Thesis of Ph. D. dissertation

Prediction of moldy spoilage of fruits

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Budapest

2012

PhD School

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INTRODUCTION

Traditional methods, like culture growth may take several days to identify microbes, as well as the modern methods based on antibody identification need at least hours. Hence any improvement to reduce the time needed to determine a microbe presence has a place in the top research areas, such as microbial elemental composition and nowadays the volatile organic compounds (VOCs) emitted by microbes.

Controlled atmosphere techniques are a typical research field which could use such a method. Postharvest losses of fruits and vegetables arise during storage, shipping and selling. Considering the fact that these loses might occur up to 50%, the interest of decreasing this number is understandable. As a result, storehouse managers become increasingly dependent on automation to limit the demand for manual labour that carries out these on-site inspections. Thus, the demand for an automated system to detect pathogen infections at an early stage has increased.

The aim of the study is to investigate the possible marker compounds in the fruit spoilage process by measuring the volatile organic compounds and the possibilities of data handling. The causing agents of post harvest losses are microbes – moulds in case of agricultural cultivars – which are not only a financial but a health risk as well. The final goal of the project is to develop a measurement system which predicts the microbial spoilage from air sampling.

The theory behind the method is that the produced volatile organic compounds during the spoilage process are proved to be able to identify microbial strains. The question is whether without any destructive method, can we tell by solid phase microextraction (SPME) sampling above fruit atmosphere followed gas chromatography - mass spectrometry (GC-MS) hyphenated system that the product is spoiled or not. During my PhD work I was investigating the practical and data handling side of this idea.

Challenges

Untargeted or non-targeted analysis are when the purpose of the analytical method is not to separate the analyte but to collect as much information, i.e. as many compounds from a mixture as possible. If the analytes are not known the procedure is to measure all the compounds and find those that make the difference in biological interest. Microbial volatile organic compounds are not known but we can have an idea by looking at the possible metabolic pathways.

One of the challenges in this field is that both the MVOCs and aroma compounds of fruits are highly dependent on environmental factors. On top of that VOCs and MVOCs are similar to each other in chemical characteristics; they can be alkanes, alkenes, alcohols, esters, aldehydes, and many other small organic compounds. A big quantity of similar compounds has to be separated then chosen within them the ones that are important in the spoilage process. These reasons close out the option for the traditional analytical process and need to imply new automated methods for comparing the results of different experiments and handling data. Hence every research that shortens the time that needs to process data is opportune.

Clear, reproducible strategies for metabolite identification and exchange of identifications between laboratories will facilitate further developments, such as the extension of profiling technologies towards metabolic signals and other technically demanding trace compound analysis. My research approach differs from the ones in literature, that I investigate parallel more fruits and more microbes for modeling spoilage. I am also monitoring the spoilage and not just compare a spoiled and a healthy candidate.

MATERIAL AND METHODS

VOC analysis was made by SPME-GC-MS (solid phase micro extraction sampling hyphenated with gas chromatography mass spectrometry) techniques. The SPME fiber was PDMS-DVB with 65 µm coating, extraction time was 30 minutes. Compounds were tentatively identified by comparison of the chromatographic peaks to NIST 2.0 reference library. Quantitative analyses were not carried out; integrated peak areas were used as half-Quantitative data for the dependent variable, which is named as concentration in this booklet for easier reading.

Penicillium expansum (P1) and *Bothyritis cinerea* (P2) was used for inoculation (source: BCE, Collection of Microbiological and Biotechnological Department, Corvinus University Budapest, Hungary). The tribes were isolated from Japanese plum by selective media. Same microbes were used throughout the whole work. The motivation to select Penicillium Expansum and Botrytis cinerea as our model microbes was that these pathogens are one of the most comprehensively studied, and thus extensive body of literature exists). As model fruit plums and apples were chosen and purchased from commercial market. Sterilization and inoculation of samples were carry out in the Microbiological Department, BCU, than samples was shipped in the sample holders (Figure 1) to the Applied Chemistry Department where the measurements were take place.

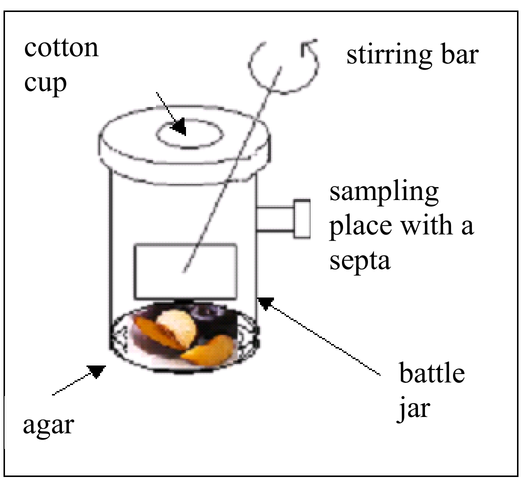


Figure 1: Sample storage

Critical point is the possibility to compare the very peak-rich GC-MS chromatograms in case of VOCs. Such manual processing would typically incur high costs associated with labour. This aspect was identified as a limiting factor for the effective application of GC-MS based food health monitoring. However, developments in computer science technology and software have increased the opportunity to automatically process GC-MS data at an affordable price. Manual analysis results in serious problems in the accuracy of peak identification and annotation depending on the knowledge and expertise of individual researchers. Automated peak detection, alignment and metabolite identification is the only way in case of untargeted analysis. To identify an individual metabolite in a certain sample properties can b used, here retention time and the mass fragments. Than the algorithm choose those information packages which are likely to form the same compound in different chromatograms. The matched, tagged compounds will form a table, where variables (compounds) are in columns and samples in rows hence, peak areas in the cells. This dataset now is available for different multivariate techniques to point out the differences find the corresponding compounds.

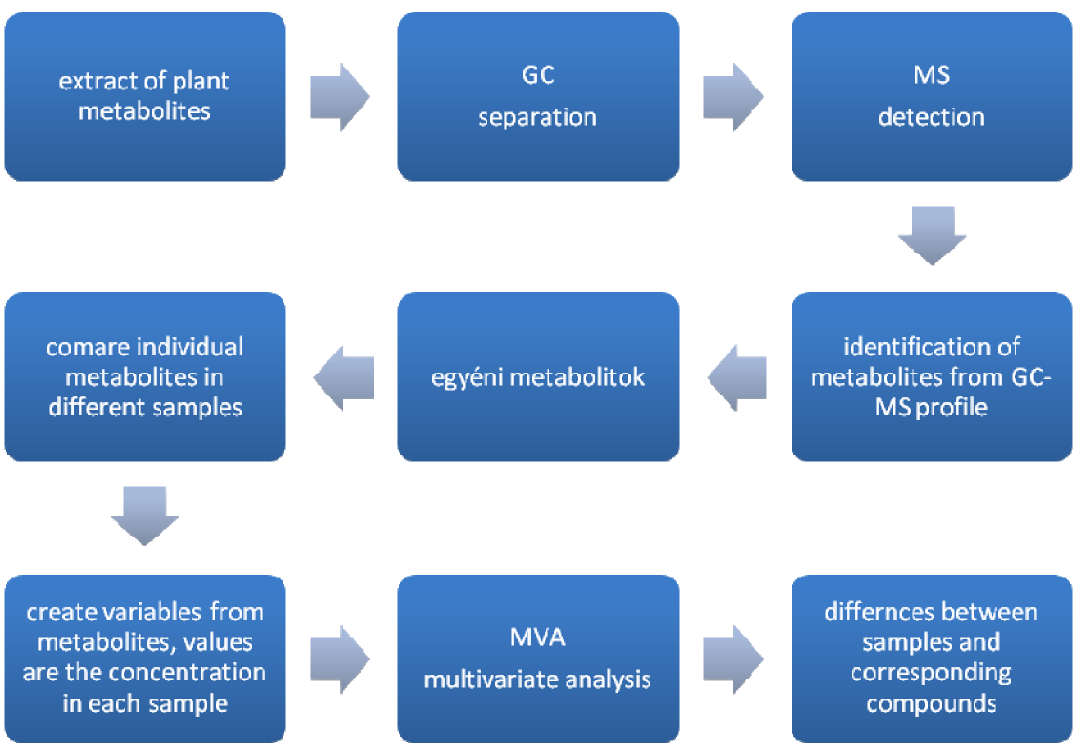


Figure 2: Flowchart of the task (MVA: multivariate analysis)

SCOPE AND DELIMITATION

A novel approach to discover a pathogen attack would be the detection of specific volatile organic compounds (VOCs) emitted from pathogen infected fruits. The main research objective of this study was to investigate whether plant emitted VOCs can be used to detect a pathogen infection in a storage house. This study is focused on the detection of the moldy diseases of apples and plums. The final goal, to make a predictive system for spoilage is too big for a PhD study, I concentrated on the data processing option and training system which are the basis of an indicator method applied by storage house industry. I have chosen the way for applying common marker compounds, compounds which are both can be an indicator in case of different agricultural products or different microbes. The research approach is a system model system to train the data processing and measurement of VOCs and provide possibility to search for common marker compounds. My goal was to investigate the properties of measurement system for VOCs and the different data handling methods for possible further usage.

Specified research questions:

- What aroma compounds can be measured for healthy fruits? How constant are these compounds through different experiments and within an experiment?
- Does there exist a significant and permanent difference between healthy and spoiled fruits? What are the important compounds in differentiating? What are the effective methods to find these marker compounds?
- Is there a common marker compound for apples and plums inoculated with *Penicillium expansum?*
- Is there a common marker compound for *Penicillium expansum* and *Botrytis cinerea?*
- Can marker compounds be used for prediction of spoilage?

RESULTS

A model system, an analytical measurement method and an experimental approach were developed to search for untargeted marker VOCs in air samples. The system is useable for monitoring VOCs. Esters and terpenes were the most important compounds in both the healthy and unhealthy fruits. Decreasing concentration of esters were indicating spoilage however changes in terpenes concentration were not useful as the different terpene isomers are impossible to differentiate in this MS system. Styerene, benzoic acid, ethyl ester and 1-methoxy-3-methylbenzene was found as marker compounds in both fruits. Markers can be detected in the first day of the visual signs of spoilage. No proper model building marker was found to be common in case of *Penicillium expansum* and *Bothyritis cinerea.* Hence, the opportunity of creating an indicator system for fungal fruit spoilage was proven. Healthy and spoiled fruits in different spoilage stage can be distinguished and there is a possibility to make classes based on spoilage stages. Proper data processing software extents the use of GC-MS instruments to other agricultural application such as quality control. This dataset shows how time and information consuming is the manual data processing even though automated methods needs training.

The process can be seen on the next page:

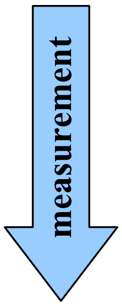
Figure 3: Sampling of the atmosphere of the mould inoculated fruits.

Figure 4: VOCs measured by SPME-GC-MS.

Figure 5: Multivariate analysis of concentration of VOCs in different samples to locate differences.

Figure 6: Selection of marker compound.

Changes in VOC s of fruits by *Bothyritis cinerea* and *Penicillium expansum*



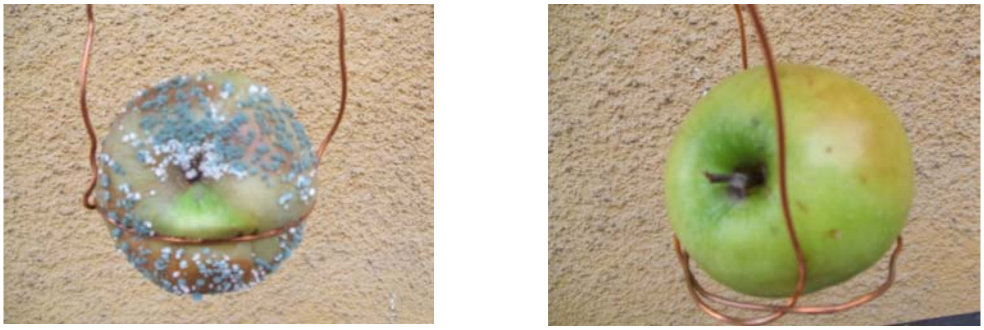
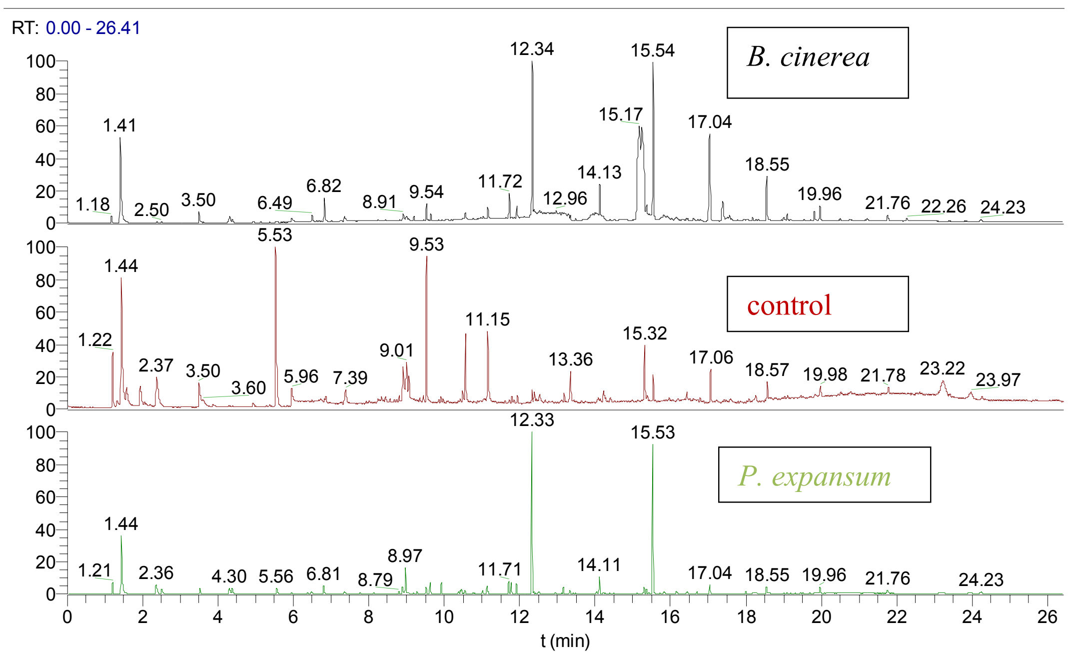


Figure 3: Spoiled and healthy apples after one week.



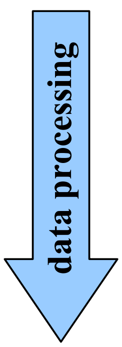


Figure 4: Chromatograms of healthy and spoiled fruits.

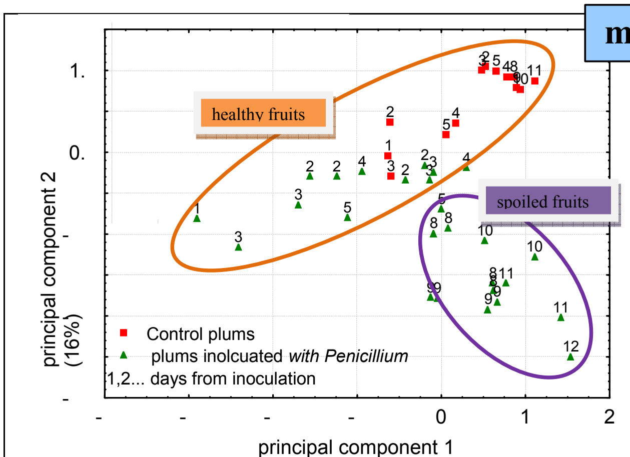
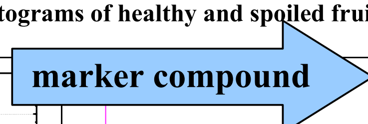


Figure 5: Principal component analysis of the concentration of the athompsehere of samples



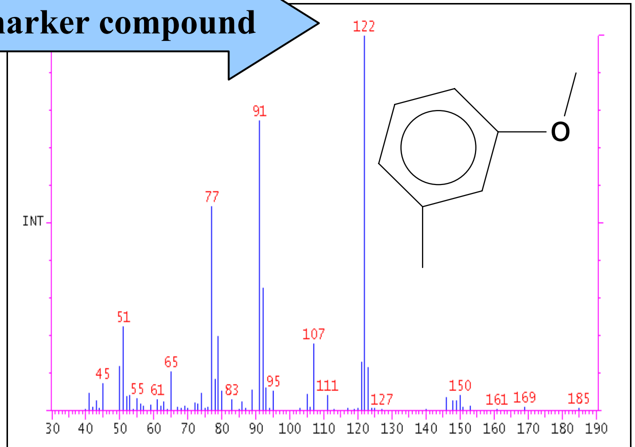


Figure 6: mass spectra and structure of methoxy-3-methylbenzene

NEW SCIENTIFIC RESULTS

1. New scientific results by investigating smapling system of plums

An experimental model system was developed which is appropriate for monitoring the changes in concentration of volatile organic compounds and able to indicate spoilage. Butanoic acid, methyl ester, styrene, 3-carene, 1-methoxy-3-methylbenzene, 1-decin and unidentified compound were the important compounds in case of spoiled plums. Qualitative information about styrene and 1-methoxy-3-methylbenzene was adequate to differentiate plums into healthy or unhealthy groups based on air samples above the fruits. This result was able to extend to two different parallel experiments with different dates.

2. New scientific results by evaluating volatile organic compounds relased by apples.

Concentrations of apple aroma compounds are not permanent in this sampling system, it changes with time. The changes are similar to different compounds so it is useable for comparing with the volatile compounds emitted by unhealthy, inoculated apples. By studying VOCs emitted by unhealthy apples it can be stated that esters and terpenes concentration changes during the spoilage process. Experimental data proves that microbes use some of the ester compounds during the spoilage process hence decreasing concentration of these esters in contrary of increasing concentration in healthy fruits can be used as spoilage indicators. PLS method was used successfully to estimate spoilage time from concentration of ester compounds.

3. New scientific results by investigating the volatile organic compounds produced by 'Granny Smith' apples inoculated with *Penicillium expansum*

Concentrations of pentanoic acid, ethyl ester and propanoic acid, hexyl ester are decreasing while concentration of butanoic acid, methyl-3,3-dimethyl ester, hexanoic acid, ethyl ester and 1-methoxy-3-methylbenzene are increasing during the spoilage process among the compounds differentiating healthy and *Penicillium expansum* inoculated apple samples. Styrene concentration is possible to predict from the nr of days from inoculation by a model built with Support vector regression method.

4. New scientific results in case of plums (*Prunus Salinica)* inoculated with *Penicillium expansum*.

Volatile organic compounds differentiating healthy and spoiled plums were chosen by principal component analysis, and then were compared with marker compounds for apple spoilage. Marker compounds emitted by apples and plums inoculated with *Penicillium expansum* are: styrene, benzoic acid, ethyl ester, 1-methoxy-3-methylbenzene. We can assume that these compounds can be produced by other fruits inoculated by *Penicillium Expansum* as well as they were common for plums and apples.

5. New scientific results in case of plums (*Prunus Salinica)* inoculated with *Botyritis cinerea.*

I found that butanoic acid, 2-2-methylhexyl ester, hexanoic acid, butyl ester, acetic acid, hexyl ester, isopropyl alcohol, 2-methy-4-pentenal are the important compounds in differentiating healthy and spoiled (inoculated with *Botrytis cinerea)* plums. I found only one Common marker compound in case of plums and apples that was: isopropyl alcohol, but because of the short retention time it cannot be used for prediction in this model system.

PUBLICATIONS

Papers with impact factors:

- S.F.Sim and V.Sagi-Kiss, Multiple Self Organising Maps (mSOMs) for simultaneous classification and prediction: Illustrated by spoilage in apples using volatile organic profiles, Chemometrics and Intelligent Laboratory Systems, 109 (2011) 57–64
- V. Sági-Kiss and P. Fodor, Development of a SPME-GC-MS method for spoilage detection in case of plums inoculated with Penicillium Expansum, Acta Alimentaria, Vol. 40 (Suppl.), pp. 188–197 (2011)
- Sim S. Fong, Virág Sági-Kiss and Richard G. Brereton, 2010, Self-Organizing Maps and Support Vector Regression as aids to coupled chromatography: Illustrated by predicting spoilage in apples using volatile organic compounds, Talanta,83, 1269-78 (2011)

Poster or oral presentations in international and Hungarian conferences:

- Virág Sági-Kiss, Sim Fong, Andrea Pomázi, Péter Fodor, Richard G Brereton, Anna Maráz, 2009. Prediction of fungal fruit spoilage via detection of volatile organic compounds, 2nd Central European Forum for Microbiology (CEFORM), Keszthely, Hungary, 7-9 October 2009 (English), abstract: Acta Microbiologica et Immunologica Hungarica 56, page 167-168 (oral presentation abstract)
- Sági-Kiss Virág, Fodor Péter, Pomázi Andrea, Maráz Anna, 2009. Predicting spoilage in storehouses based on microbial metabolic profiling, Conference of Chemical Engineering ’09, Veszprém, Hungary, 21-23 April 2009 (Hungarian), abstract: Conference Proceeding, page 103. (oral presentation abstract)
- Virág Sági-Kiss, Sim S. Fong, Andrea Pomázi, Péter Fodor, Richard G. Brereton, Anna Maráz, 2010, SPME, GC-MS integrated with chemometrics for

prediction of fungal fruit spoilage, 7th Aegean Analytical Chemistry Days, 29 Sept – 03 Okt, Lesvos, Greece (poster abstract)

- Sági-Kiss Virág, Fodor Péter, Pomázi Andrea, Maráz Anna, 2009. Predicting mouldy spoilage in storehouses based on microbial metabolic profiling, Conference of Chemical Engineering '10, Veszprém, Hungary, 27-29 April 2010 (Hungarian), abstract: Conference Proceeding, page 103 (poster abstract)
- Virág Sági-Kiss, Sim Fong, Andrea Pomázi, Péter Fodor, Károly Héberger, Richard G Brereton, 2009 Pattern recognition and GCMS peak detection as an aid to predicting spoilage in fruit from production of volatile organic compounds (VOC). Conferentia Chemometrica 2009, Siófok, (English) , **legjobb poszter díj** (poster abstract)

ACKNOWLEDGEMENTS

As a cooperative study I want to say thank to the employee of Microbiological Department of Corvinus University of Budapest, especially to Professor Anna Maráz and Professor Andrea Pomázi for developing the microbiological system and for preparing the samples. I want to say thank you to the Centre for Chemometric, Britol University, especially for Professor Richard Brereton and Dr. Sim Siong Fong for creating data sets and the help in the multivariate analyses.

I want to thank my family to make it possible for me to be here and my friends for continuous support. I want to say thanksto my supervisors, to Professor Peter Fodor who accepted me to the department and provided the financial and scientific knowledge that my work is based on, and to Professor Károly Heberger for providing the information for data analysis and being a mentor for me in my chemometrics studies. I want to thank all of the employees of the Applied Chemistry Department, the PhD students, my Masters students and everyone who helped me in my work.