infections on the compost because bisporic bottom mushroom is very sensitive to different diseases (such as viruses, bacteria or moulds) and pests.

Early detection of mould infections is crucial to keep the good quality of the compost and therefore the mushroom. Today, there isn't a single online analytical system which would be able to identify mould infections without direct sampling of the batch. A possible solution could be the solid-phase microextraction (SPME) which enables to detect the presence of mould directly from the air above the sample.

SPME sampling is usually followed by some kind of analytical system. When the aim is to analyze the volatile organic compounds of the headspace, gas chromatography – mass spectrometry (GC-MS) is a good choice to couple with SPME. The main advantage of SPME-GC-MS coupled system is its fast and simple usage, moreover it gives a detailed picture about the microbial volatile organic compounds (MVOCs) produced by different mould species. In order to identify different species based on their MVOC patterns, an online database is needed which contains all the marker compounds which are able to clearly describe the microorganisms or determine the exact date of the infection, too. Furthermore, such a database can be broadened with volatile compounds of other species which enables it to use in different fruit storages and modified atmosphere storages to detect microbial infections.

II. MAIN OBJECTIVES

I aimed to **1)** explore the volatile biomarkers of mushroom diseases-related mould species, **2)** analyze the volatiles of the most frequently detected disease (*Trichoderma aggressivum* f. *europaeum*) of mushroom production on different carbohydrate substrata and **3)** implement a fast statistical technique to differentiate mushroom diseases-related mould species.

1. The major aims, during the method development to monitor and detect the volatile biomarkers of mushroom disease-related mould species, were the following:

- Development of a fast analytical method to detect the volatile components of *Trichoderma aggressivum* f. *europaeum*; choosing the proper retention time standards and development of their fit for purpose.
- Using the created method, determination and monitoring of microbial volatile organic compounds (MVOCs) of mushroom disease-related moulds and prediction of their infection based on their MVOC pattern.
- Differentiation of mould species based on multivariate statistical methods.

2. The major aims, during the analysis of the volatiles of *Trichoderma aggressivum* f. *europaeum* on different carbohydrate substrata, were the following:

- Mapping the MVOC pattern of *T. aggressivum* and analysis of its growth on different media.
- Determination and identification of volatile secondary bimarkers of *T. aggressivum* on different media; and creating a methodology for the proper identification of components having different intensity values.
- Modification of the mushroom compost-containing agar in order to identify further biomarkers.

3. The major aims, during the implementation a fast statistical technique to differentiate mushroom diseases-related mould species, were the following:

- Application of chemometric methods (DFA, PCA, CA) to differentiate the analyzed moulds in order to substitute the traditional, often time consuming evaluation methods.

III. MATERIALS AND METHODS

3.1. Microorganisms and samples

Four different mushroom disease-related mould were examined: *Mycogone perniciosa* (wet bubble disease), *Lecanicillium fungicola* (dry bubble disease), *Trichoderma aggressivum* f. *europaeum* (green mould disease) and *Trichoderma* DOFE (green mould disease, exact species unknown), which was isolated by a mushroom grower. Bisporic button mushroom (*Agaricus bisporus* A15) was also examined for comparative purposes (comparison of volatile organic compounds emitted by button mushroom and its main mould diseases). The microorganisms were provided by the Corvinus University of Budapest, Faculty of Horticultural Science, Department of Vegetable Growing. In order to test the applied statistical methods (DFA, PCA), highly different samples were also included (which will be referred as outliers in the following): commercially available red wine (Sauska Siller, Villány/2011) and mushroom compost (Biofungi Kft.).

3.2. Substrata

Mould samples and button mushroom were inoculated on PDA (*potato-dextrose agar*, 39 g/L concentration) medium under sterile box (Figure 1.). The substrata contained potato-peptone (4 g/L), glucose (20 g/L) and agar (15 g/L). The open-air inoculations were placed into a container, having 0.5 L closed airspace.

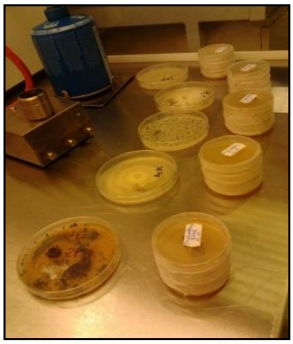

Figure 1.: The five different samples and its three parallel inoculations (Picture taken by the author)

Green mould was inoculated on four different substrata in a new experiment, where the substrata were also manufactured at Corvinus

University of Budapest, Faculty of Horticultural Science, Department of Vegetable Growing. Green mould was inoculated on MEA (malt extract agar), PDA (potato-dextrose agar) and on two nutrient-decreased media (KA: compost agar and WA: water agar). In the second part of experiment, modified dried mushroom compost agar with different carbohydrates (dextrose, maltose and mannitol) were used. Substrata were made according to the recipe in the Table 1. The samples were analyzed in three parallel measures and control samples (media without mould inoculation) were also measured.

Table 1.: List of substrata ingredients for 500 mL media. MEA (malt extract agar), PDA (potato-dextrose agar), KA (compost agar), WA (water agar)

Name	Ingredient		Agar	Distilled water
MEA	7.5 g malt extract		8.0 g	500 mL
PDA	19.5 g PDA agar			500 mL
KA	5.0 g dried mushroom compost		8.0 g	500 mL
WA	-		8.0 g	500 mL
KA + dextrose	5.0 g dried compost	1.0 g dextrose	8.0 g	500 mL
KA + maltose	5.0 g dried compost	1.0 g maltose	8.0 g	500 mL
KA + mannitol	5.0 g dried compost	1.0 g mannitol	8.0 g	500 mL

3.3. SPME sampling

To extract the volatile compounds from the headspace three different fibers were used; 100 µm PDMS (polydimethylsiloxane), 65 µm PDMS/DVB (polydimethylsiloxane/divinylbenzene) and 85 µm PA (polyacrylate). The process of headspace sampling is shown on Figure 2.

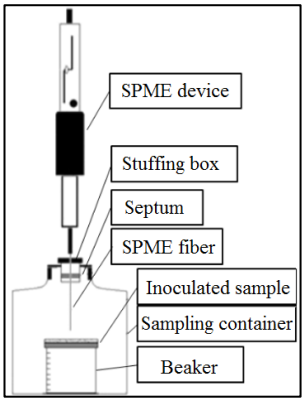

Figure 2.: Schematic figure of headspace sampling

0.3 L red wine and 0.1 kg mushroom compost were placed into a sampling container for 24 hours in order to capture volatiles from the headspace above outlier samples. The equilibrium time was set to 1 day. The samples were also analyzed in analytical (parallel measures) and biological (parallel cultures) replicates (Figure 3.). The temperature of the sampling system was maintained at 23 °C and the extraction time was set to 15 minutes.

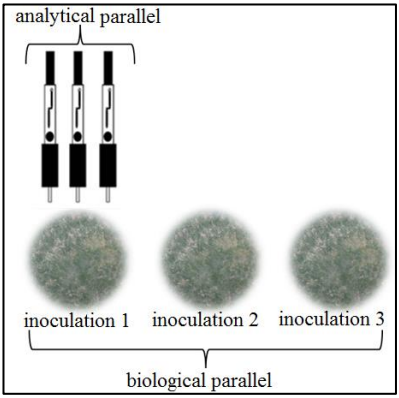

Figure 3.: Differences between analytical and biological parallel measures

3.4. Retention time standards

Different alkane standards; n-heptane (C7: C_7H_{16}), n-nonane (C9: C_9H_{20}), tetradecane (C14:$C_{14}H_{30}$), pentadecane (C15: $C_{15}H_{32}$) and heptadecane (C17: $C_{17}H_{36}$) were used as standards to maintain the retention time during measures.

3.5. Instrumental parameters

An Agilent 6890 Gas Chromatograph coupled with a 5975 C MSD Mass Spectrometer was used to analyze volatile organic compounds. A non-polar HP-5MS ((5 %-phenyl)-methylpolysiloxane) capillary column was used, which column is excellent for active and volatile compounds. The split valve was closed during desorption period and the inlet temperature was held at 250°C. Optimished column program was used; the program began at 50 °C and increased to 150 °C at 20 °C/min, then to 170 °C at 40 °C/min, to 190 °C at 25 °C/min, to 280 °C at 40 °C/min, and finally to 300 °C (2 min hold) at 50 °C/min. Hydrogen 6.0 was used as a carrier gas with a constant 1.2 mL/min flow to accelerate the separation on the column.

The mass fragments were detected by quadrupole MS, where the ion source temperature was set to 230 °C and the quadrupole temperature was held at 150 °C. Positive electron ionization (EI+) was used, with an electron energy level of 70 eV. The MS was tuned using perfluorotributylamine (PFTB) every day before the measurements. The transferline between GC and MS were held at 300 °C (equal at final column temperature). Mass-to-charge ratio (m/z: mass number/charge number) was examined between 33-500 m/z value.

3.6. Applied software

Agilent Enhanced MSD ChemStation software handled the GC and MS parameters. Agilent Enhanced Data Analysis and Agilent MassHunter Qualitative Analysis B.06.00 software were used for evaluation (deconvolution, background correction) and comparison of the chromatograms. The collected spectra were analysed using NIST Mass Spectral Search Program (NIST 2011, Wiley 10th edition). XL-Stat statistical software was used for principal component analysis, linear discriminant analysis and partial least square regression model. Sliding window DFA was carried out using the Physionet statistical software, other computations were carried out using R-project 3.0.2 stats and cluster packages.

IV. RESULTS

4.1. Method development to monitor and detect the volatile secondary metabolites of mushroom disease-related mould species

Trichoderma aggressivum f. europaeum mould was used for the optimization of the chromatographic separation. After optimization of the temperature program, finally a nearly 11 minutes long, effective chromatographic separation was achieved. Three different SPME fibers were examined to capture microbial volatiles, and finally the 65 μm PDMS/DVB fiber proved to be the most efficient.

The determination of the optimal sampling time was evaluated between 10 and 50 minutes. The results showed that the equilibrium set after 30-40 minutes sampling, but to ensure fast analysis, a shorter, 15 minutes long sampling time was accepted. The qualitative analysis of the major volatile compounds can be done after 15 minutes, too.

To ensure quality control, retention time standards were used to maintain the chromatograms. Four standards were chosen (n-nonane, tetradecane, pentadecane and heptadecane), which eluted on a component-poor section of the chromatogram. The retention standards were added by taking advantage of the memory effect of the sampling system, so there was no need to add standards to the samples. This technique ensured the presence of the retention standards on the chromatograms for approximately four days.

4.2. Determination of the volatile biomarkers of the mushroom disease-related moulds

The developed method enabled to map and identify the volatile biomarkers of mushroom disease-related moulds (*Trichoderma aggressivum*, *Mycogone perniciosa*, *Lecanicillium fungicola*). The next step was to determine the maker compounds, which clearly describe the species one-by-one. Marker compounds can clearly characterize the given microorganism, so they appear on the chromatogram only, when the given mould is present (Figure 4.). Table 2 lists the found marker compounds of the mushroom disease-related moulds.

