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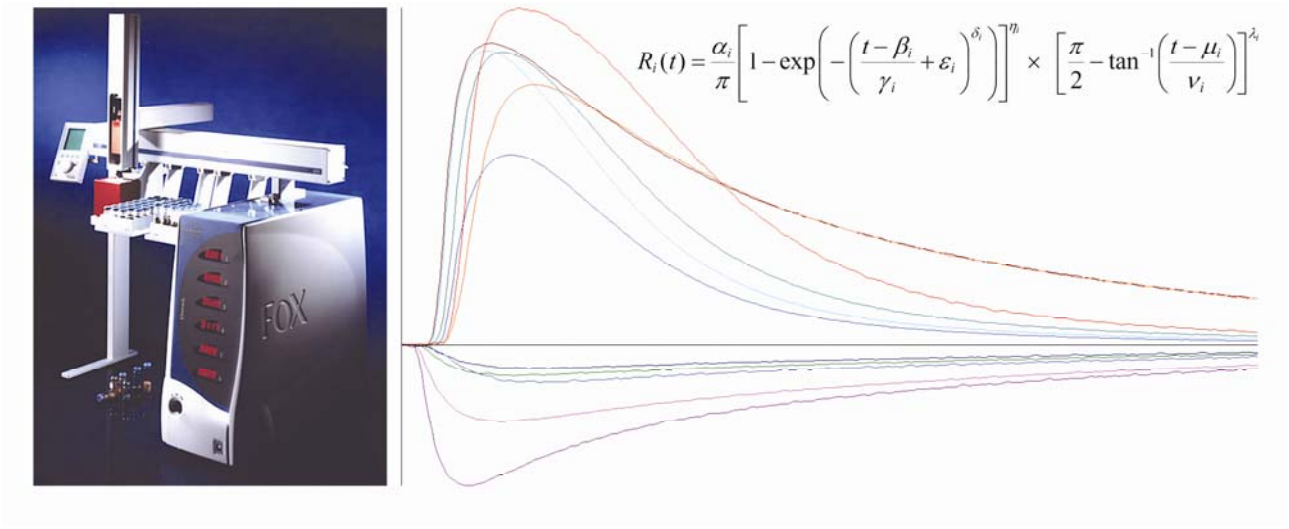

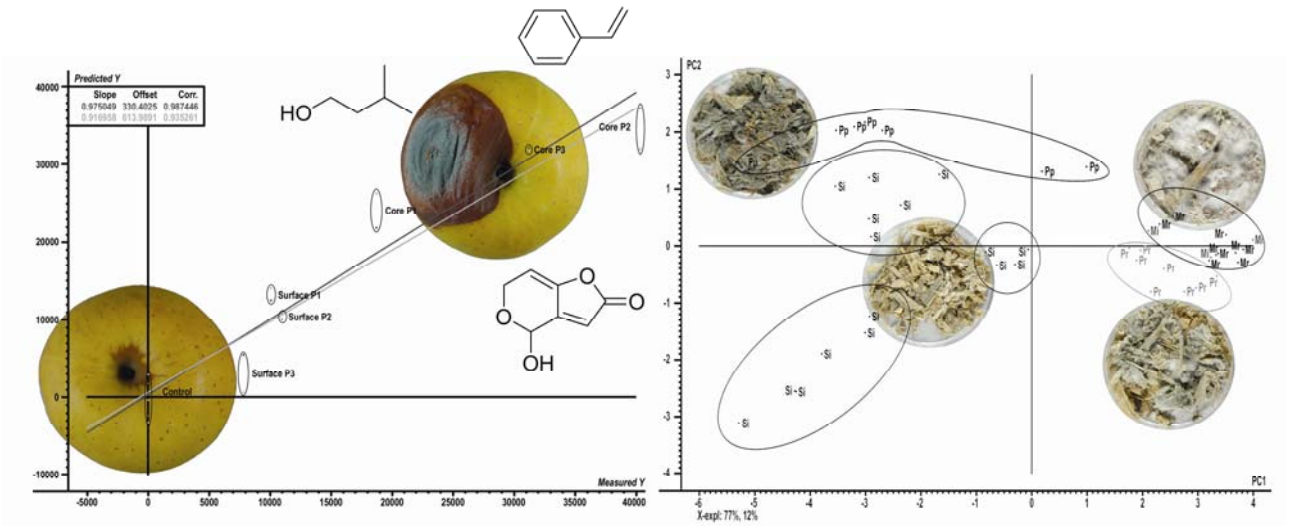

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PREFACE

The project was carried out at Center for Microbial Biotechnology at BioCentrum-DTU, Technical University of Denmark and financed by Directorate for Food, Fisheries and Agro Business, grant no. FSK03-DTU-4, under the research programme *Food Technology, Safety and Quality*.

I would like to thank my supervisors Thomas O. Larsen and Per V. Nielsen for our stimulating and encouraging talks and guidance throughout the study. Furthermore I would like to thank Thomas O. Larsen for inspiring me particularly in the field of analytical chemistry, especially gas chromatography and mass spectrometry and Per V. Nielsen for his great support on chemometrics.
I am also grateful to Kristian F. Nielsen for introducing me to the more advanced sides of sample preparation and liquid chromatography including mass spectrometry. Jeorgos Trihaas is acknowledged for introducing me to the electronic nose and for inspiration in the field of chemometrics. I would like to thank Hanne Jakobsen, Ellen K. Lyhne (Kir), Mirella Simkus and Anni Jensen for helping me in the lab, with the instruments and cultures. I thank Jens C. Frisvad for stimulating talks on mycology.

My office mates and fellow PhD students are also greatly appreciated for their inspirational talks and support.

Last but not least I would like to thank my wife, family and friends for their support and encouragement.

Kristian Karlshøj

SUMMARY

Fungi from genus *Penicillium* play an important role in food and feed spoilage such as *Penicillium expansum* infection of apples (model system 1) and fungal spoilage of silage (model system 2). It is known that the incidence of *P. expansum* spoilage of apples is increased by damage to the apples hence *P. expansum* spoilage mainly occurs on windfall apples. Storage of windfall apples prior to industrial processing is therefore likely to be the main cause of *P. expansum* spoilage and thus mycotoxin content. *Penicillium expansum* spoilage of, and hence patulin content in, apples is a well known problem for the apple juice industry as apples are commonly stored prior to production, due to processing capacity limitations. Fungal spoilage of silage potentially poses a great problem in the agricultural industry. Spoilage constitutes a production loss to the farmer in the form of loss of biomass discarding of spoiled feed. The presence of mycotoxins may furthermore affect yield and health of the livestock.

The focus of this study has been to investigate the possibility of prediction of mycotoxin production by detection of volatile metabolites via e-nose analysis. The foremost aim has been to differentiate between fungal species and genera as well as to construct prediction models by correlating e-nose data with mycotoxin concentrations both on synthetic media and in actual food and feed matrices. In all cases knowledge of the given habitats associated Funga has been applied.

As a preliminary study it was confirmed, by GC-MS analysis, that it is possible to distinguish very closely related fungi from the *Penicillium roqueforti* group; *P. roqueforti* and the patulin producing *P. carneum* and *P. paneum*, grown on artificial media by volatile metabolite profiling of mainly sesquiterpenes.

In the initial e-nose analysis study a cultivation method using artificial media was developed, in which fungi could be analyzed repeatedly with an e-nose throughout the various growth stages. This method was used to analyze the headspace of several closely related cheese associated fungi, mainly from genus *Penicillium* and *Geotrichum candidum*; *P. camemberti*, *P. nordicum*, *P. roqueforti* and its closely related species *P. paneum* and *P. carneum*, as well as the non-cheese associated fungus *P. expansum*. Headspace samples were also taken for GC-MS analysis to generate an overview of the composition of the headspace of the cultures. This study proved that it was possible to differentiate between these closely related fungi by chemometric analysis of the e-nose analysis data even if the volatile metabolite profiles, of in particular *P. roqueforti* and *P. paneum*, were very similar. HPLC analysis confirmed that the expected mycotoxins were produced under the given growth conditions.

The first food / feed stuff model system chosen was apples used for apple juice production, as apple spoilage by *Penicillium expansum* and hence, contamination of apples by patulin is of major concern for the apple juice industry. Apples of the Golden Delicious and Jonagold varieties inoculated with *P. expansum* at the core- and surface were analyzed with an e-nose both prior to and during different

stages of laboratory scale apple juice production, mimicking the industrial apple juice production steps. Samples were analyzed by HPLC to determine the patulin concentration during various stages of apple juice production as well. The headspace of the whole apples was furthermore analyzed by GC-MS in order to identify significant volatile biomarkers for *P. expansum* spoilage in apples. The volatile biomarkers were styrene, 3-methyl-1-butanol, 3-methyl-1-butanol acetate, methyl propanoate and 1-methoxy-3-methyl benzene. Chemometric analysis of the e-nose analysis data proved to be usable for distinguishing between contaminated and non-contaminated apples and it was possible to construct a prediction model, predicting the concentration of patulin via the e-nose measurements.

The second food / feed stuff model system was chosen to be silage which is typically spoiled by fungi such as *P. roqueforti*, *P. paneum* and *Monascus ruber* resulting in mycotoxin contamination of the silage. Silage from one source was contaminated with the three mentioned fungi and the headspace of the contaminated silage was analyzed with an e-nose. Furthermore silage from 15 other locations was analyzed to investigate the influence of silage variety on e-nose analysis. The headspace was furthermore analyzed by GC-MS to determine the volatile biomarkers for the various silage types as well as the volatile biomarkers for species specific fungal infection of silage. Chemometric analysis of the e-nose analysis data proved to be usable for classification of silage as being non-spoiled, spoiled by *M. ruber*, spoiled by *P. paneum* or spoiled by *P. roqueforti*. The chemometric analysis of e-nose data also showed that the 16 silage samples clustered in 3 separate groups of which the headspace, analyzed by GC-MS, of one group differed significantly from remaining two.

Finally it was investigated what effect data parameterization of the e-nose signal data could have on analysis. The parameters for the mathematical function describing e-nose response curves were determined and several parameters such as total curve area, curve slope and wavelet decompositions of the signal were extracted. Chemometric analysis of data generated in the previous experiments was performed on the "raw data" generated by the e-nose, specifically the maximum resistance measured (Y_{max}) and compared to chemometric analysis based on the parameters generated through mathematical modelling. The generated parameters were optimized to span the greatest possible variation with the least number of parameters. Both classification and regression modelling was performed with these two datasets. It was shown that with the datasets at hand there was no advantage in parameterization of the data.

In conclusion it has been shown that prediction of mycotoxin production by e-nose analysis is possible even in sample matrices containing a complex headspace such as apples and that it is possible to identify the spoilage organism in matrices with an equally complex headspace such as silage.

The future perspectives for the application of e-nose analysis in food and feed stuff quality control seem promising. There is still work to be done on construction of prediction models but the results of

this study strongly indicate that e-nose technology has a great potential in e.g. screening samples in order to make spot sampling more efficient.

RESUMÉ

Skimmelsvampe af slægten *Penicillium* spiller en vigtig rolle i fordærv af fødevarer og foderstoffer så som *Penicillium expansum* infektion af æbler (modelsystem 1) og skimmelsvampinfektion af ensilage (modelsystem 2). Det er velkendt, at forekomsten af *P. expansum* fordærv i æbler stiger, hvis æblerne er skadet, derfor forekommer *P. expansum* råd i æbler først og fremmest i nedfaldsæbler. Lagring af nedfaldsæbler inden industriel processering er derfor sandsynligvis den vigtigste årsag til *P. expansum* fordærv og dermed mykotoksinindhold. *Penicillium expansum* fordærv af æbler og dermed tilstedeværelsen af patulin, er et velkendt problem for æblejuiceindustrien, da æbler almindeligvis bliver lagret inden produktion på grund af begrænset processkapacitet. Skimmelsvampeinfektion af ensilage er et potentielt stort problem for landbrugsindustrien. Fordærvet medfører et produktionstab for landmanden i form af tabt biomasse, da fordærvet foder kasseres. Tilstedeværelsen af mykotoksiner kan ydermere potentielt påvirke kvægets helbred og dermed landmandens udbytte.

Fokus for dette studie har været at forudsige mykotoksindannelse ved detektion af flygtige metabolitter ved hjælp af e-næseanalyse overordnede mål har været at kunne skelne mellem skimmelsvampearter og -slægter såvel som at konstruere forudsigelsesmodeller ved at korrelere e-næsedata med mykotoksinkoncentrationer både på syntetiske medier og i fødevarer og foderstoffer. I alle tilfælde er viden om det givne habitats associerede funga blevet benyttet.

Som et indledende studium blev det ved GC-MS-analyse bekræftet, at det er muligt at skelne mellem meget tæt beslægtede skimmelsvampe fra *Penicillium roqueforti* gruppen (*P. carneum*, *P. paneum* og *P. roqueforti*) dyrket på syntetiske medier ved profilering af de flygtige metabolitter, hovedsageligt sesquiterpener.

Som introduktion til e-næseanalyse blev en dyrkningsmetode udviklet, med hvilken skimmelsvampe gentagent kunne e-næseanalyseres under alle vækststadier. Denne metode blev brugt til at analysere headspace fra adskillige tæt beslægtede, osteassocierede skimmelsvampe hovedsageligt fra slægten *Penicillium*: *Geotrichum candidum*, *P. camemberti*, *P. nordicum*, *P. roqueforti* og dens tæt beslægtede arter *P. paneum* og *P. carneum*, samt den ikkeosteassocierede *P. expansum*. Headspace prøver blev også analyseret med GC-MS for at danne et overblik over sammensætningen af kulturernes headspace. Det viste sig muligt at skelne imellem disse tæt beslægtede skimmelsvampe ved kemometrisk analyse af e-næsedata, selvom profilen af flygtige stoffer, især for *P. roqueforti* og *P. paneum*, var meget ens. HPLC-analyse bekræftede, at de forventede mykotoksiner blev dannet under de givne vækstbetingelser.

Æbler til brug i æblejuiceproduktione blev valgt som det første modelsystem for fødevarer / foderstoffer , da fordærv af æbler med *P. expansum* og dermed æblernes kontaminering med patulin udgør en stor bekymring for æblejuiceindustrien. Golden Delicious og Jonagold æbler, der var

overflade- og kernehuspodede med *P. expansum*, blev analyseret med en e-næse både før og under forskellige æblejuiceproduktionstrin i laboratorieskala, der efterlignede den industrielle æblejuiceproduktion. Prøver blev analyseret med HPLC for at bestemme patulinkoncentrationen under forskellige trin af æblejuiceproduktionen. Headspace fra hele æbler blev yderligere analyseret ved GC-MS for at identificere de flygtige biomarkører for *P. expansum* råd i æbler. De flygtige biomarkører var styren, 3-methyl-1-butanol, 3-methyl-1-butanolacetat, methylpropanoat og 1-methoxy-3-methylbenzen. Kemometrisk analyse af e-næseanalysedata viste det muligt at skelne imellem kontaminerede og ikke kontaminerede æbler og det var muligt at konstruere en forudsigelsesmodel for patulinkoncentration via e-næsemålingerne.

Ensilage blev valgt til det andet fødevare / foderstof modelsystem, da ensilage typisk bliver angrebet af skimmelsvampe så som *P. roqueforti*, *P. paneum* og *Monascus ruber*, hvilket resulterer i mykotoksinkontaminering af ensilagen. Ensilage fra én lokalitet blev podet med de tre nævnte skimmelsvampe, og headspace fra den inficerede ensilage blev analyseret med en e-næse. Ensilage fra yderligere 15 andre lokaliteter blev analyseret for at undersøge indflydelsen af ensilagens variation på e-næseanalysen. Headspace blev yderligere analyseret med GC-MS for at bestemme de flygtige biomarkører for de forskellige ensilagetyper såvel som de flygtige biomarkører for artsspecifik skimmelsvampeinfektion af ensilagen. Kemometrisk analyse af e-næsedata viste, at det er muligt at klassificere ensilage som værende enten ufordærvet, fordærvet af *M. ruber*, fordærvet af *P. paneum* eller fordærvet af *P. roqueforti*. Den kemometriske analyse af e-næsedata viste også, at de 16 forskellige ensilager grupperedes i 3 grupper, af hvilke GC-MS-analyse viste, at én af grupperne skilte sig signifikant ud fra de resterende to.

Til sidst blev det undersøgt, hvilken virkning parameterisering af e-næsesignalerne kunne have på dataanalysen. Parametrene for den matematiske model, der beskriver e-næseresponskurverne, blev bestemt, og adskillige parametre, så som total areal, hældning og wavelet dekompositioner af signalet, blev ekstraheret. Kemometrisk analyse af data fra tidligere forsøg blev udført på "rådata" genereret af e-næsen, specifikt den maksimalt målte resistans (Y_{max}). Denne analyse blev sammenlignet med kemometrisk analyse baseret på de ved matematisk modelering genererede parametre. De genererede parametre blev optimeret, så de udspændte den størst mulige variation med det mindst mulige antal parametre. Både klassifikations- og regressionsmodeller blev udført for disse to datasæt. Det blev vist, at der med de forhåndenværende datasæt ingen fordel var i at benytte parameterisering af data.

Det konkluderes, at det er vist, at forudsigelse af mykotoksinproduktion ved e-næseanalyse er mulig, selv i prøvematricer, så som æbler, der indeholder et komplekst headspace, og at det er muligt at identificere den organisme, der forårsager råd i en matrice, så som ensilage, med et lige så komplekst headspace.

Fremtidsperspektiverne for benyttelsen af e-næseanalyser i fødevare- og foderstofkvalitetskontrol ser lovende ud. Der er stadig arbejde, der skal gøres for at konstruere forudsigelsesmodeller, men

resultaterne af dette studie indikerer kraftigt, at e-næseteknologi har et stort potentiale i for eksempel screening af prøver, således at stikprøvekontrol kan gøres mere effektiv.

LIST OF ORIGINAL PAPERS AND OTHER PUBLICATIONS

Karlshøj, K., Nielsen, P.V. and Larsen, T.O. Fungal Volatiles Biomarkers of Good and Bad Food Quality *in* Samson, R.A. and Dijksterhus, J. (Ed.). Food Mycology, 2007, CRC Press Boca Raton, FL. ISBN: 9780849398186.

Karlshøj, K. & Larsen, T.O. Differentiation of Species from the *Penicillium roqueforti* Group by Volatile Metabolite Profiling. *J. Agric. Food Chem.* **2005**, *53*, 708-715.

Karlshøj, K., Nielsen, P.V. and Larsen, T.O. Differentiation of Mycotoxin Producing Fungi by Use of an Electronic Nose. *J. Food Sci.* **2007**, 72, M187-M192.

Karlshøj, K. Nielsen, P.V. and Larsen, T.O. Prediction of *Penicillium expansum* Spoilage and Patulin Concentration in Apples used for Apple Juice Production by Electronic Nose Analysis. *J. Agric. Food Chem.* **2007**, 55, 4289-4298.

Karlshøj, K. and Hansen, M.E. Effect of data parameterization on data analysis of electronic nose signals. (draft)

Karlshøj, K., Drejer Storm, I.M.L., Nielsen, K.F., Nielsen, P.V. and Larsen, T.O. Prediction of Silage Spoilage by Electronic Nose Analysis. (draft)

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Journal of Agricultural and Food Chemistry, ACS Publications, Washington D.C.
Journal of Food Science, IFT (Institute of Food Technologists), Chicago, IL.

OBJECTIVE

The main objective of this study has been to investigate whether unspecific detection of species specific fungal volatiles by e-nose technology is applicable for prediction of mycotoxin production in food and feed stuffs, especially in foods and feed stuffs which exhibit a complex headspace background (Figure 17). It has therefore not been the scope of this study to explore the potential for predicting e.g. biomass by e-nose analysis.

It is evident that there is a great potential for such a method in food and feed stuff quality control. E-nose analysis as means of predicting spoilage or mycotoxin production would in some cases be a much faster analysis method compared to traditional methods, such as GC-MS analysis to detect fungal contamination and HPLC analysis to detect mycotoxin content, especially when considering the workload in sample preparation particularly for HPLC samples.

To achieve the main aim of this study the following goals were set:

- To construct a method for fast and (semi) automated e-nose analysis of the headspace of growing filamentous fungi, without accumulation of carbon dioxide and with as reduced a loss of volatile metabolites as possible.

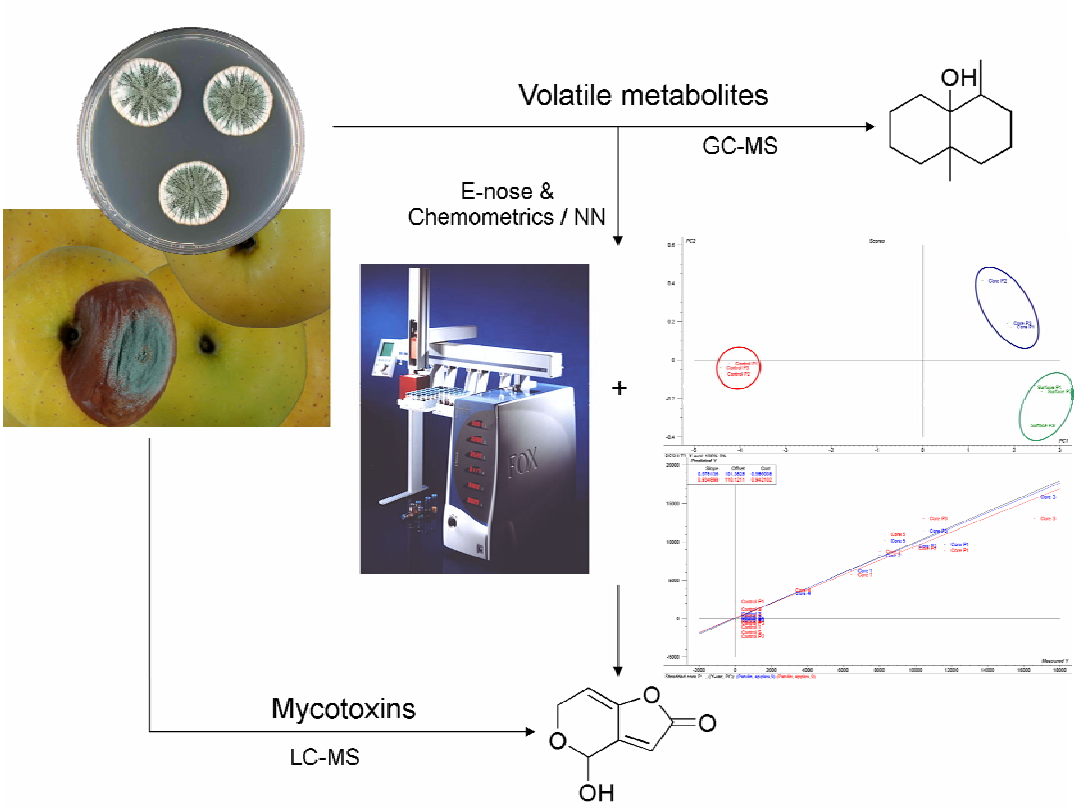

Figure 17. The principle of indirect mycotoxin detection by e-nose analysis of sample headspace.

- To determine whether it is possible to distinguish between closely related fungi by means of electronic nose analysis. A prerequisite for correct identification of spoilage organisms in prediction models.

- To determine if it is possible to enhance information extraction from e-nose data by pre-processing the data, for instance by parameterization of the e-nose signals.

- To construct classification models in order to distinguish between species of the associated funga of a food or feed stuff with a complex headspace background by means of e-nose analysis.

- To construct prediction models, in food and / or feed stuff model systems, to predict mycotoxin production within the model system by e-nose analysis.

The work in this study is based on research within the last decade on characterization of filamentous fungal volatile metabolite profiles and food quality control by e-nose analysis as well as the two decades of research on profiling species level non-volatile secondary metabolites of filamentous fungi both performed at Centre for Microbial Biotechnology at BioCentrum-DTU, Technical University of Denmark.

From: Chapter 14, "Fungal volatiles: Biomarkers of good and bad food quality. In *Food Mycology: A Multifaceted Approach to Fungi and Food***.**

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INTRODUCTION

Most people have experienced the smell of fungal spoiled foods such as bread. The typical reaction is "this really smells bad" and most people will associate this with a mouldy smell. It has been shown that the odour thresholds of some off flavour related fungal volatile compounds are very low. The odour threshold for the earthy smelling terpene alcohol geosmin (Figure 1) in water is between 0.0082- 0.018 ppb and the musty smelling 2-methyl-isoborneol (Figure 1) has an odour threshold of 0.1 ppb in water (Medsker *et al*., 1969).

Traditionally, specific fungal species have been used as starter cultures in certain fermented foods such as blue and white mould cheeses (*Penicillium roqueforti*; and *Penicillium camemberti* and *Geotrichum candidum* respectively) and soy sauce (*Aspergillus oryzae* and *Aspergillus sojae*). It is also well known that various mycotoxin producing fungi occur as contaminants in foods and feed stuffs, which is a considerable problem, in terms of food and feed quality and safety and hence economically, as in 1985 FAO estimated that 25% of world crops (Pitt and Hocking, 1985) and as much as 50% of crops in developing countries, are contaminated with mycotoxins (Waller and Brayford, 1990; Pohland, 1993). It is therefore important that it is ensured that starter cultures are pure.

Fungal detection is not only of importance in terms of food safety. For centuries, truffles, a most valuable fungal commodity, have been found by use of pigs, which can smell the truffles odorous compounds very well and find them covered under soil. In the field of medicine, it is also important to be able to detect fungal infections, such as aspergillosis, as soon as possible.

Food quality has, traditionally, often been assessed by sensory panel evaluation, for instance in quality control of cereals (Börjesson *et al*., 1996). Sensory panel analysis is a very laborious process as it requires a panel of sensory judges which is very expensive and time consuming to train to a proficient level. Even a well trained sensory judge will give an at least partially subjective score in sensory panel analysis. There are further restrictions in using sensory panel analysis, as potentially toxic samples cannot be analyzed in this manner.

Of the traditional analytical methods, GC-MS analysis is time consuming, but somewhat less expensive than sensory panel analysis or HPLC-DAD and LC-MS analysis. Data analysis

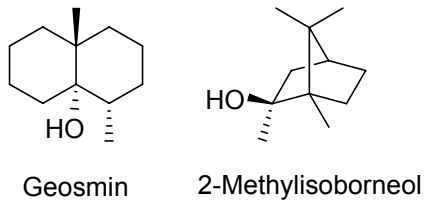

Figure 1. Chemical structure of the mouldy smelling volatile fungal biomarkers geosmin and 2-methylisoborneol, two volatile compounds with extremely low odour-threshold values.

with all these techniques is complicated and requires skill and experience. HPLC-DAD and LC-MS analysis are expensive, both in terms of equipment, running costs and time consumption (Guernion *et al*., 2001).

In recent years however, the electronic nose (e-nose) has been tested successfully for quality control of foods and feed stuffs. It has so far not been thoroughly tested whether it is possible to link e-nose analysis with mycotoxin content/production, or to establish whether it is possible to predict mycotoxin production during production or storage. This is desirable as e-nose measurements are faster, cheaper and easily automated in industrial processes (Sim *et al*., 2003). Potentially, e-nose prediction models can be constructed, and with these data analysis could principally be performed by unskilled personnel.

Following is a brief discussion of fungal biochemistry, with emphasis on volatile metabolites and mycotoxins as well as their application as biomarkers, the traditional chemical analysis methods for mycotoxins (HPLC and LC-MS) as well as for volatile organic compound (VOC) analysis (GC-MS) and finally, an in depth description of an electronic nose system and its potential applications for mycotoxin prediction. A couple of cases will be shown for illustrative purposes.

FUNGAL BIOCHEMISTRY

Fungal presence can be detected in many ways. One such way concerns the production of the sterol ergosterol, a plasma membrane component unique for fungi, and production of cell walls containing chitin. Fungi are also well known to produce a broad variety of extracellular enzymes, which are utilized in degradation of nutrient macromolecules. Some metabolites, like ergosterol, are produced by almost all fungi, but most known secondary metabolites, such as mycotoxins and volatile terpenes, have been shown to be more restricted in their distribution, for instance only being produced only by 1 to 15 species within genus *Penicillium* (Larsen and Frisvad, 1995a).

VOLATILE METABOLITES

Among the volatile metabolites produced by fungi are alcohols, aliphatic C8 compounds, alkanes, alkenes, esters, ketones, lactones, pyrazines and terpenes, an overview of the volatile metabolite pathways is shown in Figure 2.

The alcohols produced can be put into three categories according to their synthesis pathway. The first group comprises of primary alcohols which are produced in two reductive steps from fatty acid-CoA esters (Luckner, 1990). The second group, the fusel alcohols, is a product of the Ehrlich pathway. In this pathway amino acids (such as leucine, isoleucine and valine) are deaminated and the resulting β-keto acid decarboxylated, the resulting aldehyde is reduced to the alcohol by the alcohol dehydrogenase enzyme (Figure 3) (Suomalainen, 1971; Berry, 1988).

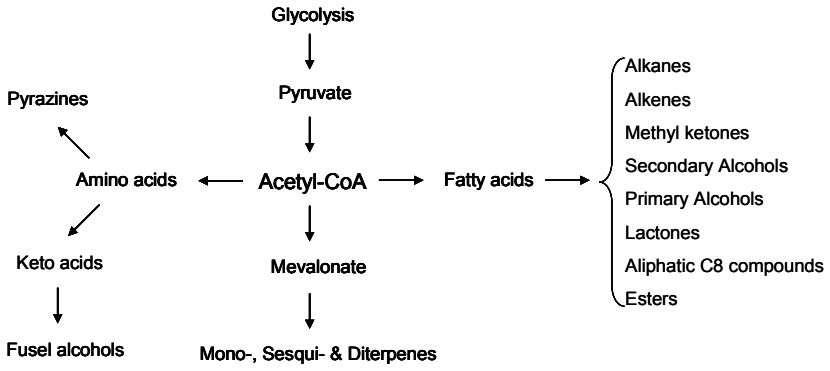

Figure 2. Overview of the biosynthesis of important fungal volatile metabolites, adapted from (Börjesson, 1993; Larsen, 1994; Jelén and Wasowicz, 1998).

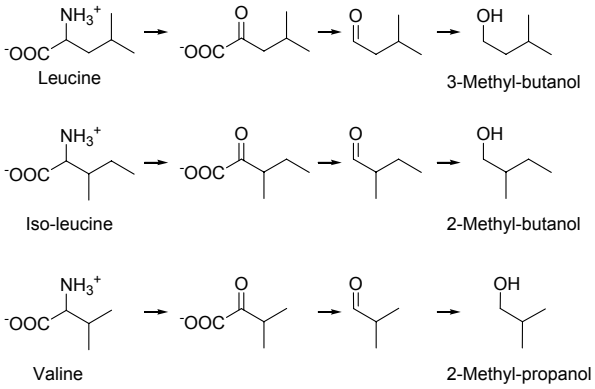

Figure 3. Fusel alcohol pathways. The amino acid is deaminated, then decarboxylated and the aldehyde reduced to the resulting alcohol (Suomalainen, 1971; Gurney, 1997).