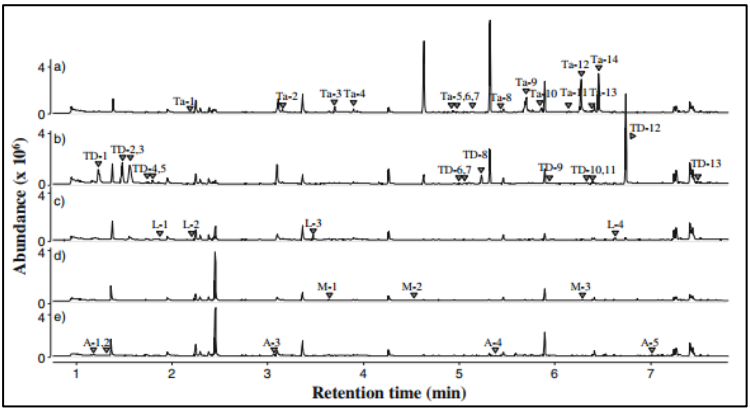

Figure 4.: Comparison of total ion chromatograms showing MVOC patterns after HS-SPME-GC-MS analysis on the third day. (a) *T. aggressivum*, (b) *T.* DOFE**, (c) *L. fungicola*, (d) *M. perniciosa*, (e) *A. bisporus*. Marked compounds are identified in the "Peak ID" column in Table 2. **Green mould, which was isolated by a mushroom grower, exact species unknown

Table 2.: Identification of marker compounds. Volatile marker compounds of mushroom disease-related microorganisms, which indicate mould presence. Moulds were successfully characterized using these biomarkers. (ID %: percentage of identification according to NIST software). Abbreviations in the column of "Peak ID" refer to the given microbial species: Ta: *Trichoderma aggressivum f. europeaum*, TD: *Trichoderma* DOFE, L: *Lecanicillium fungicola*, M: *Mycogone perniciosa*, A: *Agaricus bisporus*.

Sample	Peak ID	t_R (min)	Compound name
Trichoderma aggressivum f. europaeum	**Ta-1**	2.21	*unknown*
	Ta-2	3.41	D-*limonene*
	Ta-3	3.70	*4-nonanone*
	Ta-4	3.84	*2-nonanone*
	Ta-5	4.93	*(3*E*)-3-ethylidene-3a-methyl-2,4,5,6,7,7a-hexahydro-1*H*-indene*
	Ta-6	4.98	*1,1,4,4-tetramethyl-2,5-dimethylene-cyclohexane*
	Ta-7	5.12	*2-ethylidene-1,7,7-trimethylbicyclo[2.2.1]heptane*
	Ta-8	5.43	*1,6,6-trimethyl-7-(3-oxobut-1-enyl)-3,8-dioxatricyclo[5.1.0.0(2,4)]octan-5-one*
	Ta-9	5.70	n-*decanoic acid*
	Ta-10	5.86	*furfuryl alcohol*
	Ta-11	6.15	*(*E*)-5,8,11,14,17-eicosapentaenoic acid; (icosapent)*
	Ta-12	6.27	*6-pentyl-2*H*-pyran-2-one*
	Ta-13	6.37	*5,6-dihydro-6-pentyl-2*H*-pyran-2-one*

	Ta-14	6.46	*tetrahydro-6-pentyl-2*H*-pyran-2-one*
	Ta-15	8.31	*unidentified diterpenoid*
	Ta-16	9.01	*unknown*
Trichoderma* DOFE*	**TD-1**	1.23	*2-methyl-1-propanol*
	TD-2	1.48	*3-hydroxy-2-butanone (acetoin)*
	TD-3	1.56	*3-methyl-1-butanol*
	TD-4	1.75	*unidentified alcohol (butanediol)*
	TD-5	1.80	*2,3-butanediol*
	TD-6	5.00	*unidentified pyran compound*
	TD-7	5.10	*cycloocta-2,4-dien-1-ol*
	TD-8	5.23	*(10*R*)-10-methyl-2-oxecanone*
	TD-9	5.92	*(*R*)-1-ethenyl-1-methyl-2,4-bis-(1-methylethenyl)cyclohexane*
	TD-10	6.24	*(*E*)-12-methyloxacyclododec-9-en-2-one*
	TD-11	6.33	*(Z)-8-methyl-9-tetradecenoic acid*
	TD-12	6.74	*3,4-dihydro-8-hydroxy-3-methyl-1*H*-2-benzopyran-1-one; (3,4-dihydro-8-hydroxy-3-methylisocoumarin)*
	TD-13	7.49	*[3*R*-(3α,3aβ,7β,8aα)]-2,3,4,7,8,8a-hexahydro-3,6,8,8-tetramethyl-1*H*-3a,7-methanoazulene (cedr-8-ene)*
Lecanicillum fungicola	**L-1**	1.84	*cyclopentanone*
	L-2	2.22	*2-dodecanone*
	L-3	3.47	*methyl 2-ethylhexanoate*
	L-4	6.63	*unknown*
Mycogone perniciosa	**M-1**	3.59	*unidentified alkane compound*
	M-2	4.49	*unknown*
	M-3	6.23	*unknown*
Agaricus bisporus	**A-1**	1.15	*acetic acid*
	A-2	1.32	*1-chlorobutane*
	A-3	3.06	*3-octenone*
	A-4	5.59	*1,2,3-triacetoxypropane (triacetin)*
	A-5	7.02	*3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid)*

** Green mould, which was isolated by a mushroom grower, exact species unknown

4.3. Monitoring of the volatile biomarkers of the mushroom disease-related moulds

After inoculation, hyphae germinate from the spore and grow radially to form a circular growth of mycelium. As soon as the mycelium reach the end of substrata, the production of spores starts due to the reduction of necessary nutrients and hence the changed environmental conditions. Marker compounds can be used to determine in which of the above mentioned phases is a certain mould.

Monitoring of the markers showed that most of the marker compounds of *A. bisporus* show an increasing tendency. *M. perniciosa* and *L. fungicola* emitted the most marker compounds at the 3rd and 4th days of sampling. After

the 7th day, emission of markers decreased or stopped. These marker compounds are emitted when the moulds are in the mycelium growing phase (Figure 5. g) h)). *Trichoderma* species show different tendency. Most of the markers of *T. aggressivum* are emitted from the 7th day (Figure 5. b) c)). Markers are associated with appearing of green coloured spores by the colonies.

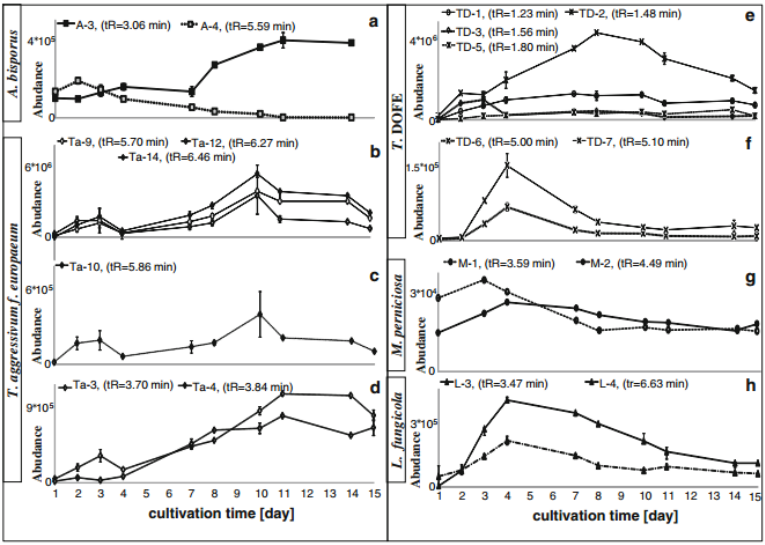

Figure 5.: Variation of metabolite expression of the studied species during cultivation period of 15 days. Compounds are identified in the "Peak ID" column in Table 2.

Determination of the marker compounds of the moulds is enough to differentiate mould species in most of the cases. But in those cases when there is not or only there is a few marker compounds are present, chemometric methods are needed to differentiate the species.

In the first step, principal component analysis (PCA) was used to differentiate the samples (using 57 common compounds' integrated area under the curve values).

Prior to the analysis, the prerequisites of PCA were checked. The first three principal components (PCs) explained 76.51 % of the total variance. PC1 explains the differences between the inoculation days while PC2 explains the differences between the samples. The third PC describes also the differences between the samples which makes the differentiation even more

detailed. Analysis of the major contributors of the PCs showed that components coming around the 7th min. are responsible for the differences between the samples. PCA was not able to completely differentiate the samples but it is known, that the major aim of the method is to describe the compounds responsible for the differences between the samples and the inoculation days.

In the second step, linear discriminant analysis (LDA) was used to differentiate the samples and the inoculation days. First, the classification of the species was done (Figure 6). The classification was successful in most of the cases, but one case (*M. perniciosa*, on the first day) was incorrectly classified as *Agaricus bisporus* (bisporic bottom mushroom).

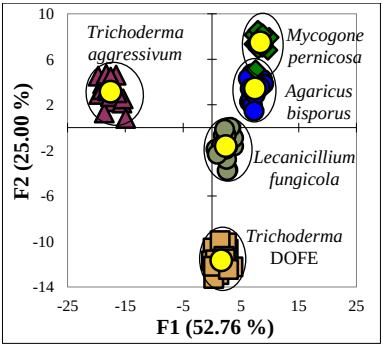

Figure 6.: Sample differentiation using LDA. F1, F2: first and second discrimination function

Secondly, LDA was used to classify the sample based on their inoculation day. According to the results, the inoculation day can be well classified until the fourth day. After that the model was unable to differntiate inoculation days of microorganisms.

4.4. Prediction of inoculation day using PLS-R model

Partial least squares regression (PLS-R) was used to predict the inoculation day of the analyzed samples. In the first step, a training data set was determined which was used to build the PLS-R model. In this case the model predicts the inoculation day and the exact day of inoculation is known; hence their difference can be calculated which gives the error of the model (mean square error: MSE and root mean square error: RMSE). Then, the model was run on a so-called prediction data set and the same indicators can be calculated (mean square error of the prediction: MSEP and root mean

square error of the prediction: RMSEP). The parameters and results of the PLS-R model is summarized by Table 3.

Table 3.: Parameters and root mean square error of prediction of PLS regression models for the mould species and *Agaricus bisporus*. R^2: coefficient of determination, MSE: mean square error, RMSE: root mean square error, RMSEP: root mean square error of prediction, $n_{(training)}$: number of samples in the training set, $n_{(prediction)}$: number of samples in the prediction set, LV: number of latent variables

Samples	R^2	MSE	RMSE (day)	RMSEP (day)	$n_{(training)}$	$n_{(prediction)}$	LV
Agaricus bisporus	0.992	0.117	0.306	0.342	16	7	3
Lecanicillium fungicola	0.991	0.187	0.432	0.654	20	9	5
Mycogone perniciosa	0.901	1.897	1.377	1.381	17	8	2
Trichoderma aggressivum	0.979	0.417	0.646	0.651	18	10	2
Trichoderma DOFE	0.983	0.334	0.578	0.771	18	8	3

The model gave high R^2 and low MSE values. The RMSE values give the error of prediction of the day of inoculation. The training set showed similar results (RMSE) compared to the prediction set (RMSEP), and there is strong positive correlation between them, which indicates that the model is able to predict the inoculation day of unknown samples.

4.5. Analysis *Trichoderma aggressivum* growth

The emitted volatile compounds of *T. aggressivum* was monitored on different media. The study was done for seven days and the changes during the growth of the green mould were evaluated. After initial mycelium growth, green spores appeared at the edge of the mycelium lawn and finally the spores suffused the white mycelium lawn. The mycelium growth on nutrient-decreased media was negligible, however the production of green spores was faster in this mycelium spots (Figure 7.).

Figure 7.: *Trichoderma aggressivum* at the 6th inoculation day. Substrata from left to right: PDA (potato-dextrose agar), KA (compost agar), MEA (malt-extract agar), WA (water agar) (Picture taken by the author)

4.6. Identification of volatile biomarkers of *Trichoderma aggressivum*

The components obtained from manual evaluation of the chromatograms were grouped based on their intensity values (Figure 8), and different methodologies were created for the proper evaluation of these compounds.

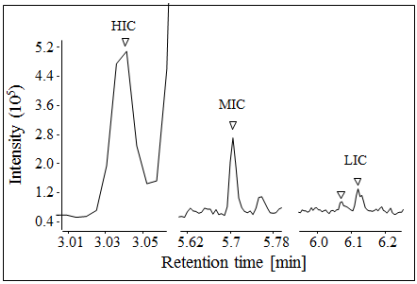

Figure 8.: High- ($\geq 4*10^5$), medium- ($1,2\text{-}3*10^5$) and low ($\leq 10^5$) intensity compounds. HIG: High Intensity Compounds, MIG: Medium Intensity Compounds, LIG: Low Intensity Compounds

High intensity compounds can be easily identified using mass spectrum libraries (for example NIST library). During the identification, reliable results were obtained. Background correction was needed in the case of medium intensity compounds. After background correction the examined compound mass spectrum is not disturbed by other compounds' fragments, therefore clean compound mass spectrum is achievable. The obtained background-free mass spectrum can be properly identified using mass spectrum libraries. Low intensity compounds caused problem in most of the cases. Manual deconvolution was used to found these compounds. During background

correction it could be a problem that the fragment ions of the examined compounds do not have much higher intensity values than the interfering background fragment ions, thus examined compound fragment ions could be lost from the mass spectra during background subtraction. The examined compounds were inspected and confirmed with a fourth parallel measure.

4.7. Analysis of the marker compounds of *Trichoderma aggressivum*

In the first step, the MVOC pattern of *Trichoderma aggressivum* was analyzed on all different substrata. As a result, 150 compounds were found, from which 44 was classified as markers of *T. aggressivum* (Table 4.). Three compounds were found from the chosen 44 biomarkers, which appeared in case of inoculated microorganism on all four different substrata. More common biomarkers (18 compounds) were found on nutrient-rich substrata (PDA, MEA). As a results, 21 (18+3) compounds were determined as common biomarkers of *T. aggressivum*. Moreover, 10 marker compounds were found during *T. aggressivum* growth on PDA substrata and 13 marker compounds on MEA substrata. These will be referred as substrata-dependent biomarkers in the following.

Table 4.: Differentiation of biomarkers according to substrata (MEA, PDA, KA, WA). *marks the unknown compound which occurred not only in the case of MEA and PDA but in the case of WA also.

Marker compounds			
	Substrata-dependent markers	**MEA-PDA markers (mycelium growth)**	**Substrata-independent markers**
PDA	*2-propanone; isobutyl chloride; 1-propanol, 2-methyl; longifolene; unknowns (t_R=5.38; 5.48; 5.58; 5.76; 5.81; 5.84 min)*	*octane; adamantan-2-ol, 4-bromo-, cis; alpha-humulene; cedr-8-ene; beta-cubebene; beta-copaene; beta-ylange; patchoulane; (5Z)-5-pentadecen-7-yne; ledane; gamma-elemene; (2Z,6E)-farnesol; unidentified $C_{15}H_{26}$ derivatives (t_R=6.30; 6.33 min);unknowns (t_R=3.10; 3.36*; 6.26; 7.67 min)*	*1,2-dimethyl benzene; 3-octanone; 2-pinen-4-ol*
MEA	*benzeneethanamine; tyrene; tetracyclo[5.3.1.1(2,6).0(4,9)]dodecane; beta-caryophyllen; 8,11-octadecadiynoic acid, methyl ester; 4-(2,2-dimethyl-6-methylenecyclohexyl)butanal; unidentified $C_{15}H_{26}$ derivatives (t_R=4,68 min);unknowns (t_R=4.33; 4.61; 5.15; 6.16; 6.48; 7.32 min)*		
KA			
WA			

After successful identification, compounds intensity changes were tracked (Figure 9.).

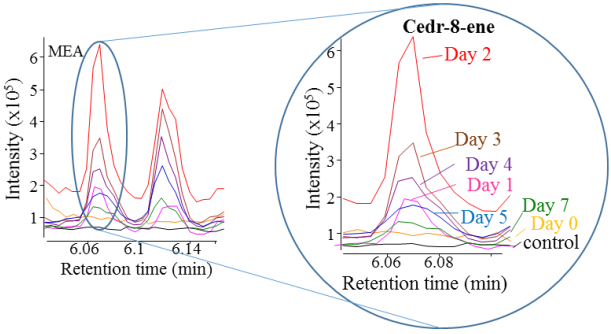

Figure 9.: Intensity changes of cedr-8-ene compound according to the examined days on MEA substrata.

Marker compounds were grouped according to their intensity change tendencies (monotone increasing, showing intensity maximum, monotone decreasing or having fluctuating intensity change tendency). The microorganisms' growth was also examined during the monitoring (Figure 10).

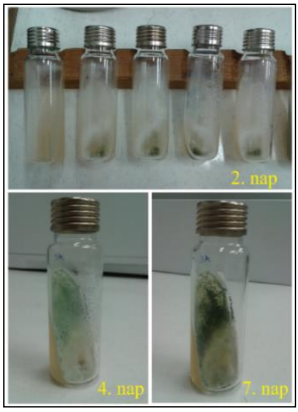

Figure 10.: Green mould growth on MEA (Picture taken by the author)

The marker compounds belonging into different group appeared in different growth phase of the microorganism. Based on these, it can be said that the volatiles emitted in the first days were produced during the mycelium growth phase of green mould. Compounds having monotone increasing tendency may refer to spore production especially those which intensity value were jumped from the third day.

4.8. Analysis of marker compounds on modified compost agar

Dried mushroom compost agar was enriched with different sugars (mannitol, maltose, dextrose), and *T. aggressivum* was inoculated on this medium again and the emitted MVOCs were also analyzed.

Instead of the expected fast growth, green spores appeared again. It can be said that the mould, inoculated on the modified media, emitted two more compounds (*tyrene* and *3-octanol*), which can be clearly connected to the presence of *T. aggressivum*; hence these are its biomarkers.

4.9. MVOC database of mushroom disease-related mould species

Using HS-SPME-GC-MS coupled technique, a database has been created which contains the biomarkers of the given microorganisms. Application of this database in future measures helps the indication or identification of infections. Database broadening, by adding new compounds and species, requires time consuming evaluation process hence the substitution of these evaluation processes are valuable.

4.10. Application of detrended fluctuation analysis to differentiate mushroom disease-related mould species

According to the traditional way of chromatogram evaluation, a visual comparison of the TIC chromatograms is the first step (Figure 11) and the aim is to identify those components which are responsible for the differences between the samples. Next step is the evaluation process, in which the differences between the samples are analyzed and explored.

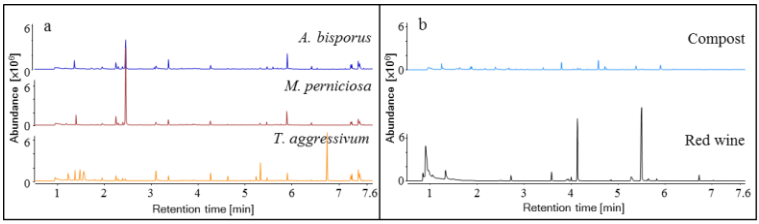

Figure 11.: a) Total Ion Chromatograms (TICs) of the analyzed samples (*A. bisporus, M. perniciosa, T. aggressivum*); b) TICs of the outliers (commercially available red wine and mushroom compost)

Using only visual comparison, marker compounds on Figure 11 cannot be properly defined, therefore sample identification and distinction is not really possible. Distinction of samples generally requires time consuming feature extraction. To reduce analysis time, different statistical methods were used to distinguish mould infection via their unprocessed TICs. In the first step, detrended fluctuation analysis (DFA) was applied to analyse the data. Since DFA is an unconventional method, its results need to be validated by principal component analysis (PCA). Two-dimensional plots can be achieved by both DFA and PCA but since the visual comparison of these plots is not reliable enough, cluster analysis (CA) was run on the PCA scores and DFA

coefficients to simplify the results. The dendrograms provide enough information to compare the methods and validate DFA. (Figure 12).

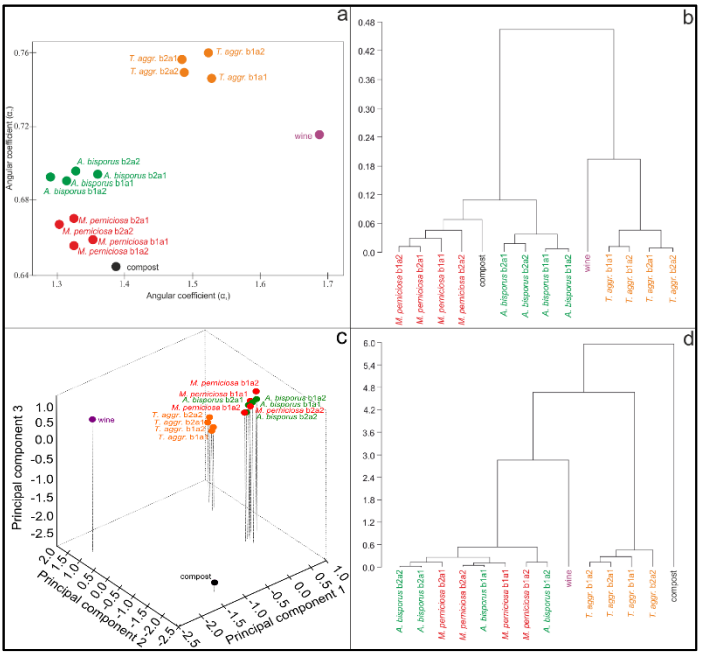

Figure 12.: Position of the samples in the two-dimensional space created by α_1 α_2 exponents of detrended fluctuation analysis (‘a’) and in the three-dimensional space created by the scores of the principal component analysis (‘c’). Dendrogram of the α_1 α_2 exponents of detrended fluctuation analysis (‘b’) and cluster analysis of the scores of the PCs (‘d’). “b” refers to the biological while “a” refers to the analytical replicates.

It can be seen that DFA differentiated the samples properly, while PCA was not able to distinguish *A. bisporus* and *M. perniciosa* samples. Therefore, DFA is suitable to differentiate samples based on their raw, unprocessed TIC chromatograms. These results enable us to create fast differentiation of samples, even online, which substitutes the time consuming, often complicated evaluation processes.

V. NEW SCIENTIFIC RESULTS

1) I have created a fast, headspace solid-phase microextraction – gas chromatography mass spectrometry-based technique (HS SPME GC MS) which is able to indicate the presence of moulds in mushroom compost and mushroom production facilities. During the method development, I introduced a new process, namely the application of memory effect-based retention time standard.

2) I have defined the microbial volatile organic compounds (MVOCs) of three mushroom disease-related moulds (*Trichoderma aggressivum*, *Mycogone perniciosa and Lecanicillium fungicola*), furthermore the MVOC pattern was defined *in vitro* circumstances. I created a PLS-R prediction method which is able to predict the inoculation day of a mould infection based on the MVOC pattern.

3) I have defined the major mushroom disease-related mould (*Trichoderma aggressivum* f. *europaeum*) substrata-dependent, fingerprint-like MVOC pattern, which was emitted during its growth. Furthermore, I mapped its MVOC emission on different carbohydrate enriched media.

4) I have defined the marker compounds (around 80 compounds) of three mushroom disease-related moulds (*Trichoderma aggressivum*, *Mycogone perniciosa* and *Lecanicillium fungicola*). These biomarkers clearly describe the presence of the examined mould. After this, I created a database consisting of microbial volatile organic compounds, which helps to identify the presence of moulds from the headspace in case of a similar experimental setup.

5) I have created a new process which differentiates the analyzed mould species based on their unprocessed (raw) total ion chromatograms. I have concluded that using time series analysis, the samples can be differentiated without time consuming, often complicated traditional evaluation processes. Hence, the created statistical process is able to be integrated into continuous online monitoring systems.

VI. CONCLUSIONS AND SUGGESTIONS

In my PhD work, I aimed to develop an analytical method, which is able to detect mushroom disease-related moulds from samples' headspace. Knowing the characteristic mould markers, they can be identified, differentiated, classified using chemometric methods or their inoculation day can be also predicted. Using analytical methods based on exact mass measurements the method can be improved. Equipments having high resolution can be also involved to the measures, thus the precision of the identification can be increased.

During my work, I have created a database which can be integrated into online systems. This can be applied to indicate the presence of moulds by monitoring of the headspace of the compost. By broadening the database, the presence of moulds can be detected on other fields in the food industry, too.

The developed HS-SPME-GC-MS method may be able to detect different food frauds. For example, during the production of noble wines, the *Botrytis cinerea* mould is necessary on the grapes. In case of inappropriate environmental conditions, *Botrytis cinerea* cannot be present on the fruit, therefore, the quality of the noble wine is questionable in the given year. Furthermore, another filed of application could be the distilling industry. Using this coupled analytical technique, the spirits made of mould infected fruits could be easily identified.

Moreover, this method is not only applicable in food industry but also in the detection of indoor moulds. The indoor moulds causing so-called sick building syndrome (SBS) can also be examined. The method can be extended from food analysis to environmental monitoring, thus several harmful diseases could be prevented such as asthma, allergic reactions or headache.

The HS-SPME-GC-MS coupled analytical system raised the attention of medical sciences, too. The most recent studies focus on the detection of different microorganisms from breath and urine proliferating in and on human body. Analysis of breath enables to identify microorganisms proliferated in throat, pharynx, stomach or in the digesting system connecting them. In this way, diabetes or tooth decay can be detected. Different microorganisms causing dermatophytoses (*e.g. Trichophyton rubrum* or *Candida intertrigo*) could be also indicated after creating a proper and reliable database.

PUBLICATIONS

Journals with impact factor:

1) Radványi D., Gere A., Sipos L., Kovács S., Jókai ZS., Fodor P. (2016): Discrimination of mushroom disease-related mould species based solely on unprocessed chromatograms. Journal of Chemometrics, Volume: 30 Issue: 4, Special Issue, Pages:197–202. DOI: 10.1002/cem.2777 (**IF: 1,50**)

2) Radványi D., Gere A., Jókai ZS., Fodor P. (2015): Rapid evaluation technique to differentiate mushroom disease-related moulds by detecting microbial volatile organic compounds using HS-SPME-GC-MS. *Analytical and bioanalytical chemistry, Volume: 407 Issue: 2 Pages: 537-545. DOI: 10.1007/s00216-014-8302-x* **(IF=3,659)**

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Conferences–Hungarian conference abstracts:

1) Radványi D, Jókai Zs és Fodor P (2013) Gombakomposzt illékony vegyületeinek vizsgálata HS-SPME-GC-MS technikával, 2. Környezetkémiai Szimpózium, Dobogókő

2) Radványi D, Csordás Zs, Jókai Zs, Fodor P (2014) Gombakomposztot károsító mikroorganizmusok vizsgálata HS-SPME-GC-MS technikával, Aktualitások a táplálkozástudományi kutatásokban című workshop, Budapest

3) Radványi D., Jókai Zs., Fodor P., DB. Chu, S. Hann (2014) Különböző beltéri penészek által kibocsátott szerves illékony vegyületek vizsgálata HS-SPM- GC-QTOF-MS kapcsolt analitikai technikával, 3. Környezetkémiai Szimpózium, Lajosmizse

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1) Radványi D. (2014) Mikrobiális eredetű illékony vegyületek vizsgálata HS-SPME-GC-EI/CI-qTOFMS kapcsolt analitikai rendszerrel, MTA-KÉT Élelmiszertudományi Albizottsági ülés, Budapest

Conferences – International conference abstracts:

1) D. Radványi, Zs. Jókai, P. Fodor, DB. Chu, K. Sterflinger-Gleixner, S. Hann (2014) Analysis of microbial volatile organic compounds of different indoor fungi using HS-SPME combined with accurate mass GC-EI/CI-qTOFMS, ASAC-Junganalytikerinnenforum, Tulln (Ausztria)

2) D. Radványi, A. Gere, L. Sipos, S. Kovács, Zs. Jókai, P. Fodor (2015) Discrimination of mushroom disease-related mould species based solely on unprocessed chromatograms, Conferentia Chemimetrica, Budapest (Magyarország)

3) D. Radványi, L. Juhász, Zs. Jókai, A. Geösel, P. Fodor (2015) Analysis of microbial volatile organic comounds emitted by Trichoderma aggressivum growing on different substrata, 7th International Symposium on Recent Advances in Food Analysis, Prague, (Csehország)

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1) L. Juhász, **D. Radványi**, Zs. Jókai, P. Fodor (2015) Determination of volatile metabolite markers using HS-SPME-GC-MS technique, 21st International Symposium on Analytical and Environmental Problems, Szeged (Magyarország)

2) D. Radványi, Zs. Jókai, P. Fodor (2015) Ionization techniques of volatile compounds. CI vs EI, 21st International Symposium on Analytical and Environmental Problems, Szeged (Magyarország)

Citations (full list):

1) Radványi D., Juhász R., Kun Sz., Szabó-Nótin B., Barta J. (2012): Preliminary study of extraction of biologically active compounds from elderberry (*Sambucus nigra* L.) pomace. *Acta Alimentaria, Volume: 42 Pages: 63-72.*

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2) Radványi D., Gere A., Jókai ZS., Fodor P. (2015): Rapid evaluation technique to differentiate mushroom disease-related moulds by detecting

microbial volatile organic compounds using HS-SPME-GC-MS. *Analytical and bioanalytical chemistry, Volume: 407 Issue: 2 Pages: 537-545.*

Sun D., She J., Gower LJ., Stokes CE., Windham GL., Baird RE., Mlsna TE. (2016) Effects of growth parameters on the analysis of *Aspergillus flavus* volatile metabolites. *Separations, Volume: 3. Issue: 2. Pages:13* DOI: 10.3390/separations3020013

Awards:

1) Magyar Állam, Köztársasági Ösztöndíj (2011.)
2) Magyar Állam, Köztársasági Ösztöndíj (2012.)

Participant of National project:

1) KMR-12-1-2012-0189

Supervisor (MSc thesis):

1) Csordás Zsófia: Gombatermesztésben kártékony mikroorganizmusok élettevékenységének vizsgálata, illékony komponenseinek feltérképezése HS-SPME-GC-MS kapcsolt technikával, Élelmiszerbiztonsági és – minőségi mérnök MSc. diplomamunka (2014) Témavezetők: **Radványi Dalma**, Fodor Péter
2) Juhász Loretta: Különböző tápagarra oltott, gombatermesztésben kártékony penész anyagcseretermékeinek vizsgálata HS-SPME-GC-MS technikával, Élelmiszerbiztonsági és – minőségi mérnök MSc. diplomamunka (2016) Témavezetők: **Radványi Dalma**, Jókainé dr. Szatura Zsuzsanna
3) Lovász Ferenc: *Trichoderma aggressivum* anyagcseremarkereinek vizsgálata különféle stressz hatására légtéri mintavételezéssel, Élelmiszerbiztonsági és – minőségi mérnök MSc. diplomamunka (2016) Témavezetők: **Radványi Dalma**, Jókainé dr. Szatura Zsuzsanna

Supervisor (TDK):

1) Csordás Zsófia: Gombatermesztésben kártékony mikroorganizmusok illékony komponenseinek vizsgálata HS-SPME-GC-MS technikával, BCE Élelmiszertudományi Kar házi TDK konferencia, Élelmiszerkémia, minőségbiztosítás szekció, **II. helyezés** (2014) Témavezető: **Radványi Dalma**
2) Juhász Loretta: Különböző tápagarra oltott, gombatermesztésben kártékony penész anyagcseretermékeinek vizsgálata HS-SPME-GC-MS technikával, BCE Élelmiszertudományi Kar házi TDK konferencia,

Beltartalmi jellemzők szekció, **III. helyezés** (2015) Témavezetők: **Radványi Dalma**, Jókainé dr. Szatura Zsuzsanna

Summary:

Publications:	16
International, publications with IF:	4
Hungarian, publications with IF:	1
Cumulative impact factor:	6.319
Hungarian publications without IF:	2
Conferences	
Hungarian abstract:	3
International full paper:	2
International abstract:	3