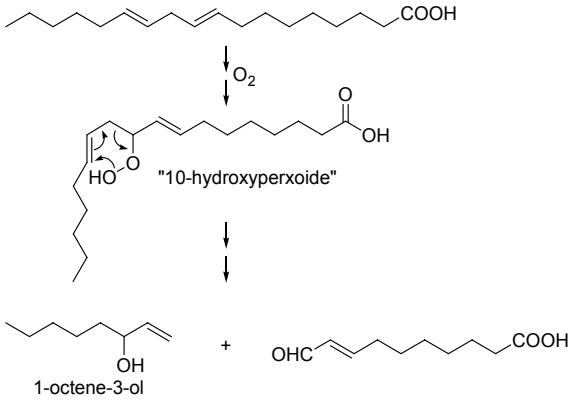

Figure 4. 1-Octen-3-ol synthesis. Linoleic acid is oxidized into a 10-hydroxyperoxide which in turn is cleaved into 1-octen-3-ol and a ten carbon fragment (Wurzenberger and Grosch, 1982; Wurzenberger and Grosch, 1984).

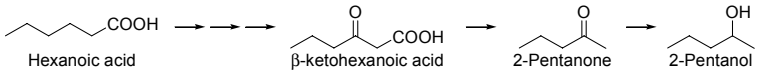

Figure 5. Methyl ketone synthesis example. The fatty acid is β-oxidatized by the usual pathway in the fatty acid metabolism. The β-keto acid is then decarboxylated to form the methyl ketone. The methyl ketone can be further reduced to the resulting secondary alcohol (Luckner, 1990).

Secondary alcohols, which comprise the third group, are formed by reduction of methyl ketones (see below and Figure 5) (Hawke, 1966; Kinsella and Hwang, 1976; Kinderlerer, 1989).

Aliphatic C8 compounds are produced by lipoxygenation; for instance 1-octen-3-ol is produced by oxidation of linoleic acid into a 10-hydroxyperxoide which is then cleaved into 1-octen-3-ol and a ten carbon fragment (Figure 4) (Wurzenberger and Grosch, 1982; Wuzenberger and Grosch, 1984).

Synthesis of alkanes and alkenes is done by decarboxylation of the corresponding fatty acids. The likely pathway for this is an α-oxidation with a β-keto acid intermediate. Further unsaturation of the alkene can be achieved by hydroxylation and dehydrogenation of the alkene (Luckner, 1990).

The acid moiety of esters produced by fungi can be formed by three possible path-

ways: by activation of monocarboxylic acids; from an intermediate from the long chain monocarboxylic acid synthesis or from oxidative decarboxylation of β-keto acids (Kempler, 1983). Methyl ketones are synthesized during fatty acid catabolism. The β-oxidation pathway is followed until β-ketoacyl-CoA has been formed, β-ketoacyl-CoA is then either both deacylated and decarboxylated to form a methyl ketone one carbon shorter than the fatty acid or the fatty acid is further β-oxidized (Figure 5) (Hawke, 1966; Kinsella and Hwang, 1976; Kinderlerer, 1989). Lactones are formed from γ-keto and δ-keto acids synthesized from fatty acids (Kempler, 1983). Synthesis of tetramethylpyrazines has been suggested to be a condensation reaction between acetoin and ammonia. Production of 2-methoxy-3-isopropylpyrazine has been proposed to be produced from valine and ethanedione whereas 2-methoxy-3-isopropyl-5-methyl pyrazine is suggested to be formed from valine and pyruvaldehyde (Kempler, 1983; Leete *et al*., 1991).

Among the most diverse volatile metabolites produced by fungi are terpenes and terpene alcohols. Terpenes are comprised by isoprene units. The synthesis of terpenes starts with acetoacetyl-CoA formation from two units of acetyl-CoA. Acetoacetyl-CoA and acetyl-CoA are synthesized into β-hydroxy-β-methyl glutaryl-CoA, which is converted into mevalonate by reduction of the carbonyl group into a primary alcohol. Mevalonate is decarboxylated yielding isopentenyl pyrophosphate. Isopentyl pyrophosphate is then polymerized into the following terpene precursors: geranyl phosphate (monoterpene precursor), farnesyl pyrophosphate (sesquiterpenes precursor) and geranylgeranyl phosphate (diterpene precursor) (Figure 6) (Lynen, 1959; Richards and Hendrickson, 1964; Herbert, 1989; Luckner, 1990). Other volatiles produced by fungi are compounds such as dimethyldisulphide (Figure 7, (Demarigny *et al*., 2000)) and other sulphur containing volatiles, which are produced by degradation of metheonine.

Knowledge of the nutrient content of a food product is therefore of importance when predicting which type of volatiles will be relevant for determination of food quality. E.g. for fat rich foods, monitoring for alcohols, alkanes, alkenes and methyl ketones will be relevant for detection of possible spoilage.

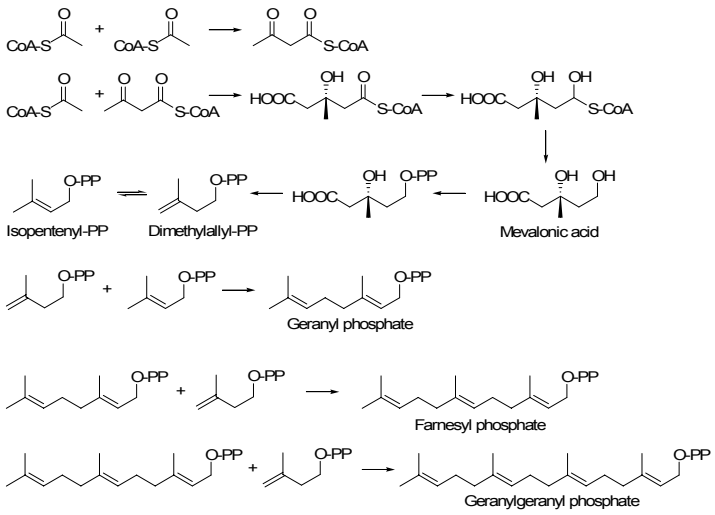

Figure 6. Synthesis pathway of terpene precursors. Acetoacetyl-CoA is formed from two units of acetyl-CoA. β-hydroxy-β-methyl glutaryl-CoA is synthesized from acetoacetyl-CoA and acetyl-CoA and then converted into mevalonate by reduction of the carbonyl group into a primary alcohol. Mevalonate is decarboxylated yielding isopentenyl pyrophosphate which is then polymerized into the following terpene precursors: geranyl phosphate (monoterpene precursor), farnesyl pyrophosphate (sesquiterpenes precursor) and geranylgeranyl phosphate (diterpene precursor) (Herbert, 1989).

Table 1. Most commonly encountered associated spoilage funga of genus *Aspergillus* and *Penicillium* for selected foods, adapted from (Filtenborg *et al*., 1996; Samson *et al*., 2002).

Foods	Fungal Species
Bread, rye	*P. roqueforti*, *P. paneum*
Cheese	*P. commune*, *P. nalgiovense*, *P. atramentosum*, *P. nordicum*, *A. versicolor*
Fruits, pomaceous and stone	*P. expansum*, *P. crustosum*, *P. solitum*
Grain, stored	*P. cyclopium*, *P. freii*, *P. hordei*, *P. melanoconidium*, *P. polonicum*, *P. verrucosum*, *P. aurantiogriseum*, *A. flavus*, *A. niger*, *A. candidus*
Nuts	*P. commune*, *P. crustosum*, *P. discolor*, *A. flavus*
Fermented sausages	*P. nalgiovense*, *P. olsonii*, *P. chrysogenum*, *P. nordicum*

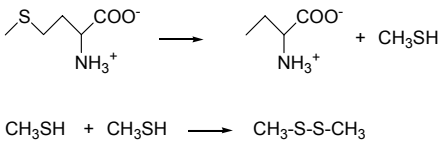

Figure 7. Dimethyldisulphide biosynthesis. Methanethiol is synthesized by via γ-demethiolase by degradation of metheonine; dimethyldisulphide is then produced by polymerization of two units of methanethiol (Demarigny *et al*., 2000).

NON-VOLATILE SECONDARY METABOLITES

The number of non-volatile secondary metabolites produced by fungi is extremely high (Samson *et al*., 2002). It has been shown that each fungal species has specific affinity for food and feed habitats thus leading to specific species occupying the different ecologic niches. The fungi most often seen in a given habitat are referred to as that habitat's *associated funga* (Filtenborg *et al*., 1996).

Species from genera *Aspergillus*, *Penicillium* and *Fusarium* are among the most frequent food contaminants. Typical contaminants, with typical habitat and key mycotoxins produced shown in brackets, are *Aspergillus flavus* (nuts and cereals; aflatoxin and cyclopaldic acid), *Aspergillus terreus* (silage; patulin and citrinin), *Penicillium carneum* (silage; patulin and roquefortin C), *Penicillium commune* (cheese; cyclopaldic acid), *Penicillium expansum* (pomaceous fruit; patulin, roquefortin C and citrinin), *Penicillium nordicum* (meat products and cheese; ochratoxin A), *Penicillium paneum* (silage; patulin and roquefortin C), *Penicillium verrucosum* (cereal and cheese; ochratoxin A and citrinin), *Fusarium graminearum* (cereals; trichothecenes), *Fusarium poae* (cereals; trichothecenes), *Fusarium sambucinum* (cereals and potatoes; trichothecenes) and *Fusarium sporotrichioides* (cereals; trichothecenes) (Samson *et al*., 2002), Table 1 grants an overview of the associated funga of genera *Aspergillus* and *Penicillium* for selected food stuffs. Detection of fungal spoilage is therefore of utmost importance.

Like the volatile metabolites, fungal secondary metabolites derive from pathways linked to key compounds formed in primary metabolism (Herbert, 1989). The plasma membrane sterol ergosterol is produced via the terpene pathway, and thus originates from acetyl-CoA converted into mevalonate (Herbert, 1989).

Many mycotoxins, such as the aflatoxins, citrinin, ochratoxin A, patulin, penicillic acid, are polyketides, and thus originate from the polyketide pathway which begins with acetyl-CoA. Alkaloid mycotoxins, such as roquefortine C and chaetoglobosin A originate from α-amino acids (lysine, ornithine, phenylalanine, tyrosine and tryptophan) and penicillins, which are β-lactams, being modified tripeptides, thus also originate from amino acids (valine, serine and α-aminoadipic acid) (Herbert, 1989). Trichothecenes are sesquiterpenes and thus originate from the terpene pathway which starts from acetyl-CoA converted into mevalonate.

As an example an overview of mycotoxins produced by fungi associated with pomaceous and stone fruits is found in Table 2. The toxicity of these mycotoxins is listed in Table 3.

Structures of selected mycotoxins are shown in Figure 8. Further information on mycotoxin production is found in Frisvad, Thrane and Samson's chapter *Mycotoxin producers*.

Table 2. Mycotoxins, capable of evoking acute or chronic diseases in vertebrate animals, produced by pomaceous and stone fruit associated fungi of genus *Penicillium*.

Species	Mycotoxins
P. crustosum	Penitrem A – F; roquefortine C; terrestric acid
P. expansum	Chaetoglobosin C; citrinin; communesins; patulin; roquefortine C
P. solitum	-

Table 3: Toxicity type of mycotoxins listed in Table 2.

Mycotoxin	Toxic activity
Chaeteoglobosins	Cytotoxic
Citrinin	Nephrotoxic
Communesins	Cytotoxic
Patulin	Carcinogenic, cytotoxic, generally toxic
Penitrems	Acutely toxic, tremorgenic
Roquefortine C	Neurotoxic
Terrestric acid	Cardiotoxic

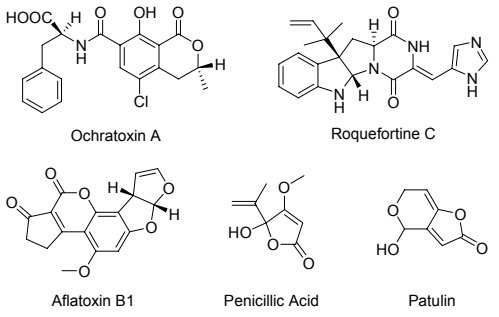

Figure 8. Chemical structure of selected mycotoxins.

BIOMARKERS AND ASSOCIATED FUNGA

In industrial food quality control, sensory panel analysis has traditionally been used. The trained panelists use terms such as musty, acidic or putrid to describe off flavours of the spoiled product. Unfortunately even the best trained sensory panel will yield subjective scores. Off flavours can also be studied by analytical chemistry, for instance by use of GC-MS. In such an analysis the level of geosmin, 2-methyl-isoborneol and other off flavour related compounds, can be determined and chemometrics can be used to classify samples according to whether they have off flavours of any kind or not. It is important to remember that sample treatment can influence the volatile metabolite profile since for instance the compound 1-octen-3-ol will be formed in large amounts when fungal mycelium is destroyed (Karahadian *et al*., 1985).

To make analysis easier it can be worthwhile to focus on compounds generally encountered in spoiled food. Two such compounds which indicate fungal spoilage are ergosterol and chitin, which thus are *biomarkers* for fungal growth/biomass and thus fungal spoilage. Chemical analysis, usually by HPLC, to determine the level of ergosterol has been proven to be a reliable measure for estimation of fungal CFU in a given sample (Cahagnier *et al*., 1983).

By applying knowledge of the associated funga of a specific product and knowledge of volatile compounds produced by these fungi, it is possible to determine the fungal species in a sample. A list of associated spoilage fungi, and their habitat, of genus *Aspergillus* and *Penicillium* is shown in Table 1. For instance, if analysis for geosmin and 2-methyl-isoborneol is done, it is possible to distinguish between *P. expansum* (geosmin producer), *P. solitum* (2-methyl-isoborneol producer) and *P. crustosum* (produces both geosmin and 2-methyl-isoborneol), three *Penicillium* species associated with apple spoilage (for production of volatile compounds by species, see Table 4). These results were obtained on synthetic media, but they indicate a possibility for differentiation of spoilage fungi in foods as well.

This distinction of course relies on the given media stimulating the production of these volatile compounds. Thus both volatile and non-volatile metabolites can be used as biomarkers. A biomarker can be more or less specific indicating fungal spoilage by pointing at a selected group, such as the terverticillate Penicillia series *viridicata* or series *verrucosa*, or even an individual species.

Table 4. Volatiles produced by the most commonly encountered food spoilage species in Table 1, adapted from (Börjesson *et al*., 1992; Zeringue, Jr. *et al*., 1993; Larsen and Frisvad, 1995a; Fischer *et al*., 1999; Karlshøj and Larsen, 2005).

Species	Volatile metabolites
A. candidus	3-methylfuran, 2-methyl-1-propanol, 1-penten-3-ol, 2-methyl-1-butanol, thujopsene, ethyl hexanoate, 1-octen-3-ol ethyl ester, 2,3,5-trimethylfuran, anisole, 3-octanone, 3-cyclohepten-1-one, 3-methyl-1-butanol, 1-octen-3-ol, 3-methyl-1-heptene, 1,3,6-octatriene and one unidentified monoterpene
A. flavus	3-methylfuran, 2-methyl-1-propanol, 1-penten-3-ol, octadiene, limonene, thujopsene, 3-methyl-1-butanol, 3-octanone, 3-octanol, 1-octen-3-ol, 1-octanol, cis-2-octen-1-ol, α-gurjunene, trans-caryophyllene, epi-bucyclosesqui-phellandrene, eremophilene, β-cubebene, valencene, epizonaren, γ-selinene, γ-cadinene, cadinene, δ-cadinene, α-muurolene, aristolen, α-copaene
A. niger	2-methyl-borane, 2-methyl-bornene, α-pinene, 3-methyl-1-butanol, 3-octanone, 3-octanol, 1-octen-3-ol, 2-octen-1-ol, 1-octanol
A. versicolor	3-methylfuran, 2-methyl-1-propanol, 1-penten-3-ol, 2-methyl-1-butanol, octadiene, limonene, thujopsene, anisole, 1-(3-methylphenyl)-ethanone, 6-methyl-2-heptanone, χ-curcumene, α-muurolene, myrcene, 3-methyl-1-butanol, 1-octen-3-ol
P. atramentosum	Ethyl acetate, methyl isobutanoate, ethyl isobutanoate, isobutyl acetate, ethyl 2-methyl-butanoate, ethyl isopentanoate, isobutyl isobutanoate, isobutyl 2-methyl butanoate, butyl isopentanoate
P. aurantiogriseum	2-methyl-1-propanol, 3-methyl-1-butanol, 1-ethyl-cyclopentene, 1,3-octadiene (two isomers), 3-heptanone, 3-octanone, γ-elemene, and two unidentified sesquiterpenes
P. chrysogenum	1-heptene, 1,3-octadiene (two isomers), 3-heptanone, 1-nonene, 1,3-nonadiene, 1-octen-3-ol, 3-octanone, 3-octanol
P. commune	Ethyl acetate, 2-methyl-1-propanol, 3-methyl-1-butanol, 3-hexanone, 1,3-octadiene (two isomers), 3-heptanone, styrene, 1-octen-3-ol, 3-octanone, 3-octanol, 2-methyl-isoborneol, β-caryophyllene, (+)-aristolochene, and seven unidentified sesquiterpenes
P. crustosum	Ethyl acetate, 2-methyl-1-propanol, ethyl propanoate, 3-methyl-1-butanol, dimethyldisulphide, ethyl isobutanoate, 1,3,5-cycloheptatriene, isobutyl acetate, ethyl butanoate, ethyl-2-methyl-butanoate, ethyl isopentanoate, Isopentyl acetate, styrene, ethyl pentanoate, 3-octanone, ethyl hexanoate, ethyl octanoate, 2-methyl-isoborneol, geosmin and an unidentified monoterpene
P. cyclopium	One unidentified sesquiterpene
P. discolor	2-methyl-3-butene-2-ol, 2-methyl-1-propanol, 3-methyl-1-butanol, isobutyl acetate, 3-octanone, 2-methyl-isoborneol, geosmin, four unidentified monoterpenes and three unidentified sesquiterpenes
P. expansum	Ethanol, 2-methyl-1-propanol, 3-methyl-1-butanol, ethyl acetate, β-pinane, 1-methyoxy-3-methyl-benzene, zingiberene, α-bergamotene, β-bisabolene, geosmin and one unidentified sesquiterpene
P. freii	2-methyl-1-propanol, 3-methyl-1-butanol, 1,3-octadiene (two isomers), 3-octanone
P. hordei	2-methyl-1-propanol, 3-methyl-1-butanol, 1,3,6-octatriene, 3-heptanone, β-phellandrene, 3-octanone, limonene, 1,8-cineol, geosmin, γ-elemene and three unidentified sesquiterpenes
P. melanoconidium	2-methyl-1-propanol, 3-methyl-1-butanol, 1-ethyl-cyclopentene
P. nalgiovense	Ethyl acetate, 2-methyl-1-propanol, 3-octanone, RI1404
P. nordicum	Acetone, 2-butanone, 2-methyl-1-propanol, 3-methyl-butanol, 2-pentanone, 2-methyl-isoborneol
P. olsonii	2-butanone, 2-methyl-1-propanol, 2-methyl-butanol, 2-heptanone, limonene, 2-nonanone
P. paneum	Acetone, 2-methyl-1-propanol, 3-methyl-1-butanol, 2-pentanone, β-elemene, β-caryophyllene, (+)-aristolochene, eremophilene, α-selinene, 14 unidentified sesquiterpenes and two unidentified diterpenes

P. polonicum	Ethyl acetate, 3-octanone, 2-methyl-isoborneol, γ-elemene, β-farnesene and three unidentified sesquiterpenes
P. roqueforti	Acetone, 2-methyl-1-propanol, 3-methyl-1-butanol, 2-methyl-butanol, isobutyl acetate, 1-octene, 2-pentanone, 3-octanone, β-myrcene, p-cymene, limonene, linalool, β-patchoulene, β-elemene, diepi-α-cedrene, β-caryophyllene, patchoulene isomer, (+)-aristolochene, RI1528, eremophilene, α-selinene, valencene, β-bisabolene, himachalene, 17 unidentified sesquiterpenes and one unidentified diterpene
P. solitum	2-methyl-1-propanol, 3-methyl-1-butanol, ethyl 2-methyl-butanoate, isobutyl 2-methyl-butanoate, 2-methyl-butyl 2-methyl-butanoate (two enantiomers), 2-methyl-isoborneol, β-elemene, (+)-aristolochene and one unidentified monoterpene
P. verrucosum	2-butanone, 2-methyl-1-propanol, 2-pentanone, 3-pentene-2-one, 3-methyl-1-butanol, 3-octanone, 2-methyl-isoborneol

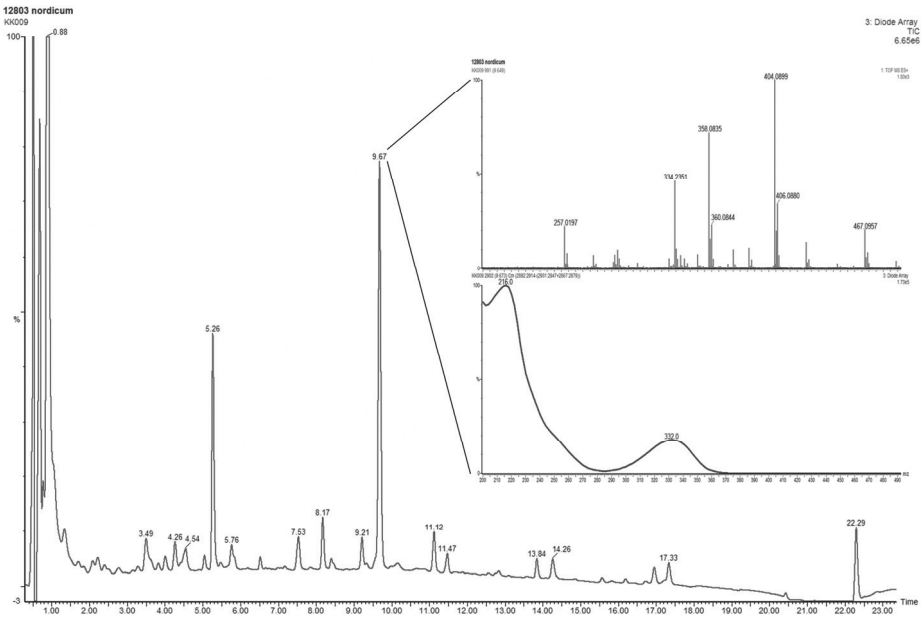

Figure 9. Positive identification of ochratoxin A by LC-DAD-MS analysis. In a LC-MS chromatogram mycotoxins in the sample can be identified by comparison of the UV spectra and the mass given by the mass spectra to literature. In this case the mycotoxin ochratoxin A is identified, the mycotoxin has a mono-isotopic mass of 403.0823, and thus a $[M+H]^+$ adduct of 404.0902, the detected mass is 404.0899, less than 1 ppm deviation from the calculated mass.

Mycotoxins form another group of compounds which are excellent as biomarkers, in the example of apple spoilage Penicillia, detection of the mycotoxin patulin will indicate that the apple spoilage has been done by *P. expansum* (Table 2, mycotoxins produced by food spoilage species from Table 1).

TRADITIONAL CHEMICAL ANALYSIS METHODS

Sampling

One of the big hurdles when an attempt is made to discover fungal growth in huge stocks of food is that fungal growth often occurs as a very local infestation, a so-called hot spot. Thus accurate sampling can be extremely difficult, particularly if dealing with spot tests, such as grain from a silo. When dealing with volatile metabolite sampling the problem lies in mak-

ing sure that the correct sampling technique is applied, so that relevant compounds can be collected for analysis.

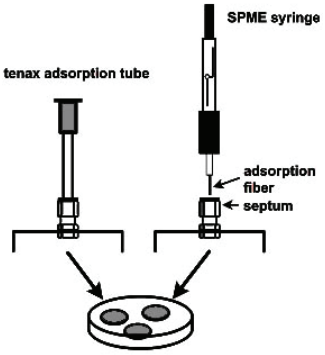

Figure 10. Collection set-up of volatile metabolites from a 3-point inoculated fungal culture on Tenax tube (left) and SPME fiber (right) (Nielsen *et al*., 2003).

How to make correct spot sampling and the sampling problem in general, is a science in itself, and going into further details is beyond the scope of this chapter. The spot sampling problem can however be avoided if head space analysis by GC-MS or electronic nose is performed. This analysis type is very suitable for large samples, such a grain in a silo.

HPLC

One of the traditional ways of analyzing non-volatile compounds is by HPLC. In order to attain good results, it is of utmost importance to have a good scheme of sample preparation. It is important to choose the right extraction procedure related to what type of compound has to be detected, i.e. a non-polar extraction method should be used to capture non-polar compounds and so forth. It is also desirable to minimize the amount of matrix within the sample, both to increase HPLC performance and sensitivity (Nielsen *et al*., 2003). The most commonly used sample preparation technique is solid phase extraction (SPE). In a typical HPLC set-up screening for mycotoxins, C18 columns are used, thus the equipment is run in the reverse phase mode.

This is ideal for separation of non-polar and semi-polar compounds. The typical detector system is a diode array detector (DAD) coupled to a mass spectrometer (MS). The combination of UV absorbance, retention index and accurate mass can be used to identify compounds by comparison to findings in literature (Figure 9) (Nielsen *et al*., 2003).

Gas Chromatography and GC-MS

Gas Chromatography (GC) is used for the analysis of volatile and semi-volatile compounds such as sugars, lipids, amino acids, sterols and trichothecenes. If analysis of semi-volatile compounds is desired, the compounds need to be derivatised prior to analysis in order to improve their volatility. In this chapter focus will be kept on GC as a tool for analysis of volatiles collected by headspace sampling. The first step in headspace analysis is sample collection and preparation. Typically samples are collected in Tenax TA (2,6-diphenylene-oxide polymer resin) adsorption tubes (Figure 10) or onto solid phase microextraction (SPME) fibers (Nielsen *et al*., 2003). For both methods, the material in the tube or on the fiber adsorbs volatile components from the headspace. Where Tenax TA adsorption most often takes place over night, sample collection on SPME fibers is done in less than an hour as the equilibrium between gas phase and SPME fiber occurs rapidly.

Sampling time depends on the level of volatiles present in the headspace, thus prior knowledge of the headspace concentration makes a good estimation of sampling time possible.

Adsorption of VOCs on Tenax TA material is dependent on the affinity of the compounds. SPME fibres consist of a fused silica core coated by a polymer adsorbent. The coating material on the fibre can be varied in polarity to suit the type of volatiles desired. Typical SPME fiber coatings are polydimethylsiloxane (for volatile compounds) and Carbowax/divinylbenzene (for alcohols and polar compounds) (Hamm *et al*., 2003); coating thickness is usually in the range of 30 to 100 μm. To achieve good separation during gas chromatography, good injection is required (Skoog *et al*., 1996). For SPME injection is done by thermal desorption directly in the GC injection port, thus SPME relies on rapid desorption to achieve good injection.

Tenax TA adsorption tubes are usually thermally desorbed, over a course up to half an hour, into a cold-trap. Once desorption of the Tenax adsorption tube is completed the cold trap is rapidly heated to release the volatile compounds which are then immediately introduced in the GC injector.

Since the capacity of capillary columns is limited, it is important that the system is not overloaded. Therefore, most injector systems are of the split/splitless type (Wilson and Walker, 1994; Skoog *et al*., 1996). This is important in case samples are collected from a concentrated source, for instance very close to the source of contamination or even immediately over a fungal culture. The split/splitless injector works by ensuring a constant flow onto the column, which is required to get reliable chromatography, while part of the sample is injected onto the column, the majority of the sample will be ejected from the system through the split/vent, thus preventing the need for diluting samples (Grob, 1993). Since the amount of volatile compounds bound to an SPME fiber will be substantially less than what is bound in a Tenax tube, GC-MS analysis from SPME samples typically start in splitless mode to get proper sample application on the GC column. The columns typically used in GC-MS systems are fused silica columns. The stationary phase on these columns can vary in polarity and film thickness and is chosen according to the polarity and volatility of the sample to be analyzed. Column polarity commonly varies between the non-polar DB-5 columns [(5%-Phenyl)-methylpolysil-oxane] and low/ medium polar DB-1701 columns [(14%-Cyanopropyl-phenyl)-methylpolysil-oxane] (Skoog *et al*., 1996). For analytical purposes the column dimensions are typically between 0.18 mm and 0.32 mm internal diameter (capillary column) by 30 m length. When selecting column film diameter, it is a choice between high separation power (thin column film) and higher capacity (thick column film). The thick film columns are better suited for separation of highly volatile compounds (Grob, 1993; Skoog *et al*., 1996). For instance for non-polar compounds such as mono- and sesquiterpenes, a non-polar column, such as a DB-5 column, is preferable for better separation of the compounds (König *et al*., 1999). GC columns are placed inside an oven for control of temperature. During a typical GC run the oven temperature is increased from 30 to 270 °C over a period between 30 to 60 minutes. By increasing the column temperature the volatile compounds in the sample will further be split by affinity to the stationary phase as a compound with high affinity to the stationary phase requires a higher temperature to leave the stationary phase than a compound with less affinity for the stationary phase.

The most common detector on any GC system is a flame ionization detector (FID). This detector utilizes a hydrogen / air flame for detection primarily of carbon containing compounds (Skoog *et al*., 1996). It is a highly robust detector, with a sensitivity level of approximately 10^{-13} g/s (Wilson and Walker, 1994; Skoog *et al*., 1996), but it does not yield any structural information, and on a single column GC instrument it cannot be used for compound identification. If identification is desired, using only an FID detector, the sample must be analyzed on two columns of differing type, and a standard of the compound must be analyzed as well for comparison of retention time / retention index. When more information about the volatile compounds is required, such as mass, for identification of the compound, mass spectrometers are used. Mass spectrometers are often used in combination with an FID. The typical mass spectrometer used for GC is a quadropole instrument. Mass spectral analysis provides structural information through the fragmentation pattern, the mass spectrum, formed by electron impact ionization (EI) this pattern can be searched in a database library for compound identification. Sensitivity in MS can be improved by a factor of around 50 from ng to pg level by scanning for few selected characteristic ions i.e. selected ion recording (SIR / SIM). Unfortunately MS cannot yield information on isomers and some compounds (Ramaswami *et al*., 1988) unless coupled to a GC system equipped with chiral columns as well as usage of chiral standards.

Case I: Identification of Penicillia and Detection of Mycotoxin Production by Volatile Metabolite Profiling and Identification

It is believed that volatile metabolites play an important role in the chemical interactions between fungi and their surrounding organisms. Recently, it has been shown that volatile metabolites produced by *Penicillium paneum* inhibit mycelial growth of different species of fungi belonging to a variety of genera (Chitarra *et al*., 2004) and it has also been shown that 1-octen-3-ol inhibits germination of spores from *P. paneum*, as well as induction of microcycle conidiation, showing that this compound is acting like a fungal hormone during fungal development in *P. paneum* (Chitarra *et al*., 2004; Chitarra *et al*., 2005).

It has been shown that the volatile metabolite profile is usually species specific within genus *Penicillium* (Larsen and Frisvad, 1995a). Fungal volatile metabolites include alcohols, ketones, esters, hydrocarbons such as small alkenes and mono and sesquiterpenes, of which the terpenes were shown to be most relevant for species identification (Larsen and Frisvad, 1995b). It is important to remember that the production of volatile metabolites is highly media specific, for instance the production of ketones and secondary alcohols derive from lipid degradation. When analyzing various series such as series *viridicata*, series *camemberti* and series *verrucosa*, within genus *Penicillium*, it has been shown to be difficult to differentiate the fungi to species level (Larsen *et al*., 2001). In those cases, the differentiation will be on a series level and the volatile compounds will be series specific instead of species specific. In some cases differentiation of species proves easier as with the very closely related species *Penicillium carneum*, *P. paneum* and *P. roqueforti* can be differentiated through volatile metabolite profiling and identification of particularly terpenes (Karlshøj and Larsen, 2005) (Figure 11). For *P. roqueforti*, for instance, the major sesquiterpenes compounds produced are β-elemene, selenine, patchuline (Larsen and Frisvad, 1995b) as well as (+)-aristolochene (Demyttenaere *et al*., 2001) whereas *P. carneum* produces far less terpenes but large amounts of 3-methyl-1-butanol.

It has been shown, using the knowledge that it is possible to differentiate species from genus *Penicillium* on basis of terpene profile, that it is possible to detect a fungal contamination at a ratio of 1000:1 of a *P. roqueforti* culture with *P. commune* within three days by analysis of volatile metabolites (Larsen, 1997), at a stage where it was very difficult to detect the contamination by morphological studies of the mixed culture.

This was achieved by combining SPME with SIR MS analysis mainly of ions characteristic to sesquiterpenes specific to the cheese associated fungi, such as 2-methyl-isoborneol and β-caryophyllene for *P. commune* and limonene, β-elemene and β-caryophyllene for *P. roqueforti*, providing a method for starter culture cross contamination checking.

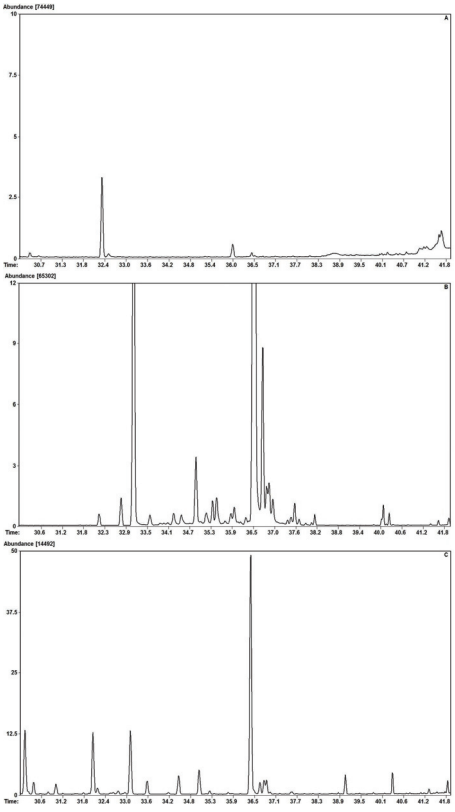

Figure 11. Chromatograms of the RI interval 1340-1800 for [A]: *P. carneum*, [B]: *P. roqueforti* and [C]: *P. paneum*. The abundance scale is in percentage of the abundance given in the top left corner of each chromatogram. The difference in volatile metabolite profile between the species is evident.

A series of studies have shown correlations between production of specific volatile metabolites and mycotoxin production. Release of specific sesquiterpenes unique to mycotoxenogenic *Aspergillus flavus* isolates, among them α-gurjunene, trans-caryophyllene and γ-cadinene, was shown to be correlated to aflatoxin biosynthesis, and the decline of aflatoxin biosynthesis was also correlated to the disappearance of the specific sesquiterpenes from the headspace (Zeringue, Jr. *et al*., 1993). Production of terpenes, among them trichodiene, has been shown to be biomarkers for production of trichothecenes in *Fusarium* species (Jelén *et al*., 1995; Pasanen *et al*., 1996; Demyttenaere *et al*., 2004) and it has been shown that it is possible to distinguish between different toxinogenic *Fusarium* species through sesquiterpene profiling (Demyttenaere *et al*., 2004). A good review on fungal volatile metabolites can be found in Jelén and Wasowicz (1998).

ELECTRONIC NOSE ANALYSIS

How the Electronic Nose Works

The electronic nose can be compared to the mammalian olfactory system where gasses stimulate receptors. The stimulated receptors send nerve signals to the olfactory cortex where they are analyzed and interpreted. It has been shown that the receptors generally are quite non-selective, though a few compounds like geosmin have a very low threshold value, thus a given receptor responds to many compounds and that many receptors respond to a given compound. This gives rise to patterns of responses for the olfactory cortex to analyze and interpret (Pearce, 1997). Like its counterpart, the electronic nose consists of a number of non-specific receptors, its sensors, whose signal patterns are analyzed, by either a neural network or chemometrics for interpretation. Like the mammalian olfactory system, the electronic nose relies on an array of receptors, a sensor array.

As seen in the following section, there are several different approaches to sensor design in terms of how the compounds are detected and thus what kind of signal pattern is generated for analysis.

Electronic nose technology has many applications. It has been used for screening for toxic gases, volatile organic compounds and food related compounds. Especially within the field of food technology, electronic noses have been applied in quality control (Maul *et al*., 2000; Werlein, 2001; Rye and Mercer, 2003; Berna *et al*., 2004; Vinaixa *et al*., 2004; García-González *et al*., 2004; Balasubramanian *et al*., 2004; Trihaas *et al*., 2005a), process control (Zondervan *et al*., 1999), maturity monitoring (Brezmes *et al*., 2005; Trihaas and Nielsen, 2005; Marrazzo *et al*., 2005; Trihaas *et al*., 2005b) etc. This has been done on both raw materials and manufactured products.

Until now the potential of the electronic nose to replace methods like GC-MS and HPLC / LC-MS for indirect mycotoxin estimation has not been explored. Obviously, for this potential to be realistic the e-nose has to be capable of separation of fungal species among a given associated funga when analyzing samples from a particular habitat. When comparing e-nose analysis with GC-MS analysis some of the key differences are that the e-nose sensors may not yield a sensitive respond to some of the compounds which can be found as key compounds, by GC-MS analysis, for a given food contamination problem (Schaller *et al*., 1998; Kohl, 2001). On the other hand, e-nose analysis has a potential for automation through construction of prediction models by chemometrics or neural network analysis. In addition, e-nose analysis is a very rapid method and thus suitable for high throughput screening.

Electronic Nose Sensors

The different sensor types used in electronic noses can be divided into four groups.

1. *Conductivity sensors*: Metal oxide semiconductors (MOS), intrinsically conductive polymer chemiresistors (ICP) and conductive polymer composite chemiresistors (CP)
2. *Electrostatic potential sensors*: Metal oxide semiconductor field effect transistors (MOSFET) and gas sensitive field effect transistor sensors (GASFET)

3. *Acoustic resonance sensors*: Thickness-shear mode / quartz crystal microbalance / bulk acoustic wave (TSM / QCM / BAW) and surface acoustic wave (SAW)
4. *Optical vapour sensors*: Polymer-deposited optical sensors (DPO) and self-encoded bead (SEB)

Conductivity Sensors

Metal Oxide Semiconductor Sensors

Metal oxide semiconductors (MOS) have been used commercially as gas alarms since the 1960s (Schaller *et al*., 1998). It took more than 20 years for the first cross-reactive MOS sensor array to be demonstrated, by Persaud and Dodd (1982). The sensors usually consist of a cylindrical ceramic former, which contains a heating element. The ceramic former is coated with a film of semiconductor material (Bartlett and Gardner, 1992; Strike *et al*., 1999; Gardner and Bartlett, 1999).

There are two types of semiconductors used, negative electron type (n-type) or positive hole (p-type). For p-type conductors, the density of holes in the valence band exceeds electron density in the conduction band the opposite is the case for n-type semiconductors. Electrical conduction in p-type semiconductors is mostly due to the movement of positive holes whereas electrical conductivity in n-type semiconductors is mostly due to the movement of electrons. N-type semiconductors, which usually consist of zinc oxide, tin dioxide, iron (III) oxide or titanium dioxide, respond mainly to reducing gases, while p-type semiconductors, which normally are oxides of nickel or cobalt, mainly respond to oxidizing compounds (Mielle, 1996). The semiconductor film can be coated as either a thick film (10-300 μm) or a thin film (6-1000 nm). Thin films, though harder to manufacture reproducibly, offer faster responses as well as higher sensitivity. Most often, commercial MOS sensors are of the thick film type (Schaller *et al*., 1998).

Sensor selectivity can be changed by various means. The semiconductor film can be doped with catalytic metals, (usually platinum or palladium), the operating temperature can be shifted (in the range of 50-400 °C) and the particle size in the semiconductor films polycrystalline structure can be changed (Watson and Yates, 1985; Morrison, 1987; Mielle, 1996; Albert *et al*., 2000; Strike *et al*., 1999).

Both reactions will lead to a change in the semiconductor material and thus to the measured change in conductivity.

Overall, the MOS sensors are less selective than for instance CP, BAW, SAW and MOSFET sensors (Mielle, 1996). MOS sensors are typically operated at high temperatures to increase reactivity and decrease the sensitivity to water (Albert *et al*., 2000).

Sensitivity to water, and therefore high operating temperature, along with a very high sensitivity to ethanol, poisoning by sulphur containing compounds as well as weak acids and slow baseline recovery when subjected to high molecular weight compounds are all drawbacks to this sensor type (Mielle, 1996; Schaller *et al*., 1998).

Conducting Polymer Sensors

Conducting polymer sensors have been applied as electronic nose sensors since the 1980s (Pelosi and Persaud, 1988; Bartlett *et al*., 1989). They are comprised of a substrate, fiberglass or silicone, coated by a conducting organic polymer between the two electrodes, which are usually gold plated (Amrani *et al*., 1995; Mielle, 1996). The polymer used is typically polypyrrole, polyaniline or polythiopene (Figure 13). The polymers act as the sensing unit of the sensor. The polymers in intrinsically conductive polymer chemiresistor sensors (ICP) are linear backboned comprised of repeating conjugated organic monomers that act as one-dimensional conductors as electrons will travel mostly through the conjugated linear backbone. In conductive polymer composite chemiresistor sensors (CP) polymers, carbon black or polypyrrole is used as the conductor, while non-conducting organic polymers serve as the insulating substrate (Strike *et al*., 1999).

To change the selectivity of the conductive polymer sensor changes in the polymerization conditions as well as the counter ion in use can be made. Furthermore, the oxidation state of the polymer can be changed after deposition on the sensor (Schaller *et al*., 1998). It has been

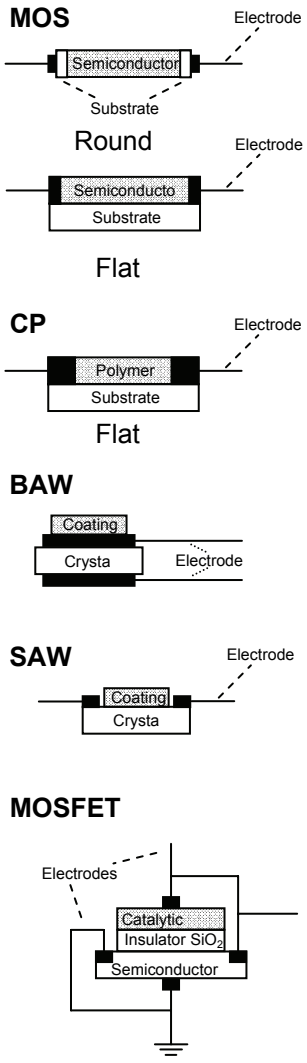

Figure 12. Schematic diagrams of five of the most common sensor types. MOS, Metal oxide semiconductor; CP, Conducting polymer; BAW, Bulk acoustic wave; SAW, Surface acoustic wave; MOSFET, Metal oxide semiconductor field effect transistor (Schaller *et al*., 1998; Albert *et al*., 2000).

shown that use of chiral material for the polymer in carbon black polymer composites (CP sensors) leads to the ability to differentiate between enantiomer compounds (Severin *et al*., 1998). It is also possible to imbed biomaterials such as antibodies, enzymes or cells in the polymer if so desired.

When a CP or ICP sensor is exposed to volatile compounds some of these will be adsorbed in the polymer causing swelling and altering the electron flow in the polymer. This will cause the measured change in conductivity (Shiers, 1995; Albert *et al*., 2000).

Conducting polymer sensors are operated at low temperatures (below 50 °C), which causes extreme sensitivity to moisture (Shiers, 1995). It has proven difficult to manufacture conductive polymer sensors reproducibly, due to the polymerization step that is hard to control (Mielle, 1996; Partridge *et al*., 1996).

Electrostatic Potential Sensors

Metal oxide semiconductor field effect transistor sensors (MOSFET) were firstly reported used by Lundström *et al*. in 1975 (Lundström *et al*., 1975; Lundström *et al*., 1990; Lundström *et al*., 1993). The MOSFET sensor is constructed of three layers: the top layer, the so-called gate, comprises of metal, underneath this is an insulating layer of an oxide, usually SiO_2, and in the bottom the semi-conducting substrate, often a p-type silicon with n-type channels on both sides of the metal gate (Schaller *et al*., 1998; Albert *et al*., 2000). In MOSFET sensors the metal gate traditionally consists of aluminium, whereas catalytic metals, such as palladium, platinum and iridium, are used, as the only difference in construction, in gas sensitive field effect transistor sensors (GASFET).

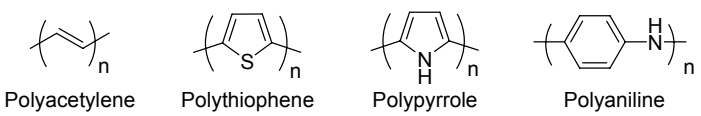

Figure 13. Structure of polymer backbone in insulating form for CP sensors (Albert *et al*., 2000).

Selectivity and sensitivity of MOSFET sensors can be changed by alterations in operating temperature, the type of metal used in the metal gate (i.e. change between catalytic and non-catalytic metals) and the microstructure of the metal in the metal gate (Lundström *et al*., 1975; Lundström *et al*., 1990; Albert *et al*., 2000). Palladium is preferable for hydrogen sensing and platinum and iridium for sensing polar compounds (Lundström *et al*., 1992; Albert *et al*., 2000).

The metal gate can be applied as either a continuous (thick) film (100-400 nm), or a discontinuous (thin) film (3-30 nm) (Müller and Lange, 1986; Sundgren *et al*., 1990; Winquist *et al*., 1992; Schaller *et al*., 1998). The thick film sensors primarily respond to compounds, which can be dehydrogenated, whereas the thin film sensors also respond to compounds such as carbon monoxide and ammonia (Lundström *et al*., 1975; Lundström *et al*., 1990; Spetz *et al*., 1992; Schaller *et al*., 1998). The dehydrogenation takes place on the metal gate, the hydrogen can adsorb to the gate and diffuse to the SiO_2 / metal interface were it forms a dipole layer. This changes the electrostatic potential of the MOSFET sensor (Bergveld, 1985; Lundström *et al*., 1992). Apart from adsorptions and reactions on the metal gate and hydrogen diffusion to the metal-insulator interface, thin film sensors also have their electrostatic potential changed on the insulator surface due to reactions of polar compounds on the metal oxide surface (Lundström *et al*., 1975; Lundström *et al*., 1990).

MOSFET sensors are silicon based and thus operate at temperatures below 250 °C (Strike *et al*., 1999). Typical operating temperatures for MOSFET sensors are in the range of 50 to 200 °C (Lundström *et al*., 1975; Lundström *et al*., 1990). These sensors, like MOS sensors, exhibit a fairly low sensitivity to moisture and furthermore are quite robust. On the down side, high manufacturing expertise is required for good sensor quality and reproducibility (Schaller *et al*., 1998).

Acoustic Resonance Sensors

King in 1964 introduced bulk acoustic wave sensors (BAW) also referred to as thickness-shear mode sensors (TSM), and by the name quartz crystal microbalance (QCM) (King, 1964). Wohltjen and Dessy introduced surface acoustic wave sensors (SAW) in 1979 (Wohltjen and Dessy, 1979a; Wohltjen and Dessy, 1979b; Wohltjen and Dessy, 1979c). Martin *et al*. reported the first use of SAW sensors in the 1980s (Martin *et al*., 1983; Martin *et al*., 1984; Martin *et al*., 1985).

Both BAW and SAW sensors consist of crystal discs, usually made of either quartz, lithium niobate or lithium thantalate coated with for instance chromatographic stationary phases, polymer films or other non-volatile compounds that adsorb vapors (Guilbault and Jordan, 1988; Nieuwenhuizen and Nederlof, 1992; Holmberg, 1997; Strike *et al*., 1999). In a BAW sensor the electrodes are positioned on top of and below the crystal, with coating on top of the electrodes. In a SAW sensor, the electrodes are both positioned on top of the crystal with the coating in between the electrodes (Albert *et al*., 2000). The coating on BAW sensors is quite thin (1 μm to 10 nm) and SAW sensors are constructed on such a minute scale as to be compatible with planar integrated circuits fabrication technology (Caliendo and Verona, 1992; Wünsche *et al*., 1995; Mielle, 1996).

In order to change the selectivity of the sensor, the coating used can be changed, and choices in coating are nearly limitless (Mielle, 1996; Hodgins, 1997; Strike *et al*., 1999).

Both BAW and SAW sensors, being vibrating crystals, exhibit resonance vibrations when an alternating current is applied to them, even at room temperature. This vibration is also dependant on the mass of the crystal. Thus when volatile compounds are adsorbed on the coating, the mass of the sensor increases which causes the resonance frequency of the sensor to change. This change is measured (Hodgins, 1997; Strike *et al*., 1999; Albert *et al*., 2000). BAW sensors generate three-dimensional waves through the crystal, perpendicular to the surface of the crystal and are operated at frequencies between 10-30 MHz. In SAW sensors the

waves generated are two-dimensional, only penetrating approximately one wavelength into the crystal. The SAW sensors are operated at frequencies between 100MHz to 1 GHz (Nieuwenhuizen and Nederlof, 1992; Holmberg, 1997; Albert *et al*., 2000). Due to its minute size, SAW sensors are very robust. They are also more sensitive than BAW sensors though both are less sensitive than the other sensor types. Because of their operating frequencies SAW sensors are much noisier than BAW sensors (Mielle, 1996; Hodgins, 1997). Unfortunately the coating technology is as yet not fully controlled, thus leading to poor batch-to-batch reproducibility. The acoustic sensors exhibit a high sensitivity to temperature and humidity fluctuations (Mielle, 1996; Doleman *et al*., 1998).

Optical Vapour Sensors

Polymer-deposited optical sensors (DPO) are comprised of an optical fibre on which an indicator is immobilized on the tip. The immobilized indicator is coated with a polymer. In self-encoded bead sensors (SEB) thousands of tiny beads (3.2 μm), of polymer or ceramic materials, are immobilized in acid-etched wells on the tip of the optical fibre. The beads consist of a ceramic or polymer material containing a vapour sensing dye. One of the dyes typically used in either sensor type is Nile Red. If a change in specificity is required the polymer, and for SEB also ceramic, material can be changed (Dickinson *et al*., 1997; Walt *et al*., 1998).

When vapour is adsorbed in the polymer the polarity of the surroundings of the vapour-sensing dye changes and the dye changes colour. In general, the more polar the vapour adsorbed, the more red-shifted the absorption and/or emission spectra of the dye will be. In PDO sensors many data can be collected simultaneously. Among those are changes in intensity, fluorescence lifetime and spectral shape (Albert *et al*., 2000). SMB sensors can be constructed in very small arrays, which will give rise to short response times and increased sensitivity. Five seconds is usually ample time for proper response and recovery of the sensor in experiments (Albert *et al*., 2000).

The most commonly used sensor types are MOS and CP sensors. Due to the high power consumption of MOS systems, almost all portable e-nose systems apply CP sensors.

Electronic Nose Signal Analysis

As with any other analysis method, proper data analysis of electronic nose signals is crucial. It is therefore important to know what you wish to achieve with your analysis in order to choose a proper data analysis technique. Apart from choice of technique, there are details to keep in mind when performing data analysis. Even the most successful analysis is worthless unless proper data analysis is performed.

Chemometrics

Use of chemometrics is the traditional data analysis tool for e-nose data. There is a broad variety of chemometric tools available and the most common ones are briefly described in the following section.

Principal Component Analysis

Principal component analysis (PCA) is frequently used to get an overview of the data obtained. One of the objectives of PCA is to reduce the dimensionality of data in order to make data analysis easier. Reduction of dimensionality also reduces the level of noise in the data, as the noise is omitted from the PCA model. PCA will also change the coordinate system of the data, moving the centre point to where the average data are (Esbensen, 2001). In PCA, data are described by principal components (PCs). The first PC is always chosen so that it explains the maximal variance, which is the same as minimizing the summed square transverse distances from the data points to the PC (least squares approach), the second PC is always orthogonal to the first PC and is chosen so it describes the second highest variance. The third PC must orthogonal to the first two and so on (Esbensen, 2001).

The maximal number of PCs in any PCA is no higher than the number of components minus one or the number of variables, which ever of the two is lower. In most cases PCA models consist of few PC's (typically one to four). Thus in PCA dividing data into structure and noise can be achieved.

In the PCA model the loadings give a relation between the real variables, for instance e-nose sensors, and the PCs. Similarly the scores show the relation between the samples/objects in the PC system. Scores and loadings are most often viewed visually in scores and loadings plots, and to see which loadings correlate positively or negatively with given objects, a bi-plot can be made (Figure 15).

The closer an object is to a specific loading in the bi-plot, the higher the positive correlation, if the object is on the opposite side of the ordinate or abscissa compared to the loading, the two are negatively correlated (Esbensen, 2001).

To check whether the PCA is modelling the data accurately object residuals can be viewed. The lower these residuals are, the better the model represents the data. To check model robustness it is important to validate the model. This is typically done by leave one out (LOO) validation, also known as full cross validation. In this validation method PCA models are made with one object removed from the model. Thus if there are 20 objects, 20 different models with 19 objects are made and compared.

Preprocessing is as important to data analysis as sample preparation is to data collection. If data are analyzed in which the scale of certain data is very different from each other, variables can be made more comparable so that no variable is allowed to dominate because of its range. This is typically done by multiplying the variable by 1/SDev (one divided by the variables standard deviation). This is not always the best solution if empirical variance is more-or-less comparable, as there is a risk of overemphasizing noise. This is overcome by using 1/(h + SDev) thus using an offset.

Finally it is important, when analyzing the results, to look for outliers. If the outlier is a known erroneous measurement it should be removed.

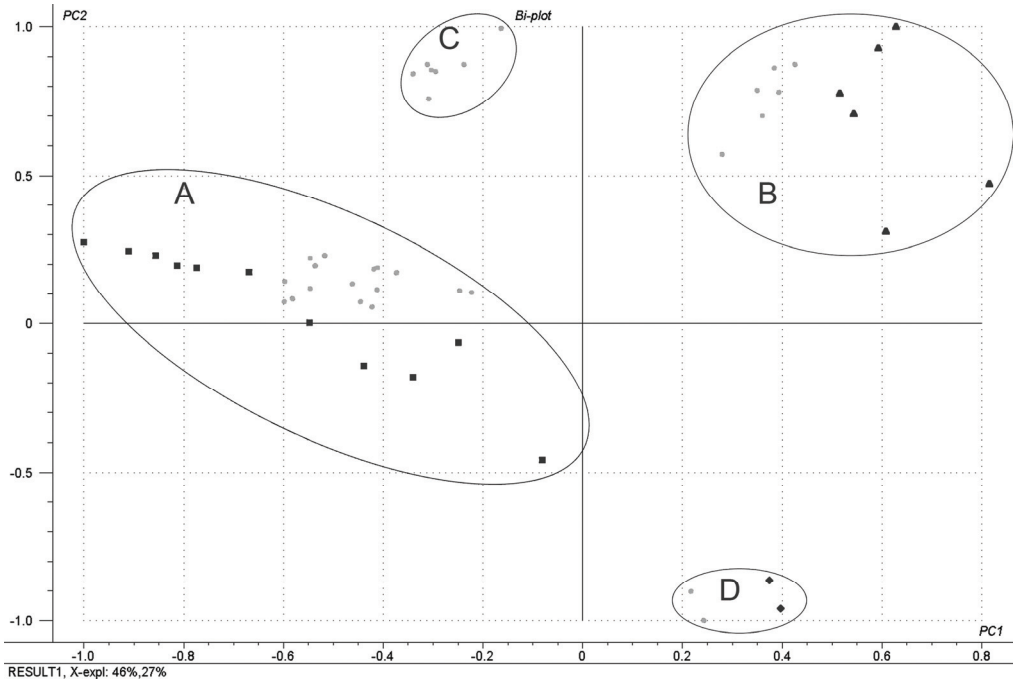

Figure 15. A bi-plot of PCA objects (dark grey squares, triangles and diamonds) and loadings (light grey circles). Loadings are positively correlated to objects which are on the same side of the ordinate or abscissa and negatively correlated to objects on the opposite side. Positive correlations are also seen between closely located loadings and objects. In this bi-plot loadings in ellipse C are positively correlated to all objects in ellipse B and all objects above the abscissa in ellipse A. All loadings in ellipse B are positively correlated to objects in ellipse B. Loadings in ellipse D are positively correlated to objects in the same ellipse, but negatively correlated to all other objects.

The influence plot can be used to check whether a sample has a high residual (poorly fitted by the model), a high leverage (high influence on the model) or both (Esbensen, 2001).

Principal Component Regression & Partial Least Squares Regression

These chemometric methods are used when prediction is desired. It is used to predict the value of Y, the dependent variable, (for instance the level of a given volatile component) from an X matrix, the independent variable, of measurements (for instance e-nose measurements) by regression. Construction of the prediction model is divided into calibration and validation. In the calibration step known X and Y values, measured in the way that is going to be used in future measurements, and which are representative for the future X measurements are used to construct the prediction model. Validation is then performed with a (or several) test set to ensure the prediction ability of the model. The test sets are important, and the use of multiple test sets for validation is advised. It is important that test sets used differ from one another (Esbensen, 2001). In Principal component regression (PCR) the prediction model is made by multi-linear regression of Y on a PCA model of X.

An approximated view of partial least squares regression (PLS) is that the PLS prediction model is constructed by multi-linear regression of a PCA model of Y on a PCA model of X

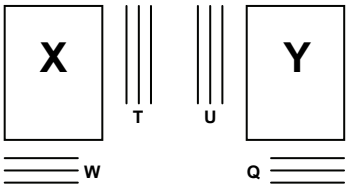

Figure 16. PLS prediction model is construction. The PCA done on X is based on a starting point score vector obtained from Y ($\mathbf{u}_1$) thus obtaining the "loading-weight" vector **w** which in turn is used to calculate the vector **t**. The PCA done on Y is based on **t** being the starting point score vector instead of **vector** u yielding the vector **q**. Multi-linear regression of the PCA model of Y on the PCA model of X is then performed yielding the PLS model (Esbensen, 2001).

This is not completely the case, as the PCA done on X is based on a starting point score vector obtained from Y and the later PCA done on Y is based on a starting point score vector obtained from the PCA on X (Figure 16). This is done to reduce the influence of large X values that do not correlate with Y (Esbensen, 2001).

Soft Independent Modelling of Class Analogy

For classification purposes, soft independent modelling of class analogy (SIMCA) is used. The classification model is usually based on individual PCA models of the classes. Construction of the classification model is a two step process consisting of training and classification. The model is trained by construction of the PCA models consisting of each class. If the classes are known, the classification is a supervised classification (Esbensen, 2001).

During the actual classification the models constructed in training are used to predict whether a new, unknown sample, belongs to any (several) of the classes described by the SIMCA model (Esbensen, 2001).

Neural Networks

Neural networks (NNs), or more accurately artificial neural networks, consist of an interconnected group of artificial neurons through which information is processed through a mathematical or computational model. This means that NNs are non-linear statistical data modelling tools with which for instance patterns in data can be found (Gurney, 1997). When using NNs for data analysis the NN has to learn how to interpret data. The typical way of teaching the NN to interpret data, for classification or prediction modelling, is to do supervised learning. This is done by feeding the NN data pairs of input data (x) and output data (y) with which the NN will seek to find the function that matches the examples. This is typically done by trying to minimize the mean-squared error between the NNs output value and the target output value (y) (Gurney, 1997). When using the learning algorithm it is important to make sure that the NN does not over-fit the training data and thus fails to find the true statistical process which generates the data.

This is particularly a problem when using small training sets (Gurney, 1997).

Case II: Discriminating between Food Spoilage Fungi by Electronic Nose Technology

Several studies have focused on food and feed quality control by electronic nose analysis. It has been shown that it is possible to classify grain samples, by use of an MOSFET and MOS sensor system coupled with neural network analysis, into three off flavour groups and a normal group (75% correct sample classification) as well as into two simple categories (90% correct sample classification), namely good or bad with higher percentage probability than the classification done by two grain inspectors (Börjesson *et al*., 1996). A more recent study has shown that it is possible to use a CP sensor e-nose to classify beef according to whether it is unspoiled ($<10^6$ cfu/g) or spoiled ($>10^6$ cfu/g) stored at 4°C and 10°C. The best classification was obtained on samples stored at 10°C where a classification accuracy of over 96% could be reached for individual data sets, and classification accuracy of approximately 70% when data sets were combined (Balasubramanian *et al*., 2004).

Differentiation or discrimination of bread spoilage organisms, grown on milled wheat agar, has been achieved on four *Eurotium* species, *Wallemia sebi* and one unknown *Penicillium* species using a 14 sensor CP system and PCA and discriminant function analysis (Keshri *et al*., 1998). Even before visible growth, 93% separation was achieved. This illustrates the potential of using electronic nose analysis for early spoilage detection in food stuffs.

Skimmed milk spoilage by *Pseudomonas fluorescens*, *Bacillus cereus*, *Candida pseudotropicalis* and *Kluyveromyces lactis* was investigated by electronic nose analysis with a 14 CP sensor system. After 60 minute incubation *Candida pseudotropicalis* could be distinguished from unspoiled milk and *Kluyveromyces lactis* was differentiated from control samples after 5 hours incubation. By discriminant function analysis it was possible to make a prediction model as to which organism was responsible for spoilage and thus correctly reclassify all samples (Magan *et al*., 2001).

In a very recent study, distinction between deoxynivalenol (DON) levels, from unknown source(s), in durum wheat (*Triticum durum*) samples was shown to be possible using a MOS sensor e-nose, classifying the samples in three groups, blank, medium and high DON levels (Cheli *et al*., 2005).

CONCLUSIONS

It is clear that volatile metabolites produced by fungi can be used as biomarkers for food quality. As previously stated, spot analysis of volatile metabolites can in many cases be more efficient than spot analysis of non-volatile metabolites such as mycotoxins (e.g. grain in a grain silo, coffee in coffee bags etc). Analysis of volatile metabolites can be used to make spot sampling of e.g. mycotoxin content more efficient since analysis of volatile metabolites present in the sample can give an overall estimate of the presence of spoilage organisms. Since it is not necessarily of interest to know exactly which compounds are present in the headspace of a given food sample, the e-nose seems to be a very promising tool for initial food quality screening. With the e-nose rapid sampling can be performed and with proper modelling it should be possible to determine whether a given sample analyzed is "good", "bad" or "unknown". The "bad" samples can immediately be discarded, whereas the "good" samples need no further testing and only the "unknown" samples need to be tested through traditional methods, thus improving speed, safety and efficiency of food quality spot sampling by using volatile metabolites as predictors of good and bad food quality. A final remark is that it would be even better if it becomes possible to predict mycotoxin content in samples by electronic nose analysis.

Acknowledgements

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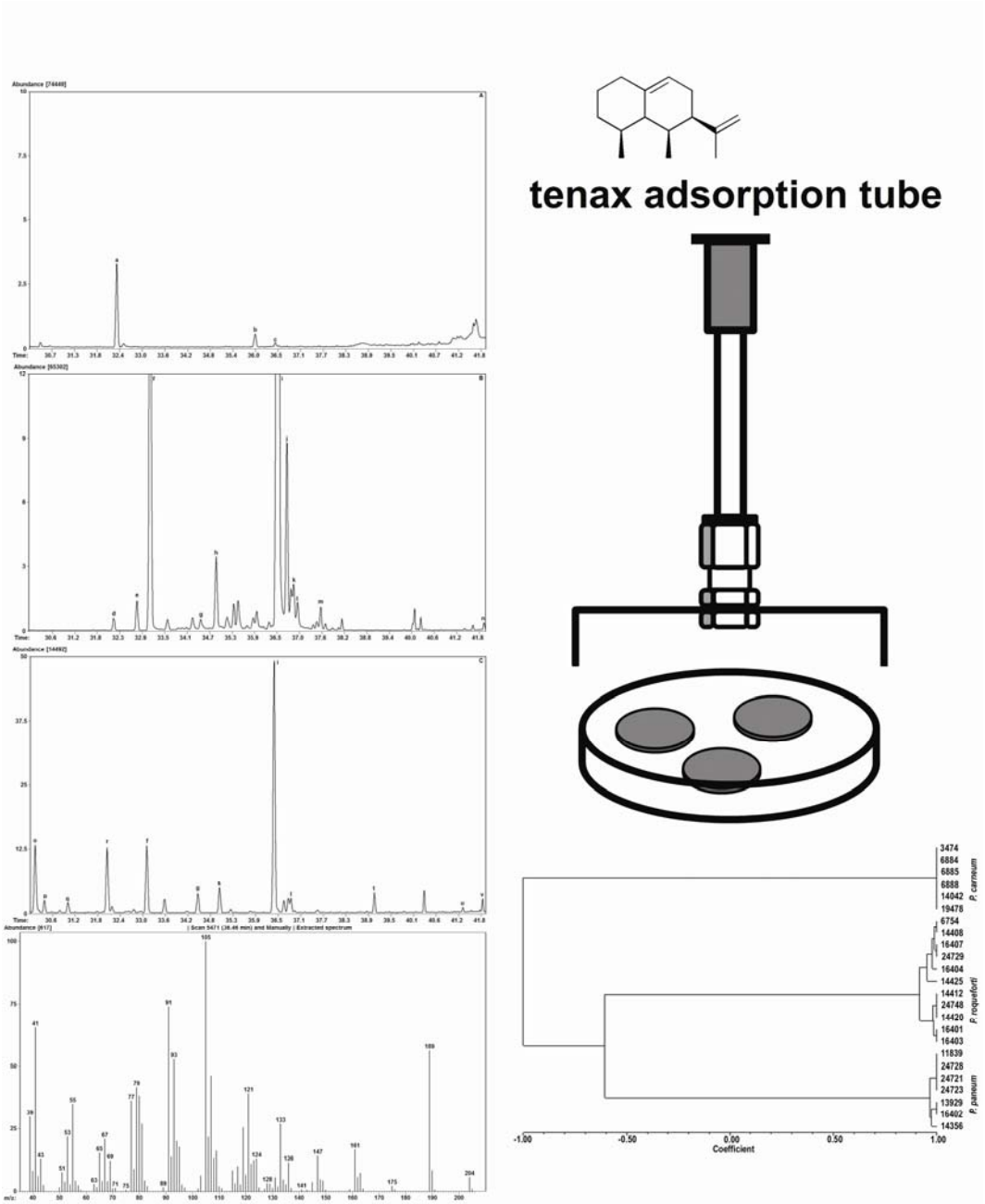
PAPER 1

Karlshøj, K. & Larsen, T.O. Differentiation of Species from the *Penicillium roqueforti* Group by Volatile Metabolite Profiling. *J. Agric. Food Chem.* **2005**, *53*, 708-715.



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Differentiation of Species from the *Penicillium roqueforti* Group by Volatile Metabolite Profiling

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Species from the *Penicillium roqueforti* group were differentiated by volatile metabolite profiling primarily of sesquiterpenes. A total of 24 isolates from species *P. roqueforti*, *Penicillium carneum*, and the recently described species *Penicillium paneum* were inoculated on yeast extract sucrose agar. Volatile metabolites were collected by diffusive sampling onto tubes containing Tenax TA, overnight between the fifth and sixth days of incubation. Volatiles were thermally desorbed and analyzed by gas chromatography coupled to mass spectrometry. The sesquiterpene area of the chromatogram was investigated, and potential sesquiterpenes were tabulated by comparison of their Kovats retention index and mass spectrum. In general, *P. carneum* isolates produced the lowest number of sesquiterpenes, all of which were unique for *P. carneum* within the *P. roqueforti* group. *P. roqueforti* and *P. paneum* produced a larger variety of volatile metabolites, some of which they have in common and some of which are unique for the two species. (+)-Aristolochene was found in samples from *P. paneum* and *P. roqueforti*. Other *Penicillium* species in which (+)-aristolochene was also detected were *P. commune*, *P. glandicola*, and *P. solitum*.

KEYWORDS: ***Penicillium roqueforti* group; *Penicillium roqueforti*; *Penicillium carneum*; *Penicillium paneum*; volatile organic compounds; volatile metabolite profiling; (+)-aristolochene**

INTRODUCTION

Historically *Penicillium roqueforti* has attracted a lot of attention due to its use as a cheese starter culture. This attention was given to a variety of topics such as aroma production and related strain development (*1*−*4*), morphology (*5*), strain differentiation by comparison of secondary metabolite patterns (*6*), mutagenicity testing (*7*), and the investigation of mycotoxin production, in both artificial media and cheese (*8*−*12*).

In 1989 *P. roqueforti* was divided into the two varieties, *P. roqueforti* var. *roqueforti* and *P. roqueforti* var. *carneum* (*13*). At some growth conditions *P. roqueforti* var. *carneum* produces the mycotoxins patulin and cyclopaldic acid (*14*). It is therefore important to distinguish between the two varieties *carneum* and *roqueforti*. A method to differentiate between *P. roqueforti* var. *roqueforti* and *P. roqueforti* var. *carneum* is the analysis of volatile metabolite production profiles (*15*).

The *P. roqueforti* varieties were reclassified as three new species, *P. roqueforti*, *P. carneum*, and *P. paneum*, based on ribosomal DNA analysis and secondary metabolite profiling (*14*). It was also shown that both *P. carneum* and *P. paneum*, given the right growth conditions, produce patulin and cyclopaldic acid (*14*). Thus, it is of importance to be able to distinguish *P. roqueforti* from both *P. carneum* and *P. paneum*.

The major aim of this study was to facilitate complete differentiation of the species in the *P. roqueforti* group by profiling the volatile organic compound (VOC) production mainly of the sesquiterpenes, because volatile production from *P. paneum* has not been investigated previously.

The versatile application of volatile metabolite production analysis by gas chromatography−mass spectrometry (GC-MS) is evident. It has been applied in fields such as chemosystematics (*16*), distinction of cheese-related fungi (*17*), screening of species-specific volatile metabolites from compost associated fungi (*18*), and the distinguishing of toxin-producing isolates from non-toxin-producing isolates in Aspergilli (*19*), *Fusarium sambucinum* (*20*), other *Fusarium* spp. (*21*), and *P. roqueforti* (*22*). Apart from volatile metabolite profile analysis by GC-MS, analysis by electronic nose technology could be of interest as electronic nose technology is increasingly used for analysis in areas such as food quality control, storage, and spoilage by bacteria and fungi (*23*−*30*) of processed as well as nonprocessed food.

It has been shown that *P. roqueforti* strains that produce PR toxin produce the volatile metabolite (+)-aristolochene (*31*), which has been found only in *P. roqueforti* within the genus *Penicillium* (*22*). (+)-Aristolochene is thus considered to be a biomarker for *P. roqueforti* within the genus (*22*, *31*). However, when volatile metabolites from 47 taxa within the genus *Penicillium* were characterized, a volatile metabolite was detected that had a mass spectrum similar to the one of (+)-aristolochene and was found in isolates of *P. commune*, *P. glandicola*, *P. roqueforti*, and *P. solitum* (*15*). The metabolite

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Table 1. Volatile Organic Compound Profiles for the *P. roqueforti* Group, RI 1340–1800[a]

		P. carneum						*P. roqueforti*											*P. paneum*						
compd	RI	3474	6884	6885	6888	14042	19478	6754	14408	14412	14420	14425	16401	16403	16404	16407	24729	24748	11839	13929	14356	16402	24721	24723	24728
1	1343																		X	X	X	X	X	X	X
2	1350																		X	X	X	X	X	X	X
3	1368																		X	X	X	X	X	X	X
4	1398																		X	X	X	X	X	X	X
5[b]	1400							X	X		X	X			X	X	X								
6	1402																		X	X		X	X	X	X
7[b]	1407	X	X	X	X	X	X																		
8	1414																		X	X	X	X	X		
9[b]	1418								*X*	*X*	*X*		*X*	*X*		*X*	*X*		*X*	*X*			*X*	*X*	*X*
10	1428							*X*	*X*	*X*	*X*	*X*	*X*	*X*	*X*	*X*	*X*	*X*	*X*	*X*	*X*		*X*	*X*	*X*
11[b]	1441							X	X	X	X	X	X		X	X	X								
12	1442																		X	X	X	X	X	X	
13	1461							X	X	X	X	X	X	X	X	X	X	X							
14[b]	1467							*X*	*X*	*X*	*X*	*X*	*X*	*X*	*X*	*X*			*X*	*X*	*X*	*X*	*X*	*X*	*X*
15[b]	1480							X	X	X	X	X	X	X	X	X	X	X							
16	1485																		X	X	X	X	X	X	X
17	1488							X	X	X	X	X	X		X	X	X								
18	1494							X	X	X	X	X	X	X		X	X	X							
19	1494																		X	X		X	X	X	X
20	1498								X	X	X		X	X		X	X								
21	1505							X	X				X			X	X								
22	1511								X	X	X		X	X		X									
23	1514							X	X	X	X	X	X	X	X	X	X								
24[b]	1517	X	X	X	X	X	X																		
25	1524							X	X	X	X		X	X		X									
26	1527								X				X			X									
27[b]	1532							*X*	*X*	*X*	*X*	*X*	*X*	*X*	*X*	*X*	*X*	*X*	*X*	*X*	*X*		*X*	*X*	*X*
28	1535	X	X	X	X		X																		
29[b]	1540							*X*	*X*	*X*	*X*	*X*	*X*	*X*	*X*	*X*	*X*		*X*	*X*			*X*	*X*	*X*
30	1545							*X*	*X*	*X*	*X*		*X*	*X*	*X*	*X*	*X*		*X*	*X*			*X*	*X*	*X*
31[b]	1547								*X*	*X*	*X*	*X*	*X*	*X*	*X*	*X*	*X*		*X*	*X*			*X*	*X*	*X*
32[b]	1551								X	X	X		X	X		X	X								
33	1567								X				X	X		X									
34[b]	1572							X	X	X	X					X	X								
35	1574								*X*	*X*	*X*		*X*	*X*	*X*	*X*	*X*		*X*	*X*			*X*		*X*
36[b]	1578								X	X	X		X	X		X									
37	1598									X	X	X	X	X		X		X							
38	1637																		X	X	X	X	X	X	X
39	1684							X	X	X	X	X	X	X		X	X	X							
40	1686							X	X	X	X	X	X	X	X	X	X	X							
41	1695							X	X	X	X	X	X	X	X	X	X	X							
42	1702																		X	X	X	X	X	X	X
43	1758																		X	X		X			
44	1773								X	X	X					X									
45	1789																		X	X		X	X		X
46	1789												X	X											
47	1790															X									
48	1800								X	X	X		X	X		X									

[a] An "X" indicates that the volatile was detected in samples from this isolate. For compounds unique for a species within the group the "X" is given in boldface type (**X**). For compounds produced by more than one of the three species the "X" is given in italic type (*X*). [b] Compound **5**, β-patchoulene; compound **7**, zingiberene; compound **9**, β-elemene; compound **11**, diepi-α-cedrene; compound **14**, β-caryophyllene; compound **15**, β-patchoulene isomer; compound **24**, geosmin; compound **27**, (+)-aristolochene; compound **29**, eremophilene; compound **31**, α-selinene; compound **32**, valencene; compound **34**, β-bisabolene; compound **36**, β-himachalene.

Table 2. Characteristic Ions of the Volatile Compounds, Six Largest Ions from *m/z* 50 to 150 and Three Ions from *m/z* 151 to 272 with Intensities Given in Parentheses

		characteristic ions								
compd	RI	*m/z* 50–150						*m/z* 151–272		
1	1343	91 (100)	133 (87)	105 (79)	106 (62)	147 (54)	148 (51)	189 (49)	204 (31)	162 (12)
2	1350	119 (100)	105 (59)	91 (54)	147 (52)	133 (43)	55 (35)	161 (55)	189 (53)	204 (30)
3	1368	121 (100)	122 (98)	91 (91)	105 (79)	119 (75)	55 (68)	175 (69)	189 (48)	204 (56)
4	1398	122 (100)	91 (82)	147 (78)	121 (69)	105 (67)	119 (65)	175 (99)	204 (69)	189 (24)
5[a]	1400	119 (85)	105 (82)	91 (73)	93 (72)	106 (48)	55 (42)	161 (100)	189 (78)	204 (38)
6	1402	91 (60)	121 (53)	105 (53)	149 (48)	107 (47)	136 (45)	161 (31)	175 (29)	189 (25)
7[a]	1407	93 (100)	119 (89)	91 (47)	69 (39)	77 (34)	105 (17)	161 (4)	204 (1)	189 (1)
8	1414	119 (27)	91 (27)	105 (20)	147 (19)	133 (17)	77 (17)	189 (57)	161 (19)	nd[b]
9[a]	1418	93 (100)	81 (96)	67 (87)	68 (77)	79 (63)	53 (59)	161 (24)	189 (14)	162 (6)
10	1428	81 (100)	93 (98)	67 (90)	68 (83)	79 (67)	53 (62)	161 (20)	189 (15)	162 (6)
11[a]	1441	119 (100)	93 (67)	91 (49)	105 (44)	77 (53)	55 (31)	161 (22)	204 (16)	189 (12)
12	1442	91 (84)	133 (80)	105 (80)	119 (68)	135 (64)	93 (57)	162 (31)	189 (27)	161 (22)
13	1461	133 (100)	91 (23)	119 (16)	105 (15)	134 (14)	55 (11)	161 (16)	204 (4)	189 (3)
14[a]	1467	93 (60)	69 (57)	91 (54)	79 (53)	133 (40)	77 (36)	161 (13)	189 (6)	175 (4)
15[a]	1480	119 (100)	91 (36)	105 (33)	93 (27)	133 (26)	77 (24)	161 (62)	189 (52)	204 (18)
16	1485	91 (100)	148 (68)	105 (63)	133 (56)	79 (47)	77/93 (39)	161 (33)	189 (23)	204 (16)
17	1488	91 (100)	133 (84)	105 (69)	119 (53)	55 (47)	79 (42)	189 (91)	175 (50)	204 (37)
18	1494	55 (74)	57 (52)	69 (45)	83 (43)	56 (41)	70 (35)	189 (3)	nd	nd
19	1494	128 (22)	143 (17)	115 (17)	129 (17)	141 (16)	142 (15)	185 (100)	200 (29)	157 (23)
20	1498	105 (100)	106 (84)	91 (54)	120 (47)	119 (43)	77 (27)	204 (23)	176 (18)	161 (11)
21	1505	91 (98)	93 (68)	133 (61)	81 (61)	105 (60)	79 (58)	189 (22)	161 (17)	nd
22	1511	91 (84)	133 (72)	105 (65)	93 (57)	79 (57)	55 (49)	189 (84)	204 (31)	161 (21)
23	1514	91 (96)	134 (79)	105 (81)	93 (74)	133 (68)	79 (68)	189 (27)	161 (17)	204 (9)
24[a]	1517	112 (100)	55 (35)	69 (16)	97 (15)	126 (15)	83 (14)	182 (2)	nd	nd
25	1524	69 (62)	93 (59)	79 (51)	91 (45)	67 (37)	55 (33)	161 (16)	189 (7)	nd
26	1527	91 (85)	133 (70)	105 (67)	79 (63)	55 (62)	93 (51)	189 (66)	204 (30)	161 (30)
27[a]	1532	105 (100)	91 (71)	93 (51)	107 (46)	79 (40)	121 (39)	189 (57)	161 (16)	204 (6)
28	1535	119 (100)	132 (77)	105 (68)	55 (46)	91 (39)	131 (29)	202 (19)	nd	nd
29[a]	1540	91 (85)	79 (84)	93 (79)	105 (76)	107 (67)	119 (56)	161 (74)	189 (29)	204 (28)
30	1545	93 (77)	79 (72)	105 (72)	91 (69)	107 (65)	67 (62)	161 (28)	189 (23)	204 (21)
31[a]	1547	93 (81)	91 (81)	107 (74)	79 (70)	81 (66)	105 (62)	189 (62)	161 (62)	204 (28)
32[a]	1551	91 (79)	79 (74)	105 (69)	93 (62)	67 (56)	55 (53)	161 (52)	189 (33)	175 (24)
33	1567	91 (100)	105 (86)	131 (65)	77 (56)	145 (52)	55 (52)	202 (52)	187 (33)	159 (21)
34[a]	1572	69 (100)	91 (55)	92 (53)	77 (48)	55 (36)	79 (30)	161 (18)	204 (9)	189 (8)
35	1574	122 (100)	107 (82)	91 (56)	79 (44)	105 (42)	55/93 (40)	161 (91)	204 (14)	162 (9)
36[a]	1578	119 (100)	91 (89)	105 (85)	93 (53)	77 (51)	79 (51)	161 (51)	189 (29)	204 (15)
37	1598	55 (78)	57 (59)	83 (48)	69 (48)	97 (39)	56 (40)	nd	nd	nd
38	1637	123 (100)	151 (75)	55 (63)	97 (59)	111 (42)	110 (37)	151 (74)	166 (61)	165 (53)
39	1684	55 (82)	67 (65)	81 (50)	82 (44)	54 (42)	69 (39)	nd	nd	nd
40	1686	67 (97)	55 (63)	81 (61)	79 (45)	54 (44)	95 (33)	nd	nd	nd
41	1695	55 (84)	57 (62)	69 (49)	83 (49)	56 (42)	97 (40)	nd	nd	nd
42	1702	81 (50)	93 (42)	55 (41)	67 (35)	79 (29)	133 (27)	151 (35)	148 (22)	147 (8)
43	1758	55 (67)	91 (40)	146 (39)	145 (27)	69 (27)	70 (27)	159 (28)	202 (26)	243 (24)
44	1773	81 (46)	55 (41)	67 (38)	71 (33)	95 (24)	69 (24)	204 (10)	189 (9)	161 (9)
45	1789	55 (84)	91 (67)	105 (52)	146 (49)	145 (40)	131 (39)	216 (94)	159 (59)	272 (41)
46	1789	55 (85)	81 (43)	69 (40)	67 (34)	91 (30)	79 (27)	189 (22)	204 (13)	nd
47	1790	55 (80)	91 (41)	69 (33)	105 (30)	67 (29)	79 (27)	216 (38)	159 (26)	229/272 (16)
48	1800	55 (42)	81 (41)	67 (40)	71 (37)	79 (26)	95 (24)	189 (19)	204 (14)	161 (14)

[a] Compound **5**, β-patchoulene; compound **7**, zingiberene; compound **9**, β-elemene; compound **11**, diepi-α-cedrene; compound **14**, β-caryophyllene; compound **15**, β-patchoulene isomer; compound **24**, geosmin; compound **27**, (+)-aristolochene; compound **29**, eremophilene; compound **31**, α-selinene; compound **32**, valencene; compound **34**, β-bisabolene; compound **36**, β-himachalene. [b] Not detected.

was not identified, but referred to by its Kovats retention index (RI), 1521, and its characteristic MS ion fragmentation pattern. Thus, volatile metabolite production of isolates from species *P. commune*, *P. glandicola*, *P. roqueforti*, and *P. solitum* were also investigated in this study, to determine whether the unidentified compound (RI 1521) in each species is in fact (+)-aristolochene.

The overall goal of this study is to differentiate among the three species of the *P. roqueforti* group by volatile metabolite profiling, mainly of sesquiterpenes, as well as to investigate whether (+)-aristolochene is a unique marker for *P. roqueforti* within the genus *Penicillium*.

MATERIALS AND METHODS

Fungi and Media. All isolates used in this study were obtained from the Fungal Culture Collection at BioCentrum-DTU (IBT collection), Technical University of Denmark, Kgs. Lyngby, Denmark. The following strains were investigated (listed by IBT number); *P. carneum*, 3474, 6884, 6885, 6888, 14042, and 19478; *P. commune*, 6373, 10763, 14135, and 21513; *P. glandicola*, 4168, 6592, and 21529; *P. paneum*, 11839, 13929, 14356, 16402, 24721, 24723, and 24728; *P. roqueforti*, 6754, 14408, 14412, 14420, 14425, 16401, 16403, 16404, 16407, 24729, and 24748; *P. solitum*, 10254 and 21545. The strains were center point inoculated from spore suspensions on 9 cm Petri dishes containing yeast extract sucrose agar (YES) medium. The YES medium consisted of yeast extract (Difco, 212750) (2%), sucrose (15%), $MgSO_4 \cdot 7H_2O$

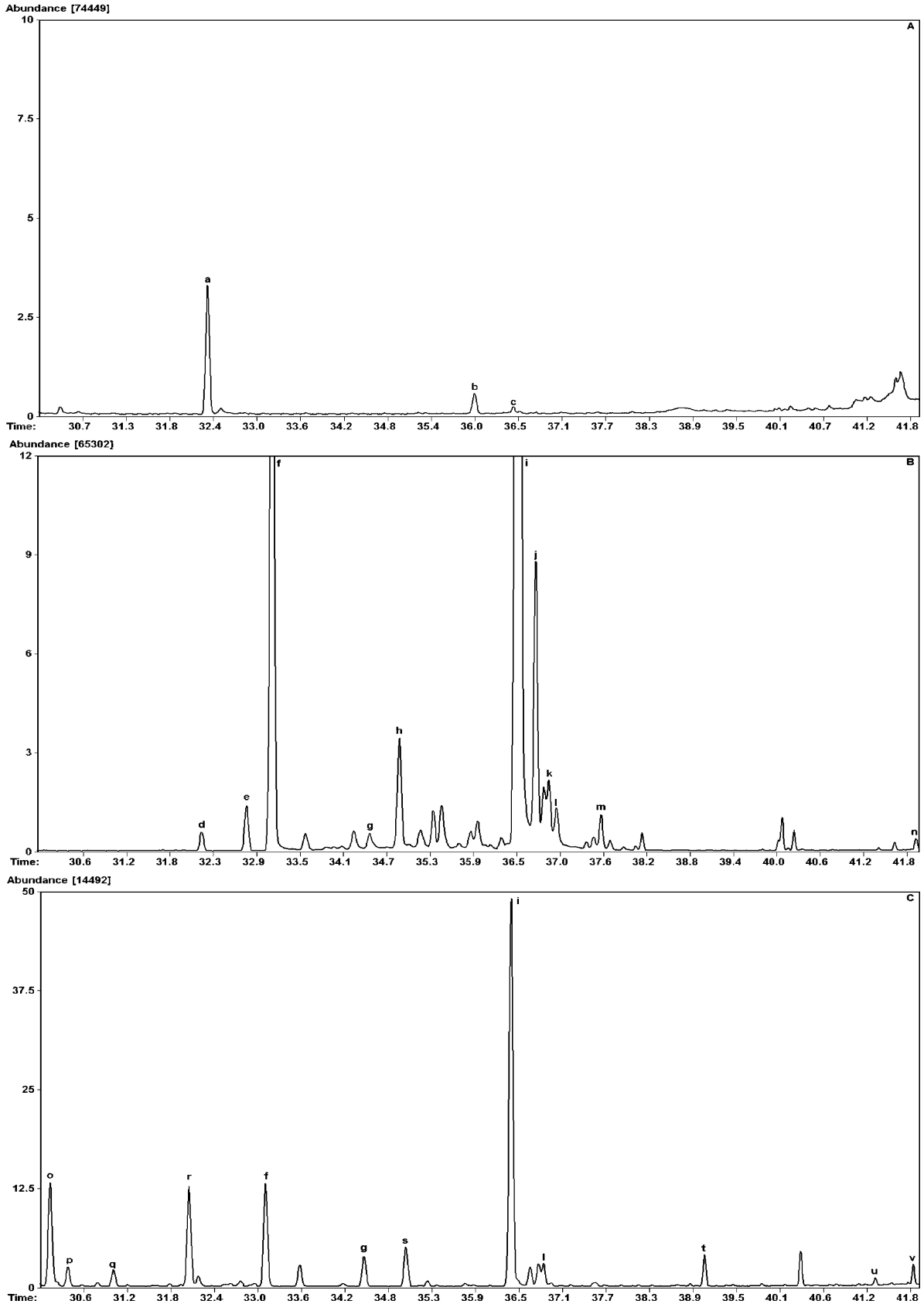

Figure 1. Chromatograms of the RI interval 1340–1800 for (**A**) *P. carneum* (IBT 19478), (**B**) *P. roqueforti* (IBT 16407), and (**C**) *P. paneum* (IBT 11839). The abundance scale is in percentage of the abundance given in the top left corner of each chromatogram. The compounds at the following peaks are noteworthy: (a) zingiberene; (b) geosmin; (c) compound **28**; (d) β-patchoulene; (e) β-elemene; (f) compound **10**; (g) β-caryophyllene; (h) β-patchoulene isomer; (i) (+)-aristolochene; (j) eremophilene; (k) α-selinene; (l) valencene; (m) compound **35**; (n) compound **47**; (o) compound **1**; (p) compound **2**; (q) compound **3**; (r) compound **4**; (s) compound **16**; (t) compound **38**; (u) compound **43**; (v) compound **45**.

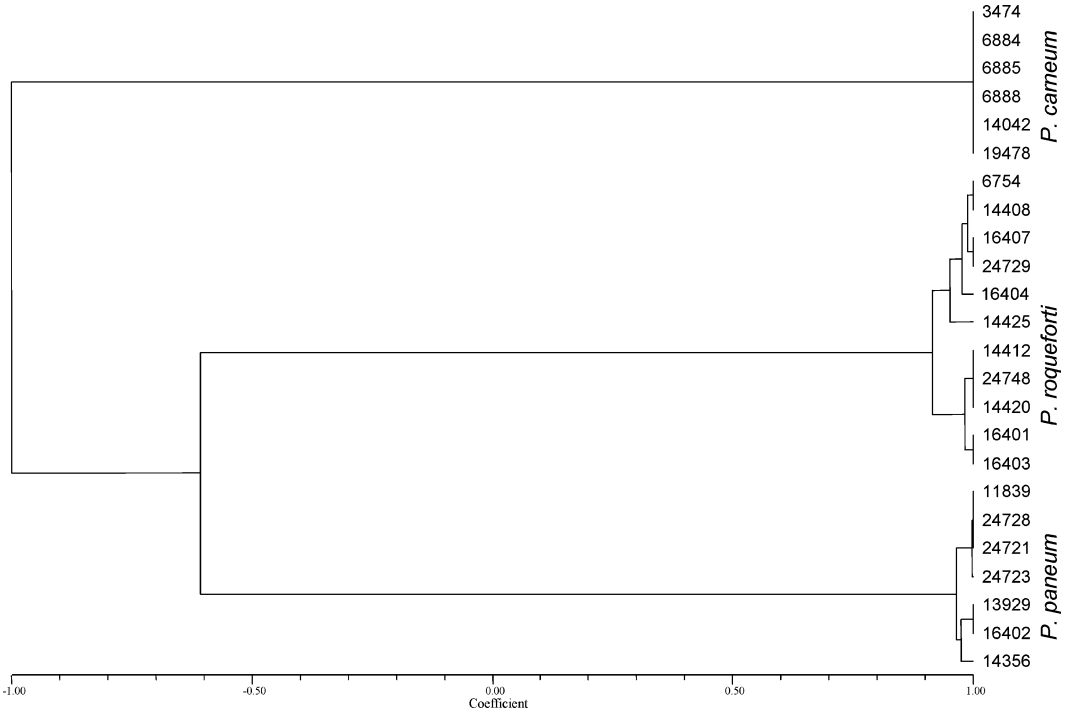

Figure 2. Dendrogram of the isolates from the three species of the *P. roqueforti* group based on volatile metabolite profile of mainly sesquiterpenes. The isolates are clearly separated into three species.

(0.05%), $ZnSO_4 \cdot 7H_2O$ (0.001%), $CuSO_4 \cdot 5H_2O$ (0.0005%), water to 1.0 L, pH 6.5, and agar (2%). The cultures were incubated in the dark at 25 °C for 5 days.

Collection and Analysis of Volatile Metabolites. Volatile metabolites were collected overnight between days five and six at room temperature. The volatiles were collected by diffusive sampling onto Tenax TA adsorption material placed in Perkin-Elmer tubes according to the method described in ref *32*. Volatiles collected were thermally desorbed on a Perkin-Elmer ATD 400 coupled to a Hewlett-Packard 5890 gas chromatograph further coupled to an HP 5972 mass selective detector. Separation of the volatiles was done on a DB-1701 (J&W) capillary column (30 m, 0.25 mm, 1.0 μm) using He as carrier gas. Initial pressure was 13 psi, and the He flow was 1 mL/min. The system was run at a 1:75 split, and the injection temperature was set to 250 °C. Chromatographic conditions were as follows: initial temperature, 35 °C for 1 min, raised at 4 °C min^{-1} to 175 °C and then at 10 °C min^{-1} to 260 °C. Separated compounds were characterized by their mass spectra generated by electron ionization (EI) at 70 eV at a scan range from *m*/*z* 33 to 330.

Data Analysis. Mass spectra from compounds with identical retention indices were compared to account for similarity. The identity of the compounds was established by comparison of mass spectra and volatile metabolite profiles with data from refs *15*, *22*, and *31*.

Cluster analysis of the volatile metabolite data was carried out with NTSYSpc (version 2.11N, Exeter software), with the data matrix set up as a qualitative, binary (1, 0) matrix of the volatiles listed in **Table 2**. The data were analyzed by UPGMA using the Yule distance coefficient to minimize the influence of biological variety between isolates from the same species as suggested by Frisvad (personal communication, 2004).

RESULTS AND DISCUSSION

The growth medium was chosen on the basis of the knowledge that it induces high production as well as high diversity in the production of both volatile and nonvolatile secondary metabolites when used for incubation of species from the genus *Penicillium* (*15*). By center point inoculating the isolates and incubating them for 5 days, an age gradient was achieved within the colony, and thus the colony produced volatile metabolites corresponding to all growth phases.

In the chromatograms of the 24 isolates of *P. roqueforti*, *P. carneum*, and *P. paneum*, a total of 48 different compounds, mainly sesquiterpenes, were detected in the interval between RI 1340 and 1800. The volatile metabolite profiles are given by species and isolate number as well as RI in **Table 1**. They are also characterized by their six tallest peaks in the interval *m*/*z* 50−150 and the three tallest in the interval *m*/*z* 151−272 (shown in **Table 2**). As reported in ref *15*, *P. carneum* produced significantly fewer volatile metabolites than the other species, namely, the three volatile metabolites zingiberene, geosmin, and compound **28**, all of which apparently are unique within the *P. roqueforti* group at the given experimental conditions. *P. roqueforti* and *P. paneum* produced up to 32 and 21 volatile metabolites, respectively. Eight of the compounds, β-elemene, compound **10**, β-caryophyllene, eremophilene, compound **30**, α-selinene, compound **35**, and (+)-aristolochene, were detected from both species. Unique markers for *P. roqueforti*, within the *P. roqueforti* group, are β-patchoulene, diepi-α-cedrene, compound **13**, β-patchoulene isomer, compounds **17**, **18**, **20**−**23**, **25**, and **26**, valencene, compound **33**, β-bisabolene, β-himachalene, and compounds **37**, **39**−**41**, **44**, and **46**−**48**, whereas the unique markers for *P. paneum* are compounds **1**−**4**, **6**, **8**, **12**, **16**, **19**, **38**, **42**, **43**, and **45**. This difference in volatile metabolite profile is visualized in **Figure 1**. The volatile metabolite profile of *P. paneum* exhibits more VOCs in the RI intervals 1340−1400 and 1700−1800 and fewer VOCs in the interval of RI 1401−1699, compared to the profile for *P. roqueforti*.

There is a clear difference in pattern within the volatile metabolite profile from the three species; thus, even with biological variation within the species, it is possible to differentiate among the three species. This is in agreement with the results from ref *15*, which showed that *P. roqueforti* and *P. carneum* are distinguishable on the basis of their VOC profiles.

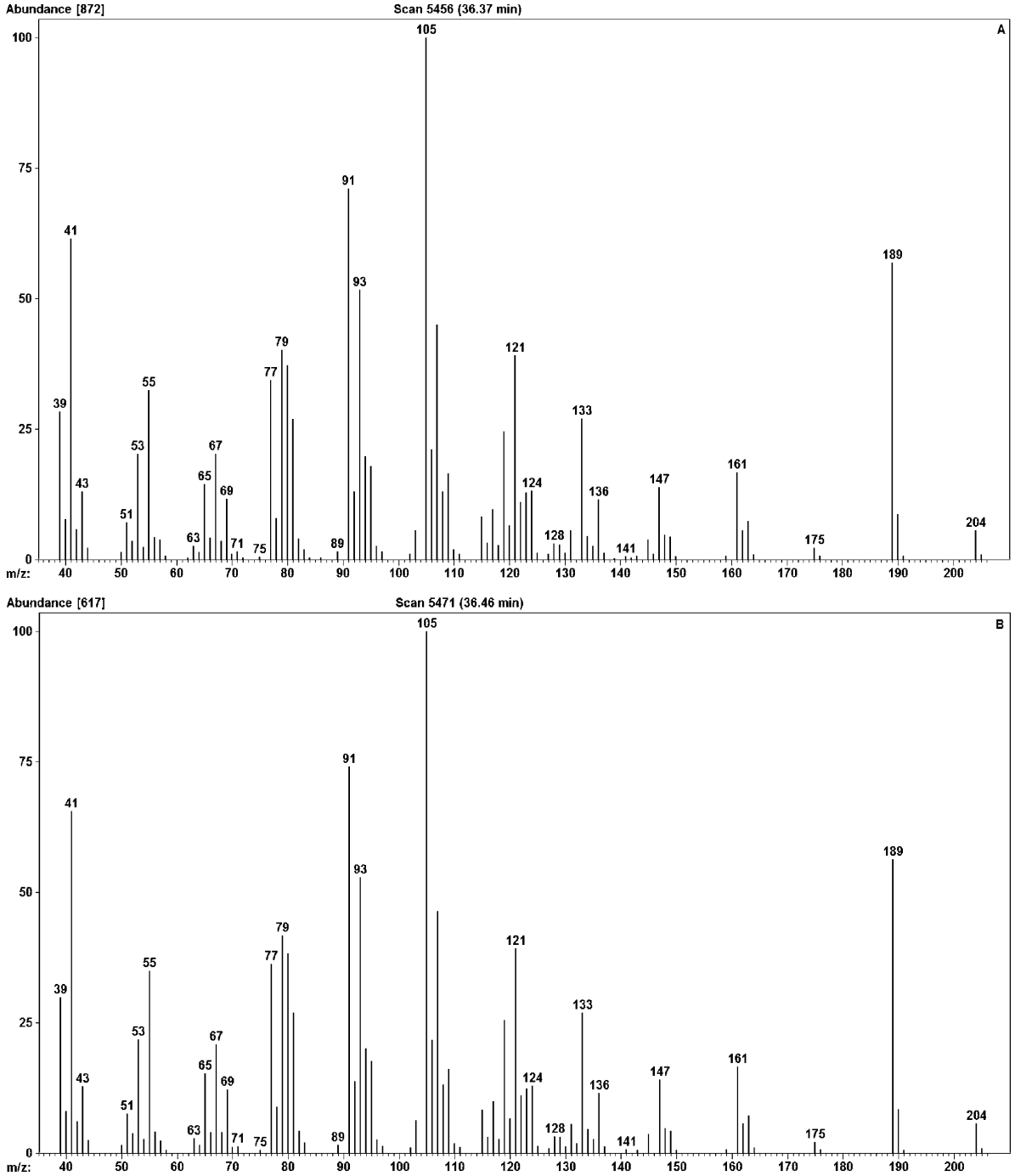

Figure 3. Mass spectrum comparison matching spectra from (**A**) (+)-aristolochene from the authentic sample and a compound of similar RI from (**B**) a sample of *P. paneum* (IBT 11839).

The three species were further investigated by hierarchical cluster analysis of the VOC data. This was desired, as it was the distinction of species rather than variety within the species, which was the objective of this study. The resulting dendrogram (**Figure 2**) shows clear separation of the three species, with *P. paneum* and *P. roqueforti* showing a closer relationship with each other than with *P. carneum*.

Because *P. paneum* and *P. roqueforti* had fairly similar volatile metabolite profiles, distinguishing them by use of an electronic nose might be more difficult than distinguishing any of the two from *P. carneum*. This of course is valid only if the sesquiterpenes play an important role in the overall volatile metabolite profiles as perceived by the electronic nose sensors. Work is in progress in our laboratory to investigate whether it is possible to distinguish the species in the *P. roqueforti* group by electronic nose as an instrument to predict mycotoxin production by species differentiation.

The presence of (+)-aristolochene was determined by comparison of RI and mass spectrum, including deconvolution of the peaks and relative peak intensities, with an authentic sample. **Figure 3** shows a comparison of matching mass spectra of (+)-aristolochene from the authentic sample and a compound with the same RI from a *P. paneum* sample (IBT 11839). Detection

of (+)-aristolochene in *P. roqueforti* matches the results published in ref *31*.

Among the 48 VOCs detected, three diterpenes, all unidentified, were found. The diterpenes were compounds **43** and **45**, found in samples from *P. paneum*, and compound **47**, found in samples from *P. roqueforti*. Volatile diterpene hydrocarbons have rarely been reported from fungi as done in ref *33*. Within the genus *Penicillium* only volatile mono- and sesquiterpenes have been described (*15*−*18*, *22*, *31*, *34*−*36*).

In conclusion, it has been demonstrated that *P. paneum* has a unique volatile metabolite profile when compared to *P. roqueforti* and *P. carneum*. This clearly supports *P. paneum* being a separate species within the *P. roqueforti* group. Second, (+)-aristolochene turned out not to be a unique biomarker for *P. roqueforti* because it was detected in volatile samples from *P. commune*, *P. glandicola*, *P. paneum*, and *P. solitum*. The fact that this important group of fungi can be distinguished by GC-MS makes it feasible that electronic nose technology can also be applied for quality control purposes in the food industry. This, of course, is of particular interest in the cheese industry during both production and storage.

ABBREVIATIONS USED

YES, yeast extract sucrose agar; GC-MS, gas chromatography−mass spectrometry; RI, Kovats retention index; VOC, volatile organic compound; EI, electron ionization.

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PAPER 2

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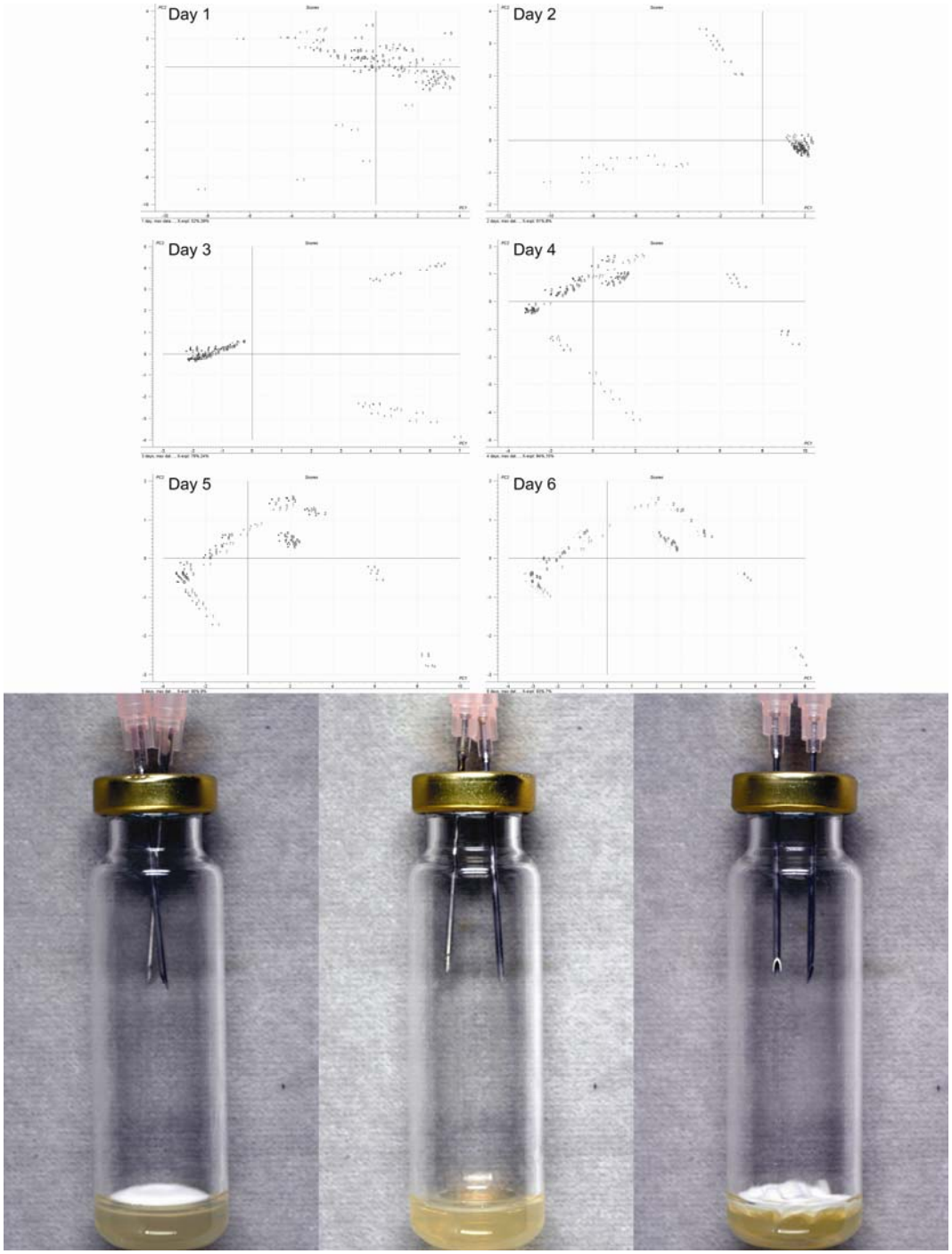

Differentiation of Closely Related Fungi by Electronic Nose Analysis

K. KARLSHØJ, P.V. NIELSEN, AND T.O. LARSEN

ABSTRACT: In this work the potential of electronic nose analysis for differentiation of closely related fungi has been described. A total of 20 isolates of the cheese-associated species *Geotrichum candidum, Penicillium camemberti*, *P. nordicum*, and *P. roqueforti* and its closely related species *P. paneum*, *P. carneum* as well as the noncheese-associated *P. expansum* have been investigated by electronic nose, GC-MS, and LC-MS analysis. The isolates were inoculated on yeast extract sucrose agar in 20-mL headspace flasks and electronic nose analysis was performed daily for a 7-d period. To assess which volatile metabolites the electronic nose potentially responded to, volatile metabolites were collected by diffusive sampling overnight onto tubes containing Tenax TA, between the 7th and 8th day of incubation. Volatiles were analyzed by gas chromatography coupled to mass spectrometry and the results indicated that mainly alcohols (ethanol, 2-methyl-1-propanol, and 3-methyl-1-butanol) and ketones (acetone, 2-butanone, and 2-pentanone) were produced at this stage. The volatile metabolite profile proved to be species specific. Nonvolatile metabolites were collected on the 8th day of incubation and mycotoxin analysis was performed by high pressure liquid chromatography coupled to a diode array detector and a time of flight mass spectrometer. Several mycotoxins were detected in samples from the species *P. nordicum*, *P. roqueforti*, *P. paneum*, *P. carneum,* and *P. expansum*. Differentiation of closely related mycotoxin producing fungi incubated on yeast extract sucrose agar has been achieved, indicating that there is a potential for predicting production of mycotoxins on food and feedstuffs by electronic nose analysis.

Keywords: biomarkers, electronic nose, fungi, mycotoxins, volatile metabolites

Introduction

Volatile metabolites produced by fungi have traditionally been analyzed by gas chromatography coupled to mass spectrometry (GC-MS) (Jelén and others 1995; Larsen and Frisvad 1995a, 1995b; Larsen 1997; Demyttenaere and others 2001, 2004; Jelén 2002, 2003). It has been shown that the profile of volatile metabolites produced by species of genus *Penicillium* can be used as a tool for chemotaxonomy (Larsen and Frisvad 1995a, 1995b)—even when it comes to closely related species (Karlshøj and Larsen 2005)—and for distinction of cheese-related fungi (Larsen 1997). This is therefore a potential tool for indirect prediction of mycotoxin production. Mycotoxins and other nonvolatile metabolites have traditionally been detected by use of high performance liquid chromatography coupled to diode array detection (HPLC-DAD) and often also to mass spectrometry (LC-DAD-MS) (Smedsgaard 1997; Chao-Ling and others 2000; Sewram and others 2000; Shephard and Leggott 2000; Ventura and others 2004). Similarly, profiling of nonvolatile metabolites, mycotoxins included, has also been used in chemotaxonomy of fungi from genus *Penicillium* (Frisvad and Filtenborg 1983, 1989). Unfortunately both LC and GC methods are time and resource consuming. It is, therefore, desirable to develop faster and cheaper tools.

In several industries a new analysis method for quality control has appeared—the electronic nose (e-nose). The nonspecific sensor response gathered by an e-nose can be used for classification and prediction purposes; for instance, it has been used for food quality control, storage, and spoilage by bacteria and fungi (Börjesson and others 1996; Jonsson and others 1997; Keshri and others 1998; Magan and Evans 1999; Magan and others 2001; Korel and Balaban 2002; Olsson and others 2002; Balasubramanian and others 2004; Vinaixa and others 2004; Cheli and others 2005; Hansen and others 2005; Trihaas and Nielsen 2005) of processed as well as nonprocessed food.

It has been shown that cheese, like most other food and feedstuffs, has an associated funga (Lund and others 1995, 1996; Filtenborg and others 1996). For cheese, this associated funga consists of species that are used as starter cultures in some cheeses (*Penicillium roqueforti* in blue mold cheeses and *Penicillium camemberti* and *Geotrichum candidum* in Camembert cheeses) (Samson and others 1977; Moreau 1980; Rousseau 1984; Bianchi-Salvadori 1987; Marth and Yousef 1991) as well as species that are solely contaminants (for example, *P. nordicum*, *P. carneum* and *P. paneum*). Contaminants are unwanted for several reasons, not least due to their ability to produce mycotoxins, which several studies have shown (Jarvis 1983; Frisvad and Filtenborg 1989; Siemens and Zawistowski 1993; Frisvad and Samson 2004). Among the mycotoxins produced by cheese-associated fungi the most important mycotoxins are Ochratoxin A (*P. nordicum*), Patulin (*P. carneum* and *P. paneum*), Mycophenolic acid (*P. carneum* and *P. roqueforti*), Roquefortine A (*P. carneum* and *P. roqueforti*), Roquefortine C (*P. paneum* and *P. roqueforti*), and Cyclopiazonic acid (*P. camemberti*) (Jarvis 1983; Frisvad and others 2004).

The overall goal of this study has been to investigate whether it is possible to classify closely related fungi incubated on yeast extract sucrose (YES) medium by use of an e-nose as well as to analyze the samples for mycotoxin production to ascertain whether mycotoxins have been produced under the growth conditions used in this study. These investigations will thus help determine whether the e-nose could be a potential tool for identification of mycotoxinogenic fungi on food and feedstuffs, and hence whether there is potential for more detailed quality control by prediction of mycotoxin production in food and feedstuffs by e-nose analysis.

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Materials and Methods

Fungi and media

All isolates used in this study were obtained from the Fungal Culture Collection at BioCentrum-DTU (IBT collection), Technical Univ. of Denmark, Kgs. Lyngby, Denmark. The following strains were investigated (listed by IBT number): *G. candidum*, 9283, 9284, and 9286; *P. carneum*, 3474, 6884, 6888; *P. camemberti*, 11570, 11571, and 11755; *P. expansum*, 3487 and 15622; *P. nordicum*, 6728, 12803, and 14875; *P. paneum*, 11839, 13929, and 24721; *P. roqueforti*, 6754, 14408, and 16404. The strains were center point inoculated, in 4 replicates, in 20-mL headspace flasks (75.5 × 22.5 mm) containing 2-mL yeast extract sucrose agar (YES) medium, from spore suspensions made from 8-d-old YES medium streak cultures. In both cases the YES medium consisted of yeast extract (2%), sucrose (15%), $MgSO_4 \cdot 7H_2O$ (0.05%), $ZnSO_4 \cdot 7H_2O$ (0.001%), $CuSO_4 \cdot 5H_2O$ (0.0005%), water to 1.0 L, pH 6.5, and agar (2%). The headspace flasks were sealed with crimp caps (3.0 mm membrane thickness silicone/PTFE caps) and 2 syringe needles (Ø 1.20 × 40 mm) were inserted through the membrane to allow oxygen diffusion into and carbon dioxide out of the flasks (Figure 1). The cultures were incubated with the flasks lying down in a rack at room temperature, between 21 and 23 °C, in the laboratory.

Electronic nose measurements

The headspace of the 82 samples was analyzed daily, from day 1 through 7, using an αFox-3000 (Alpha M.O.S., France) electronic nose (e-nose), equipped with a HS-100 auto sampler (CTC Analytics AG, Switzerland). The sensor array system consisted of 12 metal oxide semiconductor (MOS) sensors. Ten minutes prior to sampling the syringe needles were removed from the crimp cap membrane and the flask was placed in the auto sampler. The headspace flasks with the samples were incubated for 1 min at 35 °C in the autosampler oven after which 500 μL of the sample was withdrawn from the head space and injected in the sensor chamber and flushed over the sensors at a rate of 150 mL/min. Immediately after sampling, the syringes were reintroduced through the crimp cap membrane and the flask returned to the rack.

Data collection was performed every half second for 2 min after which the sensors were flushed with dry air (maximum air humidity 0.5%) for 2 min; furthermore, there was a 2-min delay before the next sample was analyzed.

Collection and analysis of volatile metabolites

Volatile metabolites were collected overnight at room temperature between days 7 and 8 by diffusive sampling onto Tenax TA adsorption material placed in Perkin-Elmer tubes. To do this the lids were taken off the headspace flasks and the tubes were lowered as far as possible into the headspace flasks and held in place with a swagelok union. Volatiles collected were thermally desorbed on a Perkin-Elmer ATD 400 coupled to a Hewlett-Packard 5890 gas chromatograph further coupled to an HP 5972 mass selective detector. Separation of the volatiles was done on a nonpolar, VB-5 (ValcoBond®, SIS, N.J., U.S.A.) capillary column (60 m, 0.25 mm, 1.0 μm) using He as carrier gas. Initial pressure was 13 psi, and the He flow was 1 mL/min. The system was run at a 75:1 split, and the injection temperature was set to 250 °C. Chromatographic conditions were as follows: initial temperature, 35 °C for 1 min, raised at 4 °C/min to 175 °C and then at 10 °C/min to 260 °C. Separated compounds were characterized (tentatively identified) by their mass spectra generated by electron ionization (EI) at 70 eV at a scan range from m/z 33 to 330.

Extraction and analysis of nonvolatile metabolites

Extraction. The nonvolatile metabolites were extracted in a slightly modified plug extraction method adapted from the method described by Smedsgaard (1997). After the volatile metabolite collection was finished 6 plugs (Ø: 6 mm) were taken from 1 headspace flask culture of each isolate. The plugs were transferred to 2-mL vials and extracted ultrasonically for 45 min with 1 mL of extraction mixture containing ethyl acetate, dichloromethane, and methanol (3:2:1, v/v) with 1% (v/v) formic acid added. The organic phase was transferred to clean 2-mL vials with a Pasteur pipette and evaporated to dryness in a rotational vacuum concentrator (RVC). Prior to analysis the residues were redissolved ultrasonically for 10 min in 300 μL methanol and filtered into clean 2-mL vials through 0.45-μm PTFE syringe filters.

Chemicals. Chemicals used in LC-MS were as follows: acetonitrile, gradient-grade; formic acid, 88% acs; water, milli-Q grade.

LC-MS instrumental. LC-MS analysis was done on an Agilent HP 1100 with a photodiode array detector (DAD) (Agilent, Germany) coupled to a LCT (Waters-Micromass, UK) orthogonal time-of-flight (oaTOF) mass spectrometer equipped with a Z-spray electrospray ionization (ESI) source and a LockSpray probe controlled by MassLynx 4.0 software (Waters, UK).

Two microliters of sample was injected for negative ESI analysis and 3 μL was injected for positive ESI analysis. The column used was a 50 × 2.0 mm Luna C18(II) column with 3-μm particles (Phenomenex, Calif., U.S.A.) and a 4 × 2 mm ODS-C18 guard column (Phenomenex). The flow rate in both analyses was 0.3 mL/min with a linear water-acetonitrile gradient starting at 85:15 (water : acetonitrile) going to 100% acetonitrile in 20 min, maintaining 100% acetonitrile for 10 min prior to returning to starting conditions (85:15) in 5 min and equilibrating for 5 min. Formic acid (20 mM) was added to the water. UV spectra were collected by a diode array detector (DAD) from 200 to 700 nm at 2.0-nm resolution.

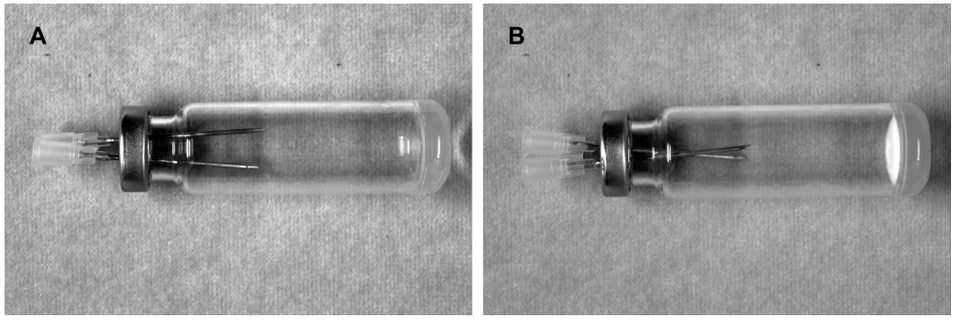

Figure 1 — Twenty-milliliter headspace flask with 2 mL YES medium, capped with a crimp cap and the membrane penetrated by 2 syringe needles to allow oxygen diffusion into the flask. A: headspace flask with control sample; B: headspace flask inoculated with *P. camemberti* (IBT 11755), incubated for 7 d.

Negative ESI was performed at a resolution > 5000 (full width half maximum [FWHM]), with the $[M-H]^-$ ion of leucine enkephalin ($[M-H]^-$ 554.2615 Da) as the lock mass. The capillary was held at 2500 V, cone 1 (skimmer 1) at 30 V, and cone 2 (skimmer 2) at 3 V.

Positive ESI was performed at a resolution > 6000 (FWHM), using $[M+H]^+$ ion of leucine enkephalin ($[M+H]^+$ 556.2615 Da) as the lock mass. The capillary was held at 3200 V, cone 1 (skimmer 1) at 30 V, and cone 2 (skimmer 2) at 5 V.

For both positive and negative ESI, the desolvation temperature was 450 °C, desolvation gas (nitrogen) at 500 L/h. The source was kept at 120 °C for negative ESI and 110 °C during positive ESI. MS spectra were collected as centroid data from *m/z* 100 to 1000 at a rate of 0.4 s per spectrum with 0.1 s interscan time. Reference spectrum was collected every 3 s.

Data analysis

Electronic nose data. The features extracted from the e-nose system responses were evaluated by principal component analysis (PCA) as well as by classification using soft independent modeling of class analogy (SIMCA) with each species (class) analyzed as a PCA group (Esbensen 2001). The e-nose data were standardized by multiplication with the inverse of the standard deviation and analyzed using systematic cross validation to account for the 4 replicates of each sample. For the analysis the software package The Unscrambler version 9.1 (CAMO, Norway) was used.

Volatile metabolites. Compounds were selected if the peak height was at least 20% of the height of the tallest peak in the chromatogram in order to only select major compounds produced. Mass spectra from compounds with identical retention indices were compared to account for similarity. The identity of the compounds was established by comparison of mass spectra and volatile metabolite profiles with data from the NIST mass spectral library (NIST92 library).

Nonvolatile metabolites. Mycotoxins were identified by comparison of UV spectra and *m/z* values with data from Nielsen and Smedsgaard (2003).

Results and Discussion

YES was selected as growth medium as it is known to induce high production of a wide range of both volatile and nonvolatile secondary metabolites when used for incubation of species of genus *Penicillium* (Larsen and Frisvad 1995a). The isolates were center point inoculated for 2 reasons. This inoculation method will resemble a point/spot infection in a given food or feedstuff; it will also induce an age gradient in the colony as it grows, thus enabling production of both volatile and nonvolatile metabolites corresponding to all growth phases.

The PCA scores plots from the e-nose analyses, days 1 through 7, reveal a growing differentiation of the fungal species, peaking at best visible separation on days 4 through 6, with the most efficient separation at day 6 (Figure 2). On day 6 measurements gave rise to a PCA scores plot in which all species, except *P. nordicum*, were separated from the control samples; furthermore, *G. candidum*, *P. carneum*, and *P. expansum* all grouped separately, showing differentiation of the mentioned species (Figure 2). *P. roqueforti* could not be completely distinguished from *P. paneum* or *P. camemberti* though slight grouping is evident.

The late, and poor, separation of *P. nordicum* from the blank samples is likely be related to the relatively slow growth of said species on YES medium compared to the other species investigated. *P. nordicum* is most often encountered on substrates rich in protein and/or fat (Frisvad and Samson 2004). The very oblong shape of the

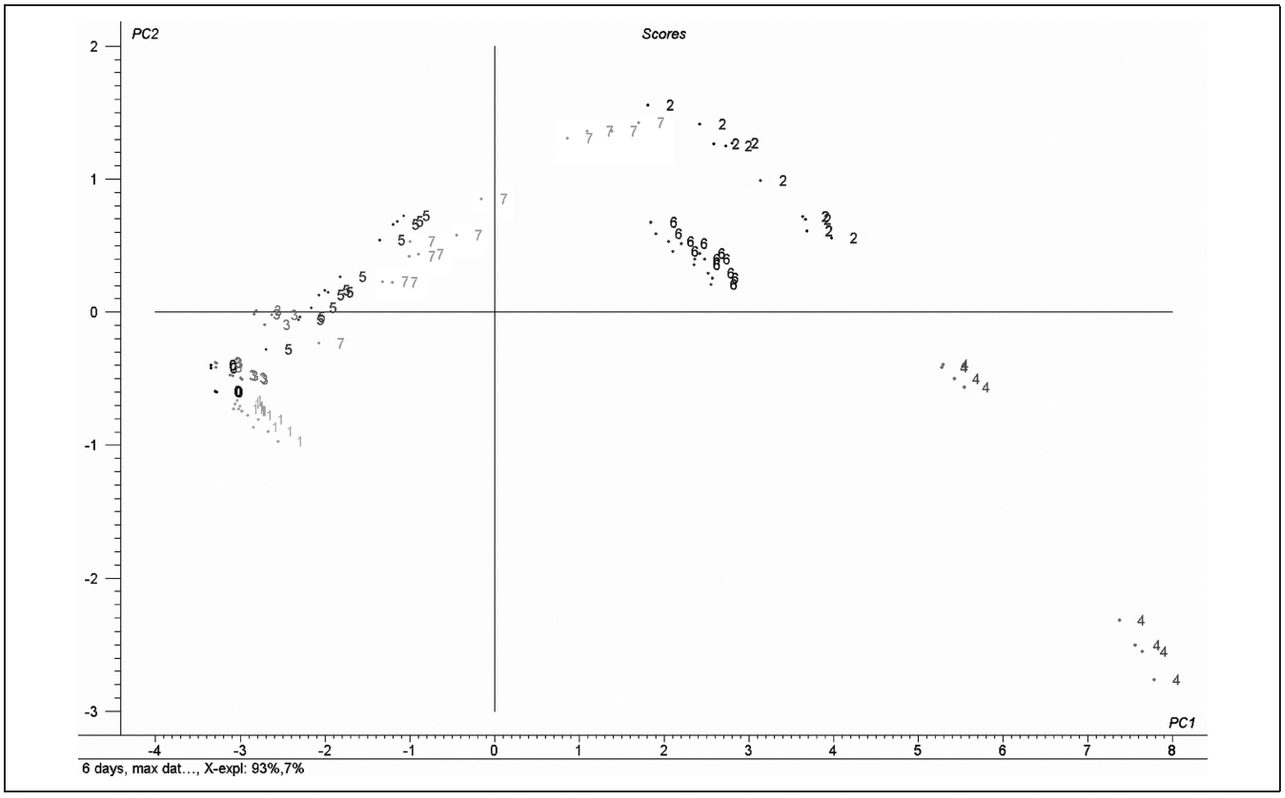

Figure 2—PCA scores plots from e-nose analysis of the 7 species, showing the separation at the 6th day analysis. 0, Blank; 1, *G. candidum*; 2, *P. camemberti*; 3, *P. nordicum*; 4, *P. expansum*; 5, *P. paneum*; 6, *P. carneum*; 7, *P. roqueforti*.

P. roqueforti group, stretching between 3 other species (*P. nordicum*, *P. paneum*, and *P. camemberti*) as seen in Figure 2, indicates a large biological variation within the species regarding volatile metabolite production, as sensed by the electronic nose.

By SIMCA analysis the total percentage of correct classification of fungal species varied between less than 10% to over 90% (Table 1), with the best classification achieved on day 6 with 93.9% correct classified. It is also seen that the control samples and species *P. nordicum* and *P. paneum* proved to be the hardest to classify overall.

The GC-MS analysis of the isolates shows that 14 major and a number of minor compounds not reported here were produced by the 7 species. The volatile metabolite profiles are given by species and isolate number as well as Kovats retention index (RI) in Table 2. As seen in the table, the identified compounds are mainly alcohols and ketones, and several of the compounds were detected in more than one of the analyzed species. For *G. candidum* production of dimethyldisulfide proved to be unique within the analyzed species. *P. camemberti* and *P. expansum* have fairly similar profiles, within the major compounds noted, as ethanol, 2-methyl-1-propanol, and 3-methyl-1-butanol were detected for both and only 1 compound was differing: ethylacetate, which was only detected from *P. expansum* isolates. *P. paneum* and *P. roqueforti* proved to have very similar volatile metabolite profiles as well, since acetone, 2-methyl-1-propanol, 2-pentanone, 3-methyl-1-butanol and an unknown compound (unknown 1 in Table 2) were detected from both. These 2 isolates differ in the sesquiterpenes detected, where 4 sesquiterpenes not detected in any of the other species were detected from *P. roqueforti* and 1 sesquiterpene, unique for the species was detected in samples from *P. paneum* in this analysis. Lastly, an unknown compound (unknown 2 in Table 2), which was not detected in *P. paneum*, was also detected in samples from *P. roqueforti*. Four of the volatile metabolites detected in samples from *P. roqueforti* and *P. paneum* were also detected in samples from *P. nordicum*; namely acetone, 2-butanone, 2-methyl-1-propanol, and 2-pentanone and no volatile metabolites unique to the species were detected. Only 2 volatile metabolites were detected in major amounts for *P. carneum*: acetone and 3-methyl-1-butanol, the latter in extreme quantities compared to any other metabolites measured in this entire analysis. All of the above findings correspond well with those reported in literature (Larsen and Frisvad 1995a; Berger and others 1999; Aldarf and others 2002).

It is interesting to note that *P. roqueforti* and *P. paneum*, which group close to one another in the PCA analysis of e-nose data, also produce very similar volatile metabolite profiles in the analysis performed after 8 d incubation. It could be interesting to investigate the change in volatile metabolite profile concurrent with e-nose measurements to determine whether there is a correlation between the detected compounds and e-nose signals.

Table 1 — Classification of fungal species by SIMCA analysis of e-nose measurements

Day	1 (%)	2 (%)	3 (%)	4 (%)	5 (%)	6 (%)	7 (%)
Blanks	0.0	0.0	75.0	0.0	0.0	50.0	0.0
G. candidum	41.7	100.0	100.0	100.0	100.0	100.0	8.3
P. camemberti	0.0	80.0	100.0	100.0	70.0	90.0	80.0
P. carneum	0.0	0.0	58.3	100.0	100.0	100.0	100.0
P. expansum	12.5	100.0	100.0	100.0	100.0	100.0	100.0
P. nordicum	0.0	0.0	58.3	33.3	58.3	83.3	25.0
P. paneum	8.3	8.3	33.3	16.7	33.3	100.0	75.0
P. roqueforti	0.0	16.7	25.0	66.7	83.3	100.0	91.7
Avg correct clas.	8.5	37.8	65.9	68.3	73.2	93.9	63.4

Rate of correct classification shown by species as well as overall per day.

Table 2 — Major volatile metabolites detected the investigated fungi

		G. candidum			*P. camemberti*			*P. nordicum*			*P. expansum*		*P. paneum*			*P. carneum*			*P. roqueforti*		
Retention index	Compound	9283	9284	9286	11570	11571	11755	6728	12803	14875	3487	15622	11839	13929	24721	3474	6884	6888	6754	14408	16404
504	Ethanol	S	S	S	S[a]	S	S				S	S	S	S	S	S	S	S	S	S	S
519	Acetone							S	S	S			S	S	S	S	S	S	S	S	S
584	2-Butanone							U[b]		U											
599	Ethylacetate										U	U									
609	2-Methyl-1-propanol				S	S	S	S	S	S	S	S	S	S	S				S	S	S
666	2-Pentanone							S					S						S		
714	3-Methyl-1-butanol				S	S	S				S	S	S	S	S	S	S	S	S	S	S
718	Unknown 1												S	S	S				S	S	S
735	Dimethyldisulfide	U	U	U																	
997	Unknown 2																			U	U
1128	(204)																				U
1144	(204)																				U
1151	(204)																				U
1296	(204)												U	U	U						

[a] S: Compounds produced by more than 1 of the 7 species.
[b] U: Compounds unique to a species.

Table 3—Mycotoxins, from the analyzed species, detected by LC-MS analysis

Retention time	Compound	*G. candidum*			*P. camemberti*			*P. nordicum*			*P. expansum*		*P. paneum*			*P. carneum*			*P. roqueforti*		
		9283	9284	9286	11570	11571	11755	6728	12803	14875	3487	15622	11839	13929	24721	3474	6884	6888	6754	14408	16404
	Patulin										nd[a]	nd	nd	nd	nd	nd	nd	nd			
	Cyclopiazonic acid				nd	nd	nd														
	Penitrem A															nd	nd	nd			
0,89	Verrucolone							U[b]	U	U											
3,27	Roquefortine A															S[c]	S	S	S	S	S
4,26	Sclerotigenin							U	U	T[d]											
5,27	Anacine							U	U	U											
5,49	Marcfortine B												U	U	U						
5,63	PR-toxin																			T	T
5,84	Marcfortine A												U	U	U						
5,92	Citrinin										U	U									
6,5	Roquefortine C										S[d]	S	S	S					S	S	S
8,25	Mycophenolic acid															S	S	S	S	S	S
9,22	Communesin A										U	U									
9,67	Ochratoxin A							U	U	U											
10,35	Chaetoglobosin C										U	U									
12,04	Communesin B										U	U									

[a]Not detected, though expected.
[b]Mycotoxins and other secondary metabolites unique to a species.
[c]Mycotoxins and other secondary metabolites produced by more than 1 of the 7 species.
[d]Trace amounts of unique mycotoxins or other secondary metabolites detected.

Analysis for mycotoxin production performed by LC-MS shows that 14 mycotoxins were detected from the 7 species. The mycotoxin profiles are given by species and isolate number as well as retention time (Rt) in Table 3.

No mycotoxins were detected in samples from *G. candidum* and *P. camemberti* though production of cyclopiazonic acid has been reported from *P. camemberti* (Le Bars 1979). The following mycotoxins or secondary metabolites were detected, with species from which they were detected noted in parentheses: Anacine (*P. nordicum*), Chaetoglobosin C (*P. expansum*), Citrinin (*P. expansum*), Communesin A (*P. expansum*), Communesin B (*P. expansum*), Marcfortine A (*P. paneum*), Marcfortine B (*P. paneum*), Mycophenolic acid (*P. carneum* and *P. roqueforti*), Ochratoxin A (*P. nordicum*), PR-toxin (*P. roqueforti*, trace amounts), Roquefortine A (*P. carneum* and *P. roqueforti*), Roquefortine C (*P. expansum*, *P. paneum* and *P. roqueforti*), Sclerotigenin (*P. nordicum*), and Verrucolone (*P. nordicum*). These findings are in agreement with literature (Frisvad and others 2004; Frisvad and Samson 2004). It was expected, based on literature reports, that Patulin would be detected in samples from *P. expansum*, *P. carneum*, and *P. paneum* and that Penitrem A would be detected in samples from *P. carneum*, but neither of the 2 metabolites was detected in the present study.

Conclusion

In conclusion the ability to correctly identify closely related fungi grown on YES medium to species level is possible by means of e-nose analysis. Since these species have also been shown to differ in mycotoxin production, it shows that the e-nose has potential as a tool for identification of mycotoxinogenic fungi on a synthetic substrate. Thus, it has been shown that the e-nose has a potential for more detailed quality control, that is, whether any production of mycotoxin is taking place in a given food or feedstuff.

To our knowledge this is the 1st study to correlate analysis of mycotoxins (by LC-MS), analysis of volatile metabolites (by GC-MS), fungal species identity, and e-nose analysis. Previous studies have either correlated only fungal species identity and e-nose analysis (Keshri and others 1998) or mycotoxin analysis and e-nose analysis (Cheli and others 2005).

Acknowledgments

We thank Jørn Smedgaard, Richard K. Phipps, and Jeorgos Trihaas for fruitful discussions regarding the analytical setup, the inoculation method, electronic nose analysis, and data processing. This work was supported by the Food Technology, Safety, and Quality research program, Ministry of Food, Agriculture and Fisheries, Denmark, grant nr FSK03-DTU-4.

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PAPER 3

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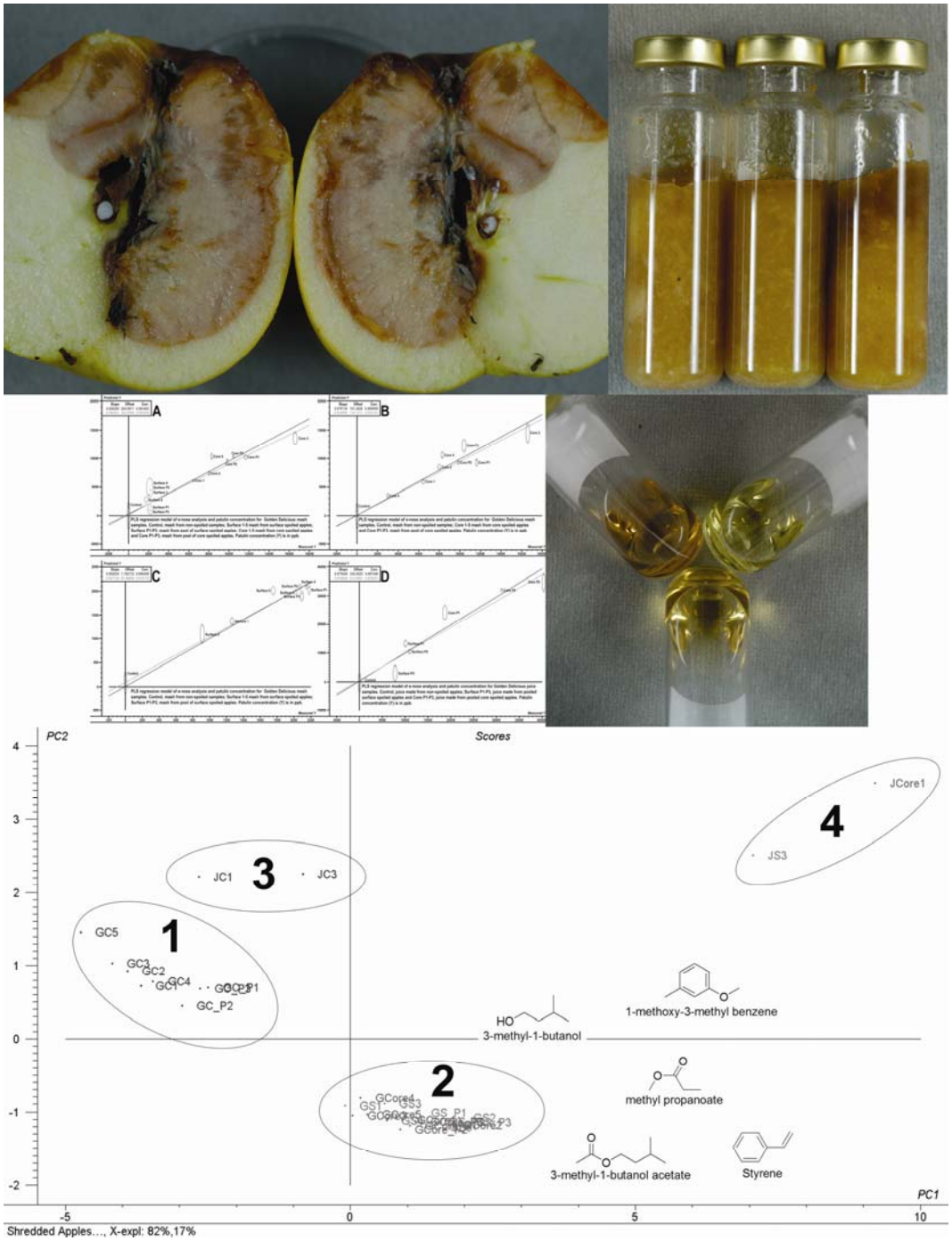

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ARTICLES

Prediction of *Penicillium expansum* Spoilage and Patulin Concentration in Apples Used for Apple Juice Production by Electronic Nose Analysis

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Classification models for *Penicillium expansum* spoilage of apples and prediction models for patulin concentration in apples usable for apple juice production were made on the basis of electronic nose (e-nose) analysis correlated to HPLC quantification of patulin. A total of 15 Golden Delicious and 4 Jonagold apples were surface sterilized and divided into three groups per variety. The Golden Delicious group consisted of five apples each. Group 1 was untreated control, group 2 was surface inoculated with *P. expansum*, and group 3 was inoculated in the core with *P. expansum*. The apples were incubated at 25 °C for 10 days. E-nose analysis was performed daily. At day 10 the Golden Delicious apples were individually processed for apple juice production. During apple juice production the mash and juice were analyzed by e-nose, and samples were taken for patulin analysis by HPLC. The volatile metabolite profile was obtained by collection of volatile metabolites, on tubes containing Tenax TA, overnight between the 9th and 10th days of incubation and subsequent analysis of the collected compounds by GC-MS. Prediction models using partial least-squares, with high correlation, for prediction of patulin concentration in shredded apples as well as apple juice were successfully created. It was also shown that it is possible to classify *P. expansum* spoilage in apples correctly on the basis of soft independent modeling of class analogy classification of e-nose analysis data. To the authors' knowledge this is the first report of a regression model between e-nose data and mycotoxin content in which actual concentrations are reported. This implies that it is possible to predict mycotoxin production and concentration by e-nose analysis.

KEYWORDS: Patulin; electronic nose; prediction models; *Penicillium expansum*; apple juice; volatile organic compounds; volatile metabolite profiling

INTRODUCTION

In the food industry quality control typically relies on random sampling of the product, description of flavor and aroma by sensory panels, volatile and/or headspace analysis by GC-MS, and mycotoxin analysis by HPLC (*1*). These methods are both time-consuming and expensive; therefore, it is desirable to improve quality control measures, for instance, by combining rapid analysis of volatiles with correlations to mycotoxin production.

The electronic nose (e-nose) has already shown its potential as a replacement for sensory panel analysis, for instance, in quality control of fruit ripeness (*2*), grain odor (*3*), packaged poultry meat (*4*), meat end-products (*5*), and Danish blue cheese (*6*−*8*) and even as a replacement for GC-MS analysis (*9*, *10*). Remaining to be done is the analysis of mycotoxin content, which is still performed by HPLC or LC-MS analysis. Only a limited number of studies (*11*, *12*) have tried to make predictions of mycotoxin contents based on e-nose response. Olsson et al. (*12*), in their combined GC-MS and e-nose analysis of grain samples, did show the possibility of predicting the mycotoxin level in spoiled grain by partial least-squares (PLS) modeling. The model could not, however, differentiate with sufficient accuracy between samples containing more or less than 5 μg/kg ochratoxin A, the threshold level established by the Swedish National Food Administration. Cheli et al. (*11*) looked at identifying mycotoxins in durum grain by e-nose analysis but did not identify the spoilage organism or go beyond principal component analysis (PCA) investigation to make a correlation model between e-nose data and mycotoxin concentrations.

To investigate the potential for mycotoxin prediction by e-nose analysis we chose to work with apples, as the associated

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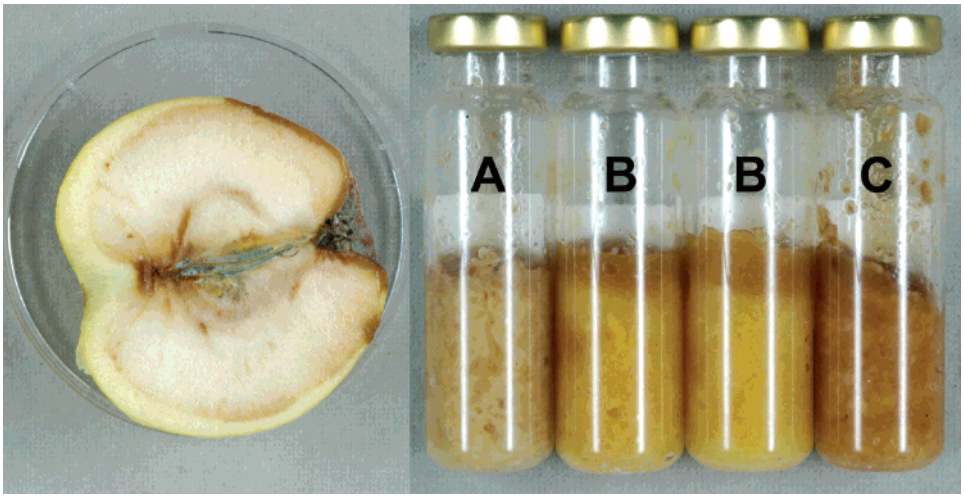

Figure 1. Discoloring of apple flesh by *P. expansum* in Jonagold apples (left) and the resulting apple mash (right). The four flasks with apple mash are from (**A**) a core-infected apple, (**B**, **B**) two control apples (mash showing browning at the surface), and (**C**) a surface-infected apple.

funga of apples is fairly limited (*13*), especially in storage contamination, where *Penicillium expansum* by far is the dominant species. It is known that the incidence of *P. expansum* spoilage of apples is increased by damage to the apples; hence, *P. expansum* spoilage mainly occurs on windfall apples. Storage of windfall apples prior to industrial processing is therefore likely to be the main cause of *P. expansum* spoilage and thus mycotoxin content. *P. expansum* is a known patulin producer (*14*), and food safety authorities, such as the U.S. Food and Drug Administration (FDA) and the Commission of the European Communities (EC), have introduced a threshold level of acceptance, 50 ppb, regarding patulin content in apple juice and other apple products (*15*, *16*).

P. expansum spoilage of and hence patulin content in apples is a well-known problem for the apple juice industry as apples are commonly stored prior to production due to processing capacity limitations; furthermore, apples used for juice production are frequently of a lesser quality than apples sold for consumption in, for instance, supermarkets. Because of the spoilage and mycotoxin problem several HPLC analysis methods have been developed for patulin quantification in apple juice samples (*17*−*21*). Several different sample preparation methods have also been developed in order to have a more robust analysis with increased sensitivity (*22*, *23*).

Because apple juice production is an industrial process it is not feasible or for that matter possible to inspect individual apple quality visually. It is therefore desirable to use screening methods that are rapid and can be used in large scale; e-nose analysis has a potential to fulfill these requirements.

The first aim of this study was to use e-nose analysis to classify both spoiled and nonspoiled samples, thus creating a classification model. Second, it was aimed to correlate the e-nose response with the mycotoxin level in a given sample, thus creating a prediction model for mycotoxin content based on e-nose analysis. It was not the aim of this study to predict biomass. The choice of model system in this study was apples used for apple juice production and the major problem in apple juice production, that is, storage spoilage by *P. expansum* and hence patulin production by *P. expansum*. The study was conducted as a "worst case" study by analysis of spoiled or nonspoiled samples. In this study the choice of apple variety for the major part of the study was Golden Delicious, whereas Jonagold apples were chosen for investigating apple variety influence on the e-nose analysis and prediction modeling.

MATERIALS AND METHODS

Fungi and Media. The *P. expansum* isolate, IBT 3487, the type culture used in this study, was obtained from the Fungal Culture Collection at BioCentrum-DTU (IBT collection), Technical University of Denmark, Kgs. Lyngby, Denmark. The apples, Golden Delicious and Jonagold, used in the experiment were obtained from the local supermarket and were surface sterilized by immersion in 96% ethanol for 1 min, then immersion in 3% hypochlorite solution for 1 min, and re-immersion in the 96% ethanol bath for 1 min, after which the apples were rinsed with autoclaved Milli-Q water and dried in a sterilized laminar flow bench.

A total of 15 Golden Delicious apples were surface sterilized and divided into three groups of 5 apples (replicates) each. Group 1 was untreated control, group 2 was surface-inoculated with *P. expansum*, and group 3 was inoculated in the core with *P. expansum*. Four Jonagold apples were surface sterilized, of which two apples were used as untreated control apples, one apple was surface-inoculated with *P. expansum*, and one apple was core-inoculated with *P. expansum*.

The surface-inoculated apples were stab wounded with a sterilized needle (7 mm deep wound), and 20 μL of spore suspension ($>10^6$ spores/mL) was put on the wound.

Core-inoculated apples were inoculated through the flower opening via a syringe, and 0.2 mL of spore suspension ($>10^6$ spores/mL) was injected. The apples were individually incubated in ethanol-sterilized 600 mL beaker glasses covered with Parafilm M (Alcan Packaging, Menasha, WI) at 25 °C in the dark for 10 days.

Apple Juice Production. Juice was only made from Golden Delicious apples. The processing steps were performed by imitating industrial apple juice production methods. After the 10 day incubation, the apples were individually shredded. Between 15 and 15.3 g was put in headspace flasks for electronic nose analysis (both Golden Delicious and Jonagold samples), and between 2 and 2.4 g was stored in headspace flasks for HPLC analysis (Golden Delicious samples only). The e-nose samples (Golden Delicious samples only) were treated with 12 μL of 10× diluted pectinase solution (Pectinex Smash) (Novozymes, Bagsværd, Denmark) and the samples left for 30 min at room temperature. The juice was then pressed using a funnel and Whatman filters (Whatman International, Brentford, U.K.) by pressing the mash with a metal spatula. The juice was collected in 20 mL headspace flasks (75.5 × 22.5 mm), and the flasks were sealed with crimp caps (3.0 mm membrane thickness silicone/PTFE caps). The juice was then pasteurized by immersion of the bottle in a 98 °C water bath for 60 s. After pasteurization, 10 μL of 3× diluted pectinase solution was added, and the samples were heated in a 53 °C cabinet for 60 min. Clearing of the juice was performed by adding 2−3 grains of Clarit WG Bentonite (Süd-Chemie AG, Moosburg, Germany), after which the sample was shaken for 5 min; then 10 μL of Gammasol Kieselsol (Gamma Chemie GmbH, Darmstadt, Germany) solution was added,

Figure 2. Discoloring of apple flesh by *P. expansum* in Golden Delicious apples (left) and the resulting apple mash (right). The three flasks with apple mash are from (**A**) a control apple (mash already showing more browning at the surface), (**B**) a surface-infected apple, and (**C**) a core-infected apple.

and the sample was shaken for another 5 min. Finally, approximately 10 μg of Rousselot gelatin 75 PS 30 was added, and the sample was shaken for another 5 min and then left overnight in the 53 °C cabinet. The cleared samples were centrifuged (15000*g*) for 5 min, and the supernatant was transferred to fresh 20 mL headspace flasks (75.5 × 22.5 mm), which were sealed with crimp caps (3.0 mm membrane thickness silicone/PTFE caps).

Collection and Analysis of Volatile Metabolites. Volatile metabolites were collected overnight from all 15 whole Golden Delicious apples between days 9 and 10. The volatiles were collected by diffusive sampling onto Tenax TA adsorption material placed in Perkin-Elmer tubes, and the tubes were placed inside glass beakers. Volatiles collected were thermally desorbed on a Perkin-Elmer ATD 400 coupled to a Hewlett-Packard 5890 gas chromatograph further coupled to a HP 5972 mass selective detector. Separation of the volatiles was done on a 60 m, 0.25 mm i.d., 1.0 μm VB-5 ValcoBond capillary column (SIS, Ringoes, NJ) using He as carrier gas. Initial pressure was 13 psi, and the He flow was 1 mL/min. The system was run at a 75:1 split, and the injection temperature was set to 250 °C. Chromatographic conditions were as follows: initial temperature, 35 °C for 1 min, raised at 4 °C/min to 175 °C and then at 10 °C/min to 260 °C. Separated compounds were characterized (tentatively identified) by their mass spectra generated by electron ionization (EI) at 70 eV at a scan range from *m*/*z* 33 to 330.

Electronic Nose Measurements. The headspace of the all 15 Golden Delicious whole apples was analyzed daily on an e-nose from days 1 through 10 by manual injection of 1500 μL of the headspace from the 600 mL beakers containing each apple using a gastight Hamilton syringe. For all of the shredded apple samples (both Golden Delicious and Jonagold) and all of the juice samples (only Golden Delicious) an HS-100 autosampler (CTC Analytics AG, Zwingen, Switzerland) was used; these samples were incubated for 1 min at 35 °C, after which 1500 μL of the sample was withdrawn from the headspace and injected in the sensor chamber and flushed over the sensors at a rate of 150 mL/min. All e-nose samples were analyzed on an αFox-3000 (Alpha M.O.S., Toulouse, France) e-nose. The sensor array system consisted of 12 metal oxide semiconductor (MOS) sensors. MOS sensors are known to be among the least moisture sensitive sensors available (*24*).

Data collection was performed every half second for 2 min, after which the sensors were flushed with dry air (maximum air humidity = 0.5%) for 2 min; furthermore, there was a 2 min delay before the next sample was analyzed.

Extraction and Analysis of Patulin. *Extraction.* A modified version of the method used by Eisele and Gibson (*23*) was used. Ten milliliters of Milli-Q water was added to each headspace flask containing 2.0−2.4 g of apple mash and left for extraction on an ultrasound bath for 30 min; 2.5 mL of supernatant from each sample was transferred to 2.5 mL Eppendorf tubes and centrifuged at 15000*g* for 5 min. Strata-X (30 mg/1 mL, 33 μm) polymeric reversed phase SPE columns (Phenomenex, Torrance, CA) were prepared for sample purification by rinsing the columns with 2 × 1 mL of methanol (Sigma-Aldrich, St. Louis, MO) and then with 2 × 1 mL of Milli-Q water. Two

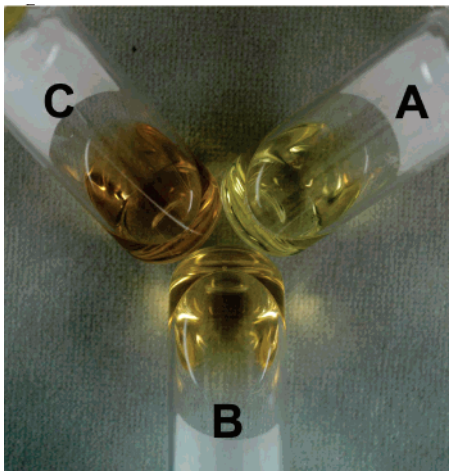

Figure 3. Apple juice from Golden Delicious apples with various amounts of *P. expansum* spoilage: (**A**) juice from control apples; (**B**) juice from surface-infected apples; (**C**) juice from core-infected apples.

milliliters of sample was loaded on each column, after which the columns were washed with 2 × 1 mL of 1% sodium bicarbonate and then 2 × 1 mL of 1% acetic acid solution. The columns were left to dry for 1 min, after which the samples were eluted into HPLC vials with 1.5 mL of 30% acetonitrile.

Quantification of Patulin. Patulin standards were made at concentrations of 10, 25, 50, 100, and 205 μg/L by diluting a standard (100 μg/mL in chloroform) (Supelco, Bellefonte, PA) in acetonitrile and analyzed by HPLC.

Patulin Recovery from Apples. Two grams of apple mash was spiked with patulin standard (100 μg/mL in chloroform) at the following levels: 2, 4, 7.5, 12.5, and 20 mg/kg (by adding 40, 80, 150, 250, and 400 μL of patulin standard to the apples, respectively). The chloroform was removed by nitrogen evaporation while the samples were kept at 40 °C on a heating block. After this, the samples were treated as the other apple samples by SPE and analyzed by HPLC.

Chemicals. Chemicals used in HPLC analysis were as follows: acetonitrile, gradient grade, and trifluoroacetic acid (Sigma-Aldrich, St. Louis, MO); water, Milli-Q grade.

HPLC-DAD Instrument. HPLC-DAD analysis was done on an Agilent HP 1100 with a photodiode array detector (DAD) (Agilent, Böblingen, Germany). Ten microliters of sample was injected, although for the patulin standards only 3 μL was injected. The column used was a 150 × 2.0 mm i.d., 3 μm CuroSil-PFP column (Phenomenex) with a 4 × 2 mm i.d. Phenyl (phenylpropyl) guard column (Phenomenex). The flow rate was 0.3 mL/min with a linear water/acetonitrile gradient starting at 96:4 (water/acetonitrile) going to 25% acetonitrile in 5 min, and then returning to starting conditions (96:4) in 3 min and equilibrating for 8 min. Trifluoroacetic acid (50 ppm) was added to

Table 1. Fifty Volatile Compounds Detected by Headspace Analysis of Whole Golden Delicious Apples[a]

compd no.	RI	compound	log av area			signif diff in log av area[b]
			control	surface	core	
1	443	acetaldehyde	2.00	1.80	1.20	
2	472	ethanol	6.54	8.10	7.53	
3	498	propanal	6.28	7.00	4.68	
4	525	methyl acetate	6.23	7.70	7.74	
5	548	1-propanol	7.23	7.37	6.99	
6	582	butanal	6.23	3.28	3.26	
7	605	ethyl acetate	8.32	8.59	8.56	
8	617	2-methyl-1-propanol	7.19	7.61	7.30	
9	622	methyl propanoate	nd[c]	4.84	3.89	surface > control core > control
10	655	1-butanol	7.85	7.86	7.68	
11	687	unknown 1	6.14	4.86	3.31	
12	707	ethyl propanoate	5.39	7.56	7.22	
13	710	propyl acetate	7.70	7.76	7.40	
14	731	3-methyl-1-butanol	1.94	6.45	7.10	surface > control core > control
15	736	2-methyl-1-butanol	7.71	7.75	7.58	
16	765	unknown 2	4.56	4.83	3.31	
17	772	2-methylpropyl acetate	7.15	7.35	7.22	
18	799	ethyl butanoate	6.44	7.59	7.25	
19	807	propyl propanoate	6.25	5.65	4.82	
20	812	butyl acetate	8.38	8.15	6.97	
21	848	ethyl 2-methylbutanoate	6.40	7.50	7.18	
22	864	1-hexanol	7.42	7.44	7.25	
23	871	3-methyl-1-butyl acetate	0.60	6.40	5.55	surface > control core > control
24	874	2-methyl-1-butyl acetate	8.38	8.20	7.99	control > core
25	891	propyl butanoate	6.35	5.63	3.95	
26	897	styrene	nd	8.36	8.27	surface > control core > control
27	900	butyl propanoate	7.39	7.31	7.07	
28	904	pentyl acetate	7.51	7.44	7.12	
29	937	unknown 3	7.09	5.70	3.40	
30	960	3-methyl-1-butyl propanoate	4.85	4.90	1.20	
31	981	butyl butanoate	7.64	7.48	7.33	
32	984	ethyl hexanoate	6.32	7.51	7.30	
33	991	unknown 4	4.52	4.90	3.99	
34	997	hexyl acetate	8.27	8.01	7.73	
35	1011	1-methoxy-3-methylbenzene	nd	7.35	7.12	surface > control core > control
36	1028	butyl 2-methylbutanoate	7.73	7.51	7.35	
37	1044	pentyl butanoate	5.59	4.96	3.98	
38	1063	α-farnesene	2.83	4.41	4.29	
39	1078	butyl hexanoate	7.22	7.29	6.32	
40	1089	hexyl propanoate	6.48	6.58	5.63	
41	1094	2-methylbutyl 2-methylbutanoate	6.37	5.75	5.54	
42	1129	unknown 5	7.19	5.74	5.49	
43	1137	butyl 2-methylpropanoate	5.56	5.75	4.75	
44	1184	hexyl butanoate	7.94	7.85	7.62	
45	1188	ethyl octanoate	2.89	5.76	3.18	
46	1208	1-allyl-4-methoxybenzene	6.54	7.41	6.42	
47	1233	hexyl 2-methylbutanoate	8.14	7.88	7.73	
48	1254	unknown 6	3.60	4.06	6.28	
49	1282	pentyl hexanoate	5.72	5.89	6.46	
50	1375	hexyl hexanoate	7.50	7.93	7.46	

[a] Compounds are listed by the average log area for the three sample groups (sample size per group was five) control apples, surface-inoculated apples, and core-inoculated apples. [b] Designating significant difference in log average area between sample groups as per Duncan's multiple-range test. [c] Not detected.

both the water and acetonitrile. UV spectra were collected by a DAD from 200 to 700 nm at 2.0 nm resolution with focus on the signal at 276 nm.

In all analysis sequences a standard of 5-hydroxymethylfurfural was included to ascertain that the retention times of this compound and patulin were different in this analysis method.

Data Analysis. *Volatile Metabolites.* All peaks that were significant enough to be integrated, using default parameters, with the ChemStation software (Agilent Technologies) were selected. Mass spectra from compounds with identical retention indices were compared to account for similarity. The identity of the compounds was tentatively established by comparison of mass spectra and volatile metabolite profiles with data from the NIST mass spectra library (NIST92 library). The significance of difference in the amount of each volatile metabolite between the three sample groups (control and core- and surface-inoculated Golden Delicious apples) was determined by using Duncan's multiple-range test (*25*) keeping the areas logarithmized.

Electronic Nose Data. The features, maximum value for resistance change, extracted from the e-nose system responses were evaluated by PCA as well as by classification using soft independent modeling of class analogy (SIMCA) with each apple condition, that is, control, surface-inoculated, and core-inoculated (class), analyzed as a PCA group (*26*). Prediction models for patulin levels were made by PLS regression analysis between the e-nose data and patulin concentrations

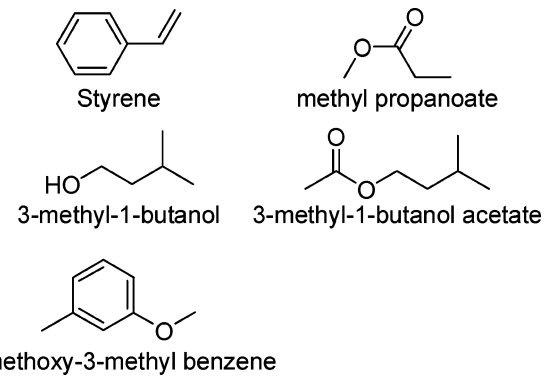

Figure 4. Chemical structures of the five volatile biomarkers for *P. expansum* contamination of Golden Delicious apples.

of the given samples by quantitative HPLC analysis (*26*). The data were standardized by multiplication with the inverse of the standard deviation and analyzed using full cross validation. For the analysis the software package The Unscrambler version 9.1 (CAMO, Oslo, Norway) was used.

Nonvolatile Metabolites. Patulin was identified by comparison of UV spectrum and retention time with HPLC analysis of a patulin standard (100 μg/mL in chloroform). A standard curve was made from the standards analyzed by HPLC. Patulin recovery (R%) from apple samples was calculated from the spiked samples (Sp) run by comparison of the attained HPLC response to the standard curve (Std) and hence expected response level, that is, R% = Sp/Std × 100. The recovery percent was used to correct the patulin levels detected from the apple sample analyses. Patulin concentration in the apples was also corrected for the sample mass extracted. Retention time of 5-hydroxymethylfurfural was checked and compared to the retention time of patulin as 5-hydroxymethylfurfural is also considered to be a quality parameter in fruit juices, and its presence is regarded an indication of quality deterioration (*20*).

RESULTS AND DISCUSSION

Golden Delicious and Jonagold apples were chosen for this study, and the apples were surface- or core-inoculated to emulate the two types of *P. expansum* spoilage commonly found in apples. The inoculation methods were deliberately strong as absolute chance of infection was desired.

It was noted that, depending on apple variety, the color of the apple flesh was either more pale with a brownish edge in the infected part of apples (Jonagold) (**Figure 1**) or dark brown (Golden Delicious) (**Figure 2**). This discoloring could be due to destruction of apple enzymes by *P. expansum*. Apple juice from Golden Delicious apples also differed in color depending on whether the juice came from control apples (light yellow), surface-infected apples (light brown), and core-infected apples (dark brown) (**Figure 3**); hence, visual identification of spoiled samples by spectrophotometric methods is variety dependent.

From the GC-MS analysis of the headspace from the whole Golden Delicious apples after 10 days of incubation 50 volatile organic compounds (VOCs) were detected (**Table 1**). The five compounds shown in **Figure 4** were both unique for apples infected by *P. expansum* and statistically significantly different from control apple samples according to Duncan's multiple-range test. Of these five compounds, four of them, namely, styrene, 3-methyl-1-butanol, 3-methyl-1-butyl acetate, and 1-methoxy-3-methylbenzene, are all well-known fungal volatile metabolites (*27*), and methyl propanoate is a well-known apple volatile metabolite (*28*, *29*). These five compounds are therefore good GC-MS analysis biomarkers for *P. expansum* infection in Golden Delicious apples, although styrene, being produced in the largest quantity, seems the best of the five. From PCA of e-nose data combined with GC-MS results and mycotoxin analysis, styrene by far had the highest positive correlation with patulin (not shown). Styrene is therefore also a relevant biomarker in e-nose analysis of potential apple spoilage by *P. expansum*. The finding of large quantities of a known apple VOC is likely due to *P. expansum* spoilage inducing large-scale release of this compound when, for instance, apple cells or peel is ruptured.

For the remaining 45 apple-related volatiles, by combined analysis of GC-MS and e-nose data, we have found that the level of the majority of the volatile compounds (30 VOCs or 60%) was relatively unchanged between surface- and core-infected apples (no statistically significant difference in VOC levels). Eighteen VOCs or 36% were detected in slightly higher levels (GC-MS), although this was not statistically significant, from surface-infected apples than from core-infected apples and were positively correlated to surface-infected apples (e-nose, PCA). Two VOCs, α-farnesene and unknown 6, corresponding to 4%, were detected at a higher level in core-infected apples compared to surface-infected apples (GC-MS), although this was not statistically significant, and were positively correlated to core-infected apples (e-nose, PCA). The profiles of volatile metabolites of the surface- and core-inoculated Golden Delicious apples, respectively, were thus quite similar and different from those of the control apples (**Figure 5**). This indicates that the differentiation between the two infection types on day 9 could be due to the fungal growth being at different stages. It could also derive from a lesser amount of volatile compounds being released from apples core inoculated with *P. expansum* compared to surface-inoculated apples, as the fungal atmospheric surface area per gram to hyphae ratio is smaller for core-inoculated apples than for surface-inoculated apples. Because of the location of the core infection, the volatile compounds might be bound in the apple tissue as long as the apple maintains structural integrity. Other studies have also indicated that the VOC profiles from apples differ according to whether the apple is whole or damaged (*30*−*32*).

SIMCA analysis of e-nose signals of the whole Golden Delicious apples showed that from days 1 through 8 the volatile metabolite background from the apple was overwhelming the detection of fungal growth by the e-nose even if growth was highly visible. At day 6 it was possible to correctly classify two of five surface-inoculated apples, whereas it was not possible to distinguish core-inoculated apples from the controls until day 7, and then only one of five samples was correctly classified. E-nose signal SIMCA analysis of the whole apples on the 9th and 10th days showed that apples at this stage of infection can be classified into spoiled and nonspoiled apples. On day 9 it was even possible to distinguish the two infection types (surface vs core), whereas it was possible to distinguish between spoiled and control apples only on day 10. In terms of classification of spoiled from control samples, it was possible to classify 40% of the spoiled samples correctly on day 6, rising to 100% correctly classified samples from day 9 onward. The early classification of spoiled apples might improve if the apples are shredded as this will induce an increased release of VOCs from both apple and mold, which could, for instance, potentially reveal core-infected apples at an earlier stage.

By analysis of e-nose data of shredded apples from both Jonagold and Golden Delicious varieties it was evident that the two varieties give rise to two different aroma profiles and hence lead to two different e-nose models (**Figure 6**). This was expected as it is known that GC-MS analysis of apple varieties yields clustering of apples according to whether they belong to

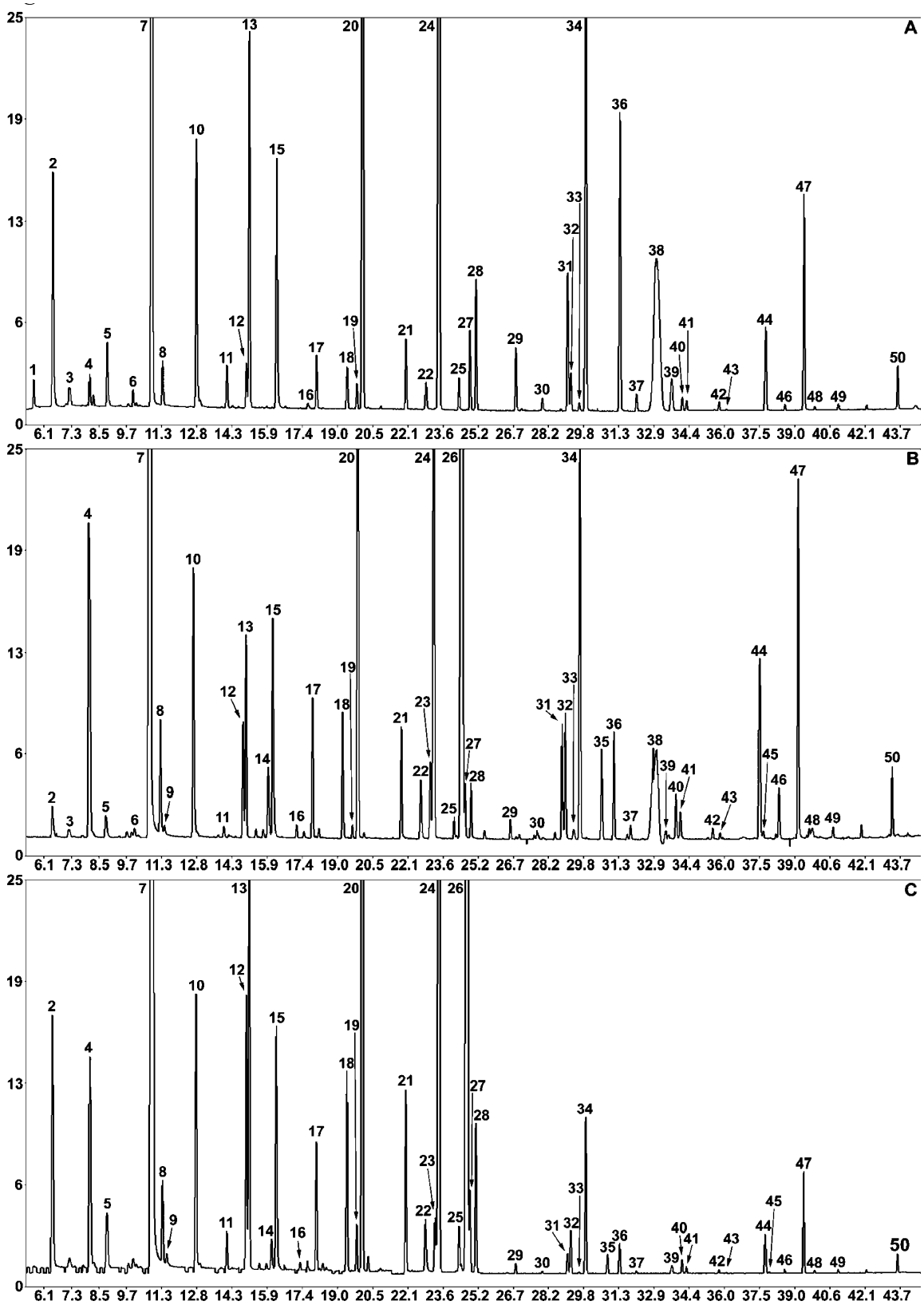

Figure 5. GC-MS chromatogram of the headspace from Golden Delicious apples: (**A**) control apple; (**B**) core-infected apple; (**C**) surface-infected apple. The abundance scale is in percentage of the abundance given in the top left corner of each chromatogram. The noteworthy compounds are numbered corresponding to the numbering of compounds in **Table 1**.

the red skin, green skin (Golden Delicious), or red and green skin (Jonagold) category (*33*).

The HPLC analysis method developed proved sufficient to distinguish between 5-hydroxymethylfurfural and patulin with retention times of 4.77 and 5.79 min, respectively (**Figure 7**). Patulin analysis revealed that patulin, in Golden Delicious apples, had been produced at quite high levels by *P. expansum* in both core and surface inoculations. The patulin concentrations were 3−4 times higher in core-inoculated apples compared to the surface-inoculated apples, probably due to the higher degree

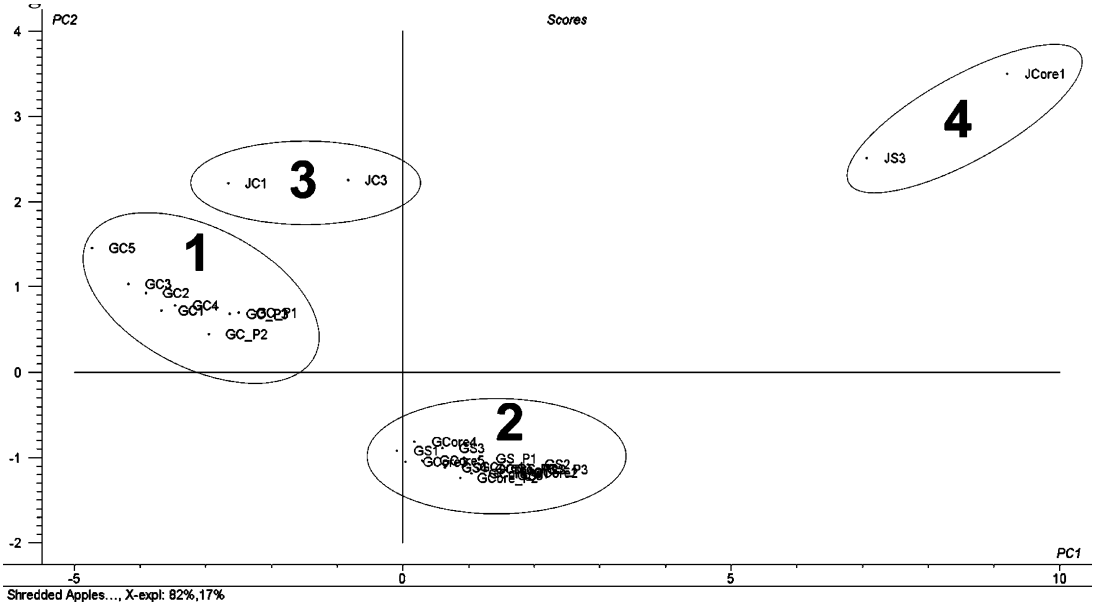

Figure 6. PCA scores plot of e-nose analysis results of shredded Jonagold and Golden Delicious apples: 1, GC1–5, Golden Delicious control apples, and GC_P1–3, pooled samples of Golden Delicious control apples; 2, GCore1–5, Golden Delicious core-inoculated apples, and GS1–5, Golden Delicious surface-inoculated apples; 3, JC1 and 3, Jonagold control apples; 4, JCore1, core-inoculated Jonagold apple, and JS3, surface-inoculated Jonagold apple.

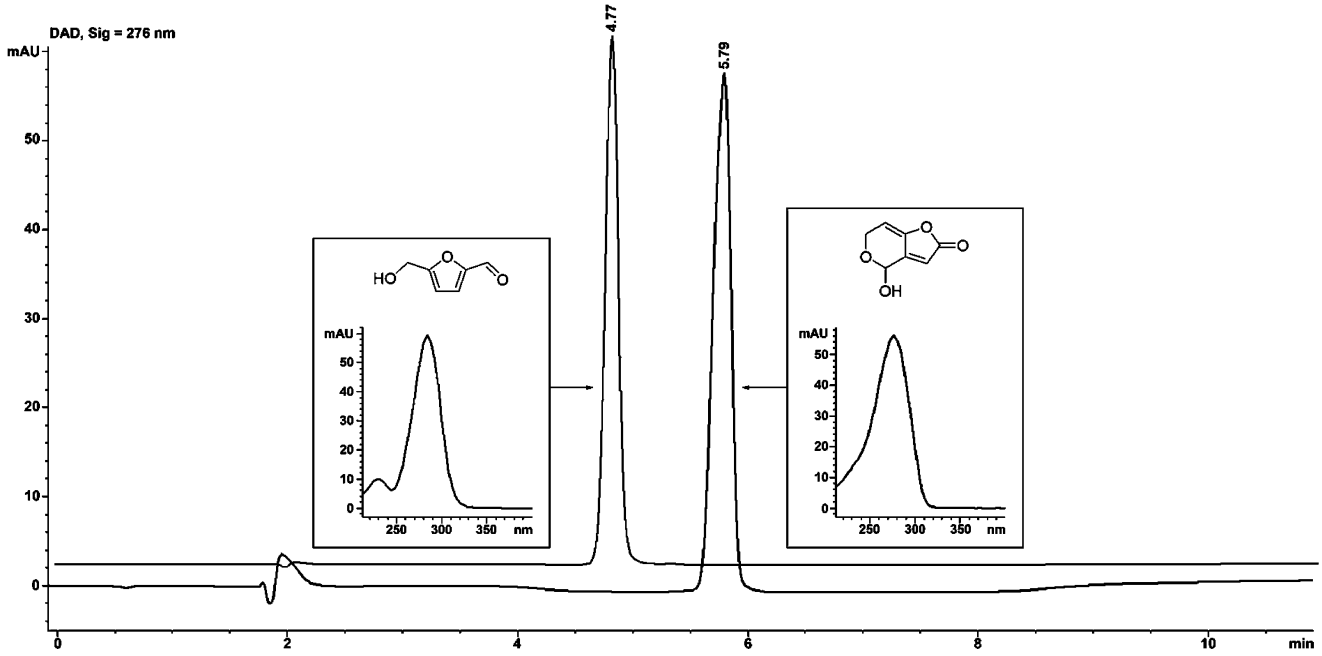

Figure 7. Overlay of HPLC chromatograms with 5-hydroxymethylfurfural, RT 4.77 min, and patulin, RT 5.79 min. To the left of the 5-hydroxymethylfurfural peak the UV spectrum and chemical structure are inserted and the UV spectrum and chemical structure for patulin are inserted to the right of the patulin peak.

of spoilage in these apples as the fungus was able to grow spherically in all directions from the core, whereas the surface-inoculated colony was limited to hemispherical growth; that is, the patulin to hyphae ratio between the two types of infection is expected to be the same. It is also evident that patulin was concentrated in the Golden Delicious apple juice through the apple juice production process, because a 3−4 times higher patulin concentration was detected in the juice compared to the mash from which it came. This concentration could be due to patulin being a very polar compound, hence being highly water soluble and therefore present in a higher concentration in the juice (*34*, *35*). Because the samples taken for analysis were approximately 2 g whether it was mash or juice, more patulin would then be sampled from a pure juice sample than from a mash sample. The resulting juice from the surface- and core-inoculated apples had patulin contents of, respectively, approximately 200 and 600 times above the allowed threshold (*15*, *16*). This indicates that the allowed level of patulin, 50 ppb, will be exceeded by producing apple juice from apples containing as little as two to five spoiled apples per thousand.

As seen in **Figure 8A**, the PLS regression analysis of e-nose data and patulin concentration for Golden Delicious apple mash gave rise to a regression model with a high fit (high correlation value, RMSEP = 14%). From the dispersement of the surface samples in the plot it is evident that it is not a perfect model for these samples. Separate PLS modeling of the data from core-

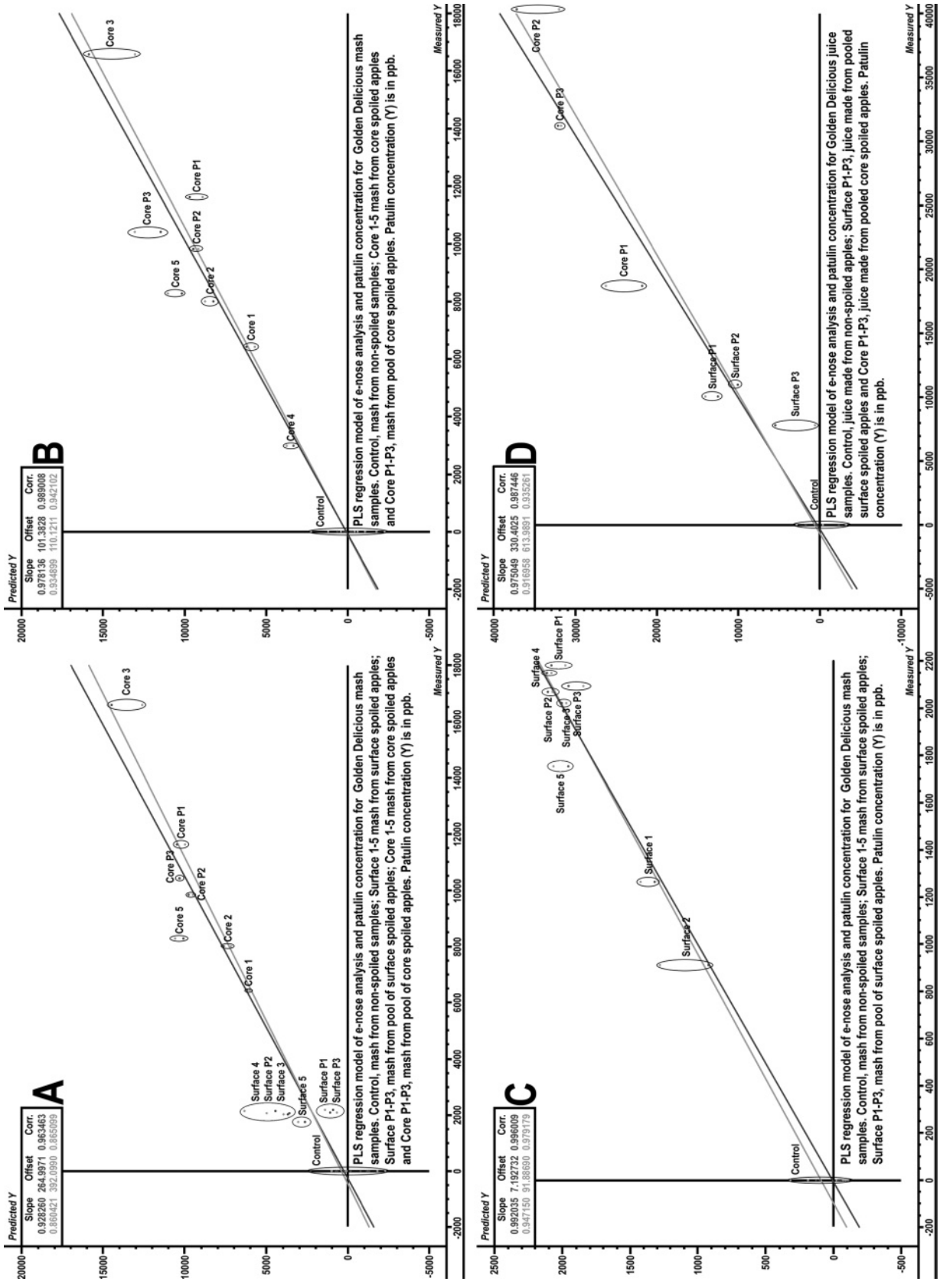

Figure 8. PLS regression models of (**A**) Golden Delicious mash samples from both core- and surface-inoculated apples, (**B**) Golden Delicious mash samples from core-inoculated apples, (**C**) Golden Delicious mash samples from surface-inoculated apples, and (**D**) Golden Delicious juice samples from both core- and surface-inoculated apples.

and surface-inoculated Golden Delicious apples show even better fits, with RMSEP values of 11 and 9%, respectively (**Figure 8B**,**C**), which leads to the conclusion that the two systems, surface- and core-inoculated apples, are indeed two separate entities which cannot easily be covered by one model. This is not too surprising because there is a big difference in the surface to hyphae ratio between the two types of spoilage. PLS regression modeling of e-nose data and patulin concentration for the Golden Delicious juice samples also gave a high-fit regression model with a RMSEP of 12% (**Figure 8D**). For the juice samples it seems that one model covers both the surface inoculation and core inoculation samples.

To our knowledge this is the first report of a regression model between e-nose data and mycotoxin content in which actual concentrations are reported. In a previous study (*11*) the investigation was limited to looking at levels (high, low) of mycotoxins, whereas the regression modeling in another study (*12*) was less accurate and less reliable.

From the results of this "worst case" study it seems very promising to use an e-nose for quality control in the apple juice industry, although additional work has to be carried out to produce prediction models for the various apple varieties, and probably also mixes of apple varieties, used in production. Because this study was a worst case scenario, additional experiments will have to be performed to determine the cutoff concentration for patulin prediction by e-nose analysis. Other factors, such as the influence of apple shredding on e-nose prediction of spoilage and patulin concentration, could also be investigated. The results indicate that it would be feasible to analyze at one of two points during production. E-nose analysis should be carried out either right after shredding of the apples or on the finished juice. The advantage of early, in terms of production, analysis is that there are several process steps in which it is possible to rectify any patulin problem, whereas analysis of the finished juice will yield information on the quality of the finished product. In either case patulin can be, and industrially is, removed by treatment with charcoal (*36*), ascorbic acid (*37*), or irradiation (*38*).

Altogether this study has proven it possible to classify spoiled from nonspoiled apples during the apple juice production steps and at some points even to distinguish between spoilage type (surface and core) by e-nose analysis. It has also been proven possible to create regression models that can be used for quite exact prediction of patulin content in Golden Delicious apples. These results of course are valid for only the chosen cultivar, Golden Delicious, but suggest that it ought to be possible to achieve similar prediction models with any given apple cultivar. To make industrially relevant prediction models it is required to measure many more samples, to use mixed samples of various types of spoilage, to make measurements on various degrees of spoilage, and of course to expand the model with different varieties of apples and mixes of apple varieties. We believe that this study is the first study to show correlation between e-nose analysis data and mycotoxin concentration. The results of this study show a great potential for e-nose prediction of mycotoxin levels even in systems with a high level of volatiles from the matrix such as silage, grain, and fruit juices.

ACKNOWLEDGMENT

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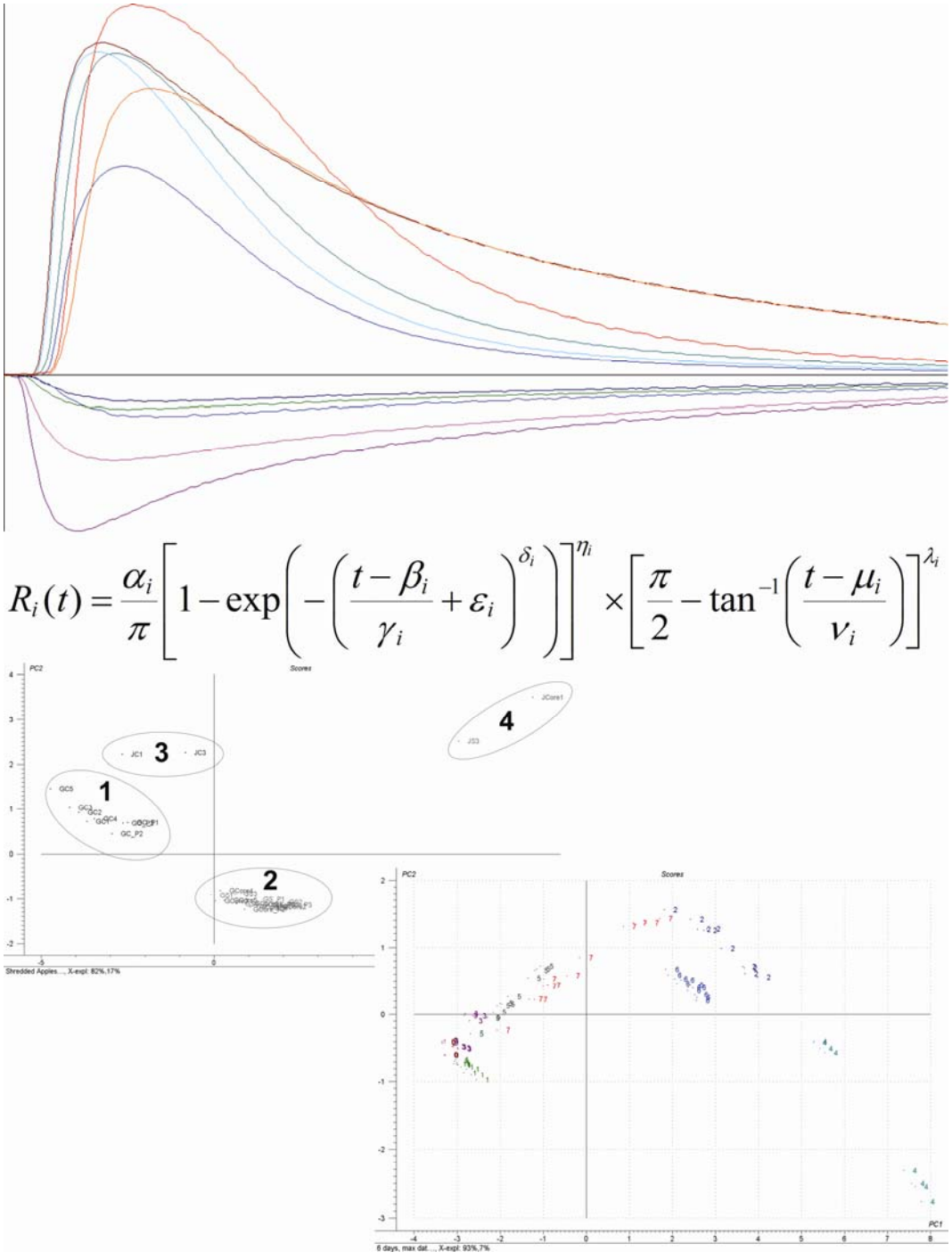
PAPER 4

Karlshøj, K., Larsen, T.O., Nielsen, P.V. and Hansen, M.E. Effect of data parameterization on data analysis of electronic nose signals. (draft)



Effect of data parameterization on electronic nose signal data analysis

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Abstract The parameters for the theoretic mathematical function describing the response curves of the metal oxide semiconductor (MOS) sensor based electronic nose (e-nose) were determined and several parameters such as total curve area, curve slope and wavelet decompositions of the signal were extracted. Chemometric analysis of fungal spoilage data generated in the previous experiments was performed on the "raw data" generated by the e-nose, specifically the maximum resistance measured (Y_{max}) and compared to chemometric analysis based on the parameters generated through mathematical modeling. The generated parameters were optimized to span the greatest possible variation with the least number of parameters. These parameters proved to be of the wavelet decomposition type. Both classification and regression modeling was performed with the two datasets, Y_{max} and wavelet parameters and it was shown that with the datasets at hand there was no advantage in parameterization of the data.

Keywords: parameterization, electronic nose analysis, chemometrics, signal analysis

Introduction

The use of an electronic nose (e-nose) for headspace analysis has become widespread, for instance in the food and feed industries, such as meat spoilage detection [1-2], shelf life effect on tomato aroma [3], fruit ripeness assessment [4], grain quality assessment [5-9], prediction of cheese quality [10-12]. E-nose analysis has also emerged in other fields such as for detection of fungal contamination of library paper [13], medical diagnostics [14-15] and prediction of patulin concentration in apples [16]. Thus great effort is made in producing analysis results with electronic

nose instruments and it is therefore desirable to maximize the data quality gained from these analyses.

Until now the main focus in improving e-nose analysis has been based on improving the entire e-nose analysis process from sensor technology [17-18] through feature extraction [19-20] and pattern recognition [21-22]. Carmel *et al.*, in 2003, reported how to achieve optimal function parameterization of e-nose signals by employing a Lorentzian model, they also tested a double-sigmoid model, which though having a better fit, lacked in robustness compared to the Lorentzian model. They found that using the best fitting parameters from this Lorentzian model as the e-nose features enhanced the performance of the classification algorithms leading to improved classification. Their work was based on data produced by e-nose analysis of 30 pure chemicals.

The aim of our work was to investigate features extracted, such as function parameters as per Carmel *et al.*'s double-sigmoid model, maximum response values (Ymax), area, derivatives (i.e. slope) etc, yields the best data analysis for classification as well as regression modeling purposes compared to the raw data delivered by the e-nose software. The samples used in this investigation were more complex compared to Carmel *et al.*'s study. Our focus was based on two systems, classification of closely related fungi grown on yeast extract sucrose (YES) agar and correlations between and prediction of the mycotoxin patulin concentration in and e-nose analysis of apples infected by *Penicillium expansum*. It is well known that fungi produce a large variety of volatile metabolites, the profiles of which, if determined by GC-MS analysis, can be used for classification [23-26]. It is also well known that apples produce a wide variety of ester and alcohol volatile metabolites [27-30] which add to the complexity of the data. Since the most commonly used e-nose sensors have been of the metal oxide semi-conductor (MOS) or conducting polymer (CP) types, in this work our focus was on a MOS sensor based e-nose systems.

Experimental

Samples 1: Classification of closely related fungi grown on YES medium

All isolates used in this study were obtained from the Fungal Culture Collection at BioCentrum-DTU (IBT collection), Technical University of Denmark, Kgs. Lyngby, Denmark. The following strains were investigated (listed by IBT number); *G. candidum*, 9283, 9284 and 9286; *P. carneum*, 3474, 6884, 6888; *P. camemberti*, 11570, 11571 and 11755; *P. expansum*, 3487 and 15622; *P. nordicum*, 6728, 12803 and 14875; *P. paneum*, 11839, 13929 and 24721; *P. roqueforti*, 6754, 14408 and 16404. The strains were center point inoculated, in four replicates, in 20 ml headspace

flasks (75.5 × 22.5 mm) containing 2 ml YES medium, from spore suspensions made from 8 day old YES medium streak cultures. In both cases the YES medium consisted of yeast extract (2%), sucrose (15%), $MgSO_4 \cdot 7H_2O$ (0.05%), $ZnSO_4 \cdot 7H_2O$ (0.001%), $CuSO_4 \cdot 5H_2O$ (0.0005%), water to 1.0 L, pH 6.5, and agar (2%). The headspace flasks were sealed with crimp caps (3.0 mm membrane thickness silicone/PTFE caps) and two syringe needles (Ø 1.20 × 40 mm) were inserted through the membrane to allow oxygen diffusion into and carbon dioxide out of the flasks. The cultures were incubated with the flasks lying down in a rack at room temperature in the laboratory.

Electronic nose analysis

The headspace was analyzed daily, from day one through seven, using an αFox-3000 (Alpha M.O.S., France) electronic nose, equipped with a HS-100 auto sampler (CTC Analytics AG, Switzerland). The sensor array system consisted of 12 metal oxide semiconductor (MOS) sensors. 10 minutes prior to sampling the syringe needles were removed from the crimp cap membrane and the flask placed in the auto sampler. Immediately after sampling, the syringes were re-introduced through the crimp cap membrane and the flask returned to the rack. The samples were incubated for 1 minute at 35 °C after which 500 µl of the sample was withdrawn from the head space and injected in the sensor chamber and flushed over the sensors at a rate of 150 ml min^{-1}.

Data collection was performed every half second for 2 minutes after which the sensors were flushed with dry air (maximum air humidity 0.5%) for 2 minutes; furthermore, there was a 2 minute delay before the next sample was analyzed.

Samples 2: Penicillium expansum *on apples*

The *Penicillium expansum* isolate, IBT 3487, the *P. expansum* type culture, used in this study was obtained from the Fungal Culture Collection at BioCentrum-DTU (IBT collection), Technical University of Denmark, Building 221, DK-2800 Kgs. Lyngby, Denmark. The apples, Golden delicious, used in the experiment were obtained from the local supermarket and were surface sterilized by immersion in 96% ethanol for one minute, then immersion in 3% hypochlorite solution for one minute and re-immersed in the 96% ethanol bath for one minute after which the apples were rinsed with autoclaved Milli-Q water and dried in a sterilized laminar flow bench.

The surface inoculated apples were stab wounded with a sterilized needle (7 mm deep wound) and 20 µL spore suspension (> 10^6 spores / mL) was put on the wound.

Core inoculated apples were inoculated through the flower opening and via a syringe 0.2 mL spore suspension (> 10^6 spores / mL) was injected. The apples were individually incubated in

ethanol sterilized 600 mL beaker glasses covered with Parafilm M® (Alcan packaging, USA) at 25 °C in the dark for ten days.

After the ten day incubation the apples were individually shredded. Between 15 and 15.3 g was put in headspace flasks for electronic nose analysis and between 2 and 2.4 g was stored in headspace flasks for HPLC analysis. The e-nose samples were treated with 12 µL 10x diluted pectinase solution (Pectinex® Smash, Novozymes, Denmark) and the samples left for 30 minutes at room temperature. The juice was then pressed using a funnel and Whatman® filters (Whatman international, UK) pressing the mash with a metal spatula. The juice was collected in 20 ml headspace flasks (75.5 × 22.5 mm) and the flasks were sealed with crimp caps (3.0 mm membrane thickness silicone/PTFE caps). The juice was then pasteurized by immersion of the bottle in a 98 °C water bath for 60 seconds. After pasteurization 10 µL 3x diluted pectinase solution (Pectinex® Smash, Novozymes, Denmark) was added and the samples heated in a 53 °C cabinet for 60 minutes. Clearing of the juice was performed by adding 2-3 grains of Bentonite (CLARIT® WG, Süd-Chemie AG, Germany) after which the sample was shaken for five minutes, then 10 µL Kieselsol (Gammasol, Gamma chemie GmbH, Germany) solution was added and the sample shaken for another five minutes. Finally approximately 10 µg gelatin (Rousselot™ gelatin 75 PS 30) was added, the sample shaken for another five minutes and then left over night in the 53 °C cabinet. The cleared samples were centrifuged (15000x g) for 5 minutes and the supernatant was transferred to fresh 20 mL headspace flasks (75.5 × 22.5 mm) which were sealed with crimp caps (3.0 mm membrane thickness silicone/PTFE caps).

Electronic nose analysis

The headspace of the whole apples was analyzed on an electronic nose daily, from day one through ten by manual injection of 1500 µL of the headspace using a gastight Hamilton syringe. For the shredded apple and juice samples a HS-100 auto sampler (CTC Analytics AG, Switzerland) was used, these samples were incubated for 1 minute at 35 °C after which 1500 µL of the sample was withdrawn from the head space and injected in the sensor chamber and flushed over the sensors at a rate of 150 ml min^{-1}. All e-nose samples were analyzed on an αFox-3000 (Alpha M.O.S., France) electronic nose (e-nose). The sensor array system consisted of 12 metal oxide semiconductor (MOS) sensors.

Data collection was performed every half second for 2 minutes after which the sensors were flushed with dry air (maximum air humidity 0.5%) for 2 minutes; furthermore, there was a 2 minute delay before the next sample was analyzed.

Extraction and Analysis of Patulin

A modified version of the method used by Eisele and Gibson (REF 341) was used. 10 mL Milli-Q water was added to each headspace flask containing 2.0 to 2.4 g apple mash and left for extraction on an ultrasound bath for 30 minutes. 2.5 mL supernatant from each sample was transferred to 2.5 mL eppendorf tubes and centrifuged at 15000x g for 5 minutes. 30 mg / 1mLStrata-X 33 µm Polymeric Reversed Phase SPE columns (Phenomenex, CA, USA) were prepared for sample purification by rinsing the columns with 2 x 1 mL methanol (Sigma-Aldrich, USA) and then 2 x 1 mL Milli-Q water. 2 mL sample was loaded on each column after which the columns were washed with 2 x 1 mL 1% sodium bicarbonate and then 2 x 1 mL 1% acetic acid solution. The columns were left to dry for 1 minute after which the samples were eluted into HPLC vials with 1.5 mL 30% acetonitrile.

Patulin standards were made at concentrations of 10, 25, 50, 100 and 205 µg/L by diluting a standard (100 µg / mL in chloroform, Supelco, USA) in acetonitrile (gradient-grade, Sigma-Aldrich, USA) and analyzed by HPLC for quantification of patulin in samples.

Chemicals used in HPLC analysis were as follows: Acetonitrile, gradient-grade (Sigma-Aldrich, USA); trifluoroacetic acid (Sigma-Aldrich, USA); water, Milli-Q grade.

HPLC analysis was done on an Agilent HP 1100 with a photodiode array detector (DAD) (Agilent, Germany). 10 µL of sample was injected, though for the patulin standards only 3 µL was injected. The column used was a 150 × 2.0 mm CuroSil-PFP column with 3 µm particles (Phenomenex, CA, USA) and a 4 × 2 mm Phenyl (phenylpropyl) guard column (Phenomenex, CA, USA). The flow rate was 0.3 ml min^{-1} with a linear water-acetonitrile gradient starting at 96:4 (water:acetonitrile) going to 25% acetonitrile in 5 minutes, and then returning to starting conditions (96:4) in 3 minutes and equilibrating for 8 minutes. Trifluoroacetic acid (50 ppm) was added to both the water and acetonitrile. UV spectra were collected by a diode array detector (DAD) from 200 to 700 nm at 2.0 nm resolution with focus on the signal at 276 nm.

Feature extraction

The double-sigmoid model from Carmel *et al*., 2003, was used to create the e-nose response curve functions. The base features chosen to be investigated in this study are features that can be calculated at high speed – in real-time. Assuming that each of the profiles can be described by a function y(t), the extracted features are shown in **Table 1**.

Table 1. The function extracted parameters used in this study.

Y_max_t	Maximum value (location)
Y_max	Maximum value (value)
Int	Total integral
Inta	Total integral (from time 0 to Y_max_t)
Intb	Total integral (from time Y_max_t to end)
Inta/Int	Integral ratio
Intb/Int	Integral ratio
OP_*p*	Signal (*p*-percent quantile)
OD_*p*	Differentiated signal (*p*-percent quantile)

A Wavelet is used to decompose the signal into it's time-frequency components, i.e. it shows how the signal changes during acquisition (see **Figure 1**). Wavelets (The wavelet transform, WT) [32, 33] are mathematical functions that split data into different frequency components, and then study each component with a resolution matched to its scale. Whereas the perhaps more known Fourier transformation (FT) [35] also deals with separating a signal into its frequency components, the most interesting dissimilarity between these two kinds of transforms is that individual wavelet functions are localized in "space". Fourier sine and cosine functions are not. In other words, the FT only gives what frequency components exist in the signal and the time and frequency information can not be seen at the same time. The WT splits the signal into its frequency local components, and hence overcomes these problems, and the WT breaks the signal into its "wavelets", scaled and shifted versions of a so-called "mother wavelet". This localization feature, along with wavelets' localization of frequency, makes many functions and operators using wavelets "sparse" when transformed into the wavelet domain. This sparseness, in turn, results in a number of useful applications such as data compression, detecting features, and removing noise from time series signals [36-39].

The (continuous) wavelet transform is calculated from

$$\mathrm{CWT}_x^{\psi}(\tau,\, s) = \Psi_x^{\psi}(\tau,\, s) = \frac{1}{\sqrt{|s|}} \int x(t) \bullet \psi^* \left(\frac{t-\tau}{s} \right) dt$$

$$\mathrm{CWT}_x^{\psi}(\tau,\, s) = \int X(T) \bullet \psi_{\tau,s}^*(t)\, dt$$

where is the mother wavelet (type of transformation). One of the choices that has to be made in the WT is the choice of motherwavelet.

$$\psi^*_{\tau,s}(t) = \frac{1}{\sqrt{s}}\psi\left(\frac{t-\tau}{s}\right)$$

where τ is the translation of the mother wavelet (location in the signal) and s is the scale. It is out of the scope in this paper to go into all the WT details, and therefore the reader is referred to the large amount of literature for further details [32-39].

The main point to remember in this transformation is that the signal is split into its local frequencies. These frequencies are then used in the further feature extraction. As an example please look at **Figure 1**. Here we have an e-nose signal (upper part) that has been wavelet transformed

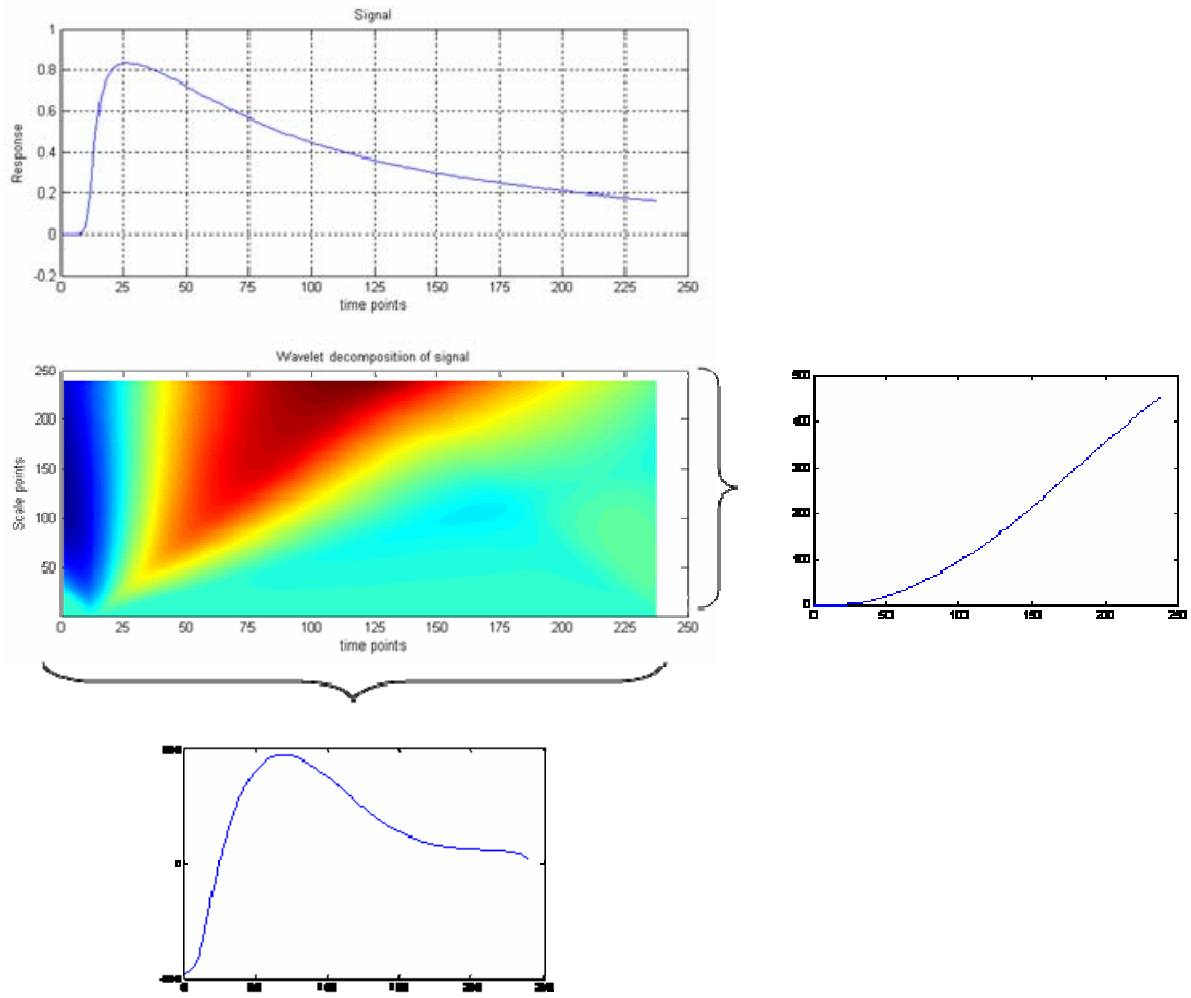

Figure 1. Overview of some of the time dependant signal changes a Wavelet decomposition of the signal can reveal.

(WT) using the Daubechies Wavelet (2nd order) as motherwavelet. The middle figure shows the wavelet transform (the coefficients) at different scales (y-axis) at different time points.

Looking at the transformation we see that at a higher scale the low frequent changes are more distinct in the decay region of the signal whereas the high frequent changes are more distinct at a lower resolution.

Since the coefficients (each time-scale pair) indicate the amount of "energy" at that specific level we will use the WT to extract features that are distinct for the e-nose sensor responses, and use those in our comparison. First we condense the WT by extracting the maximum and minimum values for the time and scale axes. The WT is calculated for both the original signal as well as for the differentiated signal. The features extracted from these two profiles are listed in **Table 2**.

Data analysis

The features, maximum value for resistance change, extracted from the e-nose system responses as well as the features extracted by parameterization of the e-nose response curves were evaluated

Table 2. The Daubechies Wavelet (2nd order) parameters used in this study.

W1_*p*	Wavelet decomposition of signal (*p*-percent quantile along scale-axis)
W1_max_t	Wavelet decomposition of signal (Max value position along scale-axis)
W1_max	Wavelet decomposition of signal (Max value along scale-axis)
W1_min_t	Wavelet decomposition of signal (Max value position along scale-axis)
W1_min	Wavelet decomposition of signal (Max value position along scale-axis)
W2_*p*	Wavelet decomposition of signal (*p*-percent quantile along time-axis)
W2_max_t	Wavelet decomposition of signal (Max value position along time-axis)
W2_max	Wavelet decomposition of signal (Max value along time-axis)
W2_min_t	Wavelet decomposition of signal (Max value position along time-axis)
W2_min	Wavelet decomposition of signal (Max value position along time-axis)
ODW1_*p*	Wavelet decomposition of differentiated signal (*p*-percent quantile along scale-axis)
ODW1_max_t	Wavelet decomposition of differentiated signal (Max value position along scale-axis)
ODW1_max	Wavelet decomposition of differentiated signal (Max value along scale-axis)
ODW1_min_t	Wavelet decomposition of differentiated signal (Max value position along scale-axis)
ODW1_min	Wavelet decomposition of differentiated signal (Max value position along scale-axis)
ODW2_*p*	Wavelet decomposition of differentiated signal (*p*-percent quantile along time-axis)
ODW2_max_t	Wavelet decomposition of differentiated signal (Max value position along time-axis)
ODW2_max	Wavelet decomposition of differentiated signal (Max value along time-axis)
ODW2_min_t	Wavelet decomposition of differentiated signal (Max value position along time-axis)
ODW2_min	Wavelet decomposition of differentiated signal (Max value position along time-axis)

by principal component analysis (PCA) as well as by classification using soft independent modeling of class analogy (SIMCA) with each condition (class) analyzed as a PCA group [31]. For the extracted parameters the best parameters for each analysis were chosen by investigating the loadings plot of an initial PCA to pick the loadings responsible for the highest variation within the data. Prediction models for patulin levels (sample 2) were made by PLS regression analysis between the e-nose data and patulin concentrations of the given samples by quantitative HPLC analysis [31]. The data were standardized by multiplication with the inverse of the standard deviation and analyzed using full cross validation. For the analysis the software package The Unscrambler version 9.1 (CAMO, Norway) was used.

Results

Samples 1: Classification of closely related fungi grown on YES medium

The results of the SIMCA analysis of both Y_{max} data and the features extracted via parameterization shown as the percentage of correctly classified samples are shown in **Table 3**.

Samples 2: Penicillium expansum *on apples*

The results of the SIMCA analysis of both Y_{max} data and the features extracted via parameterization shown as the percentage of correctly classified samples are shown in **Table 4**.

The results of the PLS regression analysis of both Y_{max} data and the features extracted via parameterization versus patulin concentration, shown as the fit (R^2) and root mean square error in percent (RMSEP) are shown in **Table 5**.

Table 3. Comparison of percentage of correct classification of fungi grown on YES medium by SIMCA classification of e-nose data; Y_{max} and the best parameter model available.

	Day 1		Day 2		Day 3		Day 4		Day 5		Day 6		Day 7	
	Y_{max}	Parameters	Ymax	Parameters	Y_{max}	Parameters	Y_{max}	Parameters	Y_{max}	Parameters	Y_{max}	Parameters	Y_{max}	Parameters
Control	0.0%	0.0%	0.0%	50.0%	75.0%	50.0%	0.0%	0.0%	0.0%	0.0%	50.0%	25.0%	0.0%	0.0%
G. candidum	41.7%	0.0%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	91.7%	8.3%	0.0%
P. camemberti	0.0%	0.0%	80.0%	80.0%	100.0%	80.0%	100.0%	100.0%	70.0%	90.0%	90.0%	90.0%	80.0%	90.0%
P. carneum	0.0%	0.0%	0.0%	0.0%	58.3%	91.7%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%
P. expansum	12.5%	0.0%	100.0%	87.5%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	50.0%
P. nordicum	0.0%	0.0%	0.0%	16.7%	58.3%	8.3%	33.3%	0.0%	58.3%	33.3%	83.3%	75.0%	25.0%	0.0%
P. paneum	8.3%	0.0%	8.3%	0.0%	33.3%	75.0%	16.7%	8.3%	33.3%	50.0%	100.0%	100.0%	75.0%	58.3%
P. roqueforti	0.0%	0.0%	16.7%	8.3%	25.0%	66.7%	66.7%	83.3%	83.3%	58.3%	100.0%	100.0%	91.7%	83.3%
Avg correct class.	8.5%	0.0%	37.8%	39.0%	65.9%	72.0%	68.3%	64.6%	73.2%	70.7%	93.9%	90.2%	63.4%	51.2%

Table 4. Comparison of percentage of correct classification of *P. expansum* spoilage of apples by SIMCA classification of e-nose data; Y_{max} and the best parameter model available.

	Ymax				Parameters			
Whole Apples	Control	Core	Surface	Spoiled	Control	Core	Surface	Spoiled
Day 1	20.0%	20.0%	0.0%	10.0%	40.0%	0.0%	0.0%	0.0%
Day 2	60.0%	0.0%	0.0%	0.0%	60.0%	0.0%	0.0%	0.0%
Day 3	60.0%	0.0%	0.0%	10.0%	80.0%	0.0%	0.0%	0.0%
Day 4	60.0%	0.0%	0.0%	0.0%	40.0%	0.0%	0.0%	0.0%
Day 5	60.0%	0.0%	0.0%	0.0%	40.0%	0.0%	0.0%	0.0%
Day 6	80.0%	20.0%	60.0%	40.0%	60.0%	0.0%	60.0%	30.0%
Day 7	100.0%	20.0%	60.0%	60.0%	80.0%	0.0%	80.0%	40.0%
Day 8	100.0%	20.0%	60.0%	70.0%	80.0%	20.0%	60.0%	70.0%
Day 9	100.0%	100.0%	100.0%	100.0%	60.0%	100.0%	60.0%	100.0%
Day 10	100.0%	40.0%	60.0%	100.0%	100.0%	20.0%	100.0%	100.0%
Shredded	100.0%	0.0%	12.5%	100.0%	100.0%	12.5%	25.0%	100.0%
Pectinated	100.0%	25.0%	25.0%	100.0%	100.0%	25.0%	87.5%	100.0%
Fresh Juice	100.0%	0.0%	66.7%	100.0%	100.0%	100.0%	100.0%	100.0%
Pasteurized	100.0%	66.7%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%
Cleared	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%

Table 5. Comparison of fit (R^2) and Root mean square error in percent (RMSEP) of regression model correlating e-nose data, Y_{max} and the best parameter model available, from *P. expansum* spoilage of apples with patulin concentration from the apple samples determined by HPLC analysis

	Ymax		Parameters	
	R^2	RMSEP	R^2	RMSEP
Shredded Apples	0.9634	14%	0.9233	15%
Shredded Core	0.9890	11%	0.9586	14%
Shredded Surface	0.9960	9%	0.9941	14%
Cleared Juice	0.9874	12%	0.9740	12%

Discussion & Conclusion

Samples 1: Classification of closely related fungi grown on YES medium

Comparison of SIMCA analysis of both Y_{max} data and the features extracted via parameterization showed similar results for the two data sets except for the day 1 and day 7 samples, which for day 1 corresponds to low growth for all fungal species except *G. candidum* whereas day 7 shows decline in growth for several fungal species, especially *G. candidum*. This implies that the parameter data are more sensitive to changes in fungal growth than the Y_{max} data acquired from the e-nose software.

Samples 2: Penicillium expansum *on apples*

Comparison of SIMCA analysis of both Y_{max} data and the features extracted via parameterization showed that for samples taken from the whole apples the SIMCA analysis of Y_{max} data performs better than the parameter data set SIMCA whereas it is opposite when dealing with processed samples (shredded apples through cleared apple juice).

For the PLS regression correlation of e-nose analysis to patulin concentration Y_{max} gave rise to higher fits as well as lower root mean square error in percent than the parameter data set analysis.

All in all the findings of this study contradict the conclusions of Carmel *et al.*, who used "30 volatile odorous pure chemicals.... intentionally chosen from many different families, so that they would represent a broad range of possible stimuli." and found that the use of the parameters describing e-nose sensor response gave rise to better classification than any other data analysis method. The data used in our study are more complex as each sample contains a mixture of VOCs and many of the samples have highly similar profiles, such as apple vs. spoiled apple where the VOC profile difference no higher than 6%.

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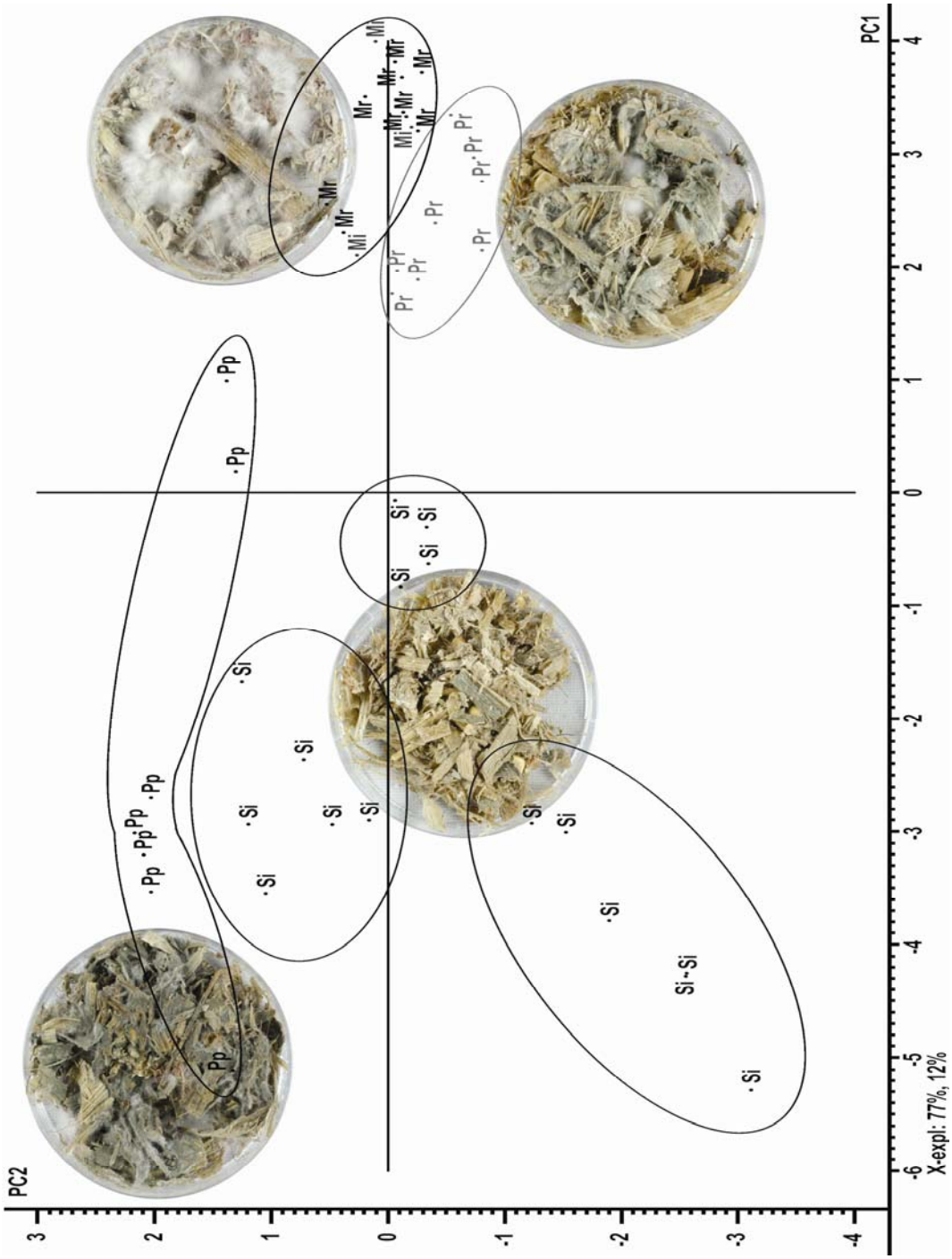
PAPER 5

Karlshøj, K., Drejer Storm, I.M.L., Nielsen, K.F., Nielsen, P.V. and Larsen, T.O. Prediction of Silage Spoilage by Electronic Nose Analysis. (draft)



Prediction of Silage Spoilage and Mycotoxin Production by Electronic Nose Analysis

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ABSTRACT: Classification models for *Penicillium paneum*, *Penicillium roqueforti* and *Monascus ruber* spoilage and mycotoxin in maize silage were made on basis of electronic nose analysis of the head space. For each of the three days analysis, two isolates of each fungal species, as well as a mixture of the three fungi were inoculated on maize silage (6 replicates each). Of the 21 control samples used, 6 were from the maize silage used for inoculation and the remaining were from 15 different maize silages. The silage was incubated at 25 °C for thirteen days. Electronic nose and GC-MS analysis of the headspace was performed on days three, six and thirteen. Selected mycotoxin and secondary metabolites: marcfortine A, mycophenolic acid, roquefortine C, PR-toxin, were measured in the silage on the three days. It was shown that it is possible to classify the spoilage by *P. roqueforti*, *P. paneum* or *M. ruber* in silage correctly to species level on basis of soft independent modeling of class analogy classification of electronic nose analysis data.

KEYWORDS: Silage, Electronic nose, prediction models, volatile organic compounds, volatile metabolite profiling.

INTRODUCTION

Maize silage is a very important feed for dairy cattle. In Denmark its use has more than five-doubled in Denmark since 1990 (*1*) and it is often used as the main part of the feed all year round. Unfortunately growth of filamentous fungi is often seen in silage stacks (*2-4*). This constitutes a production loss to the farmer due to loss of biomass during spoilage and the discarding of spoiled

feed. However most importantly presence of mycotoxins may furthermore affect yield and health of the livestock (*5-7*) and perhaps also be carried over to milk or meat. A quick, cheap and easy method for the detection of filamentous fungi and their mycotoxins in silage is therefore desirable.

Maize silage is produced by natural lactic acid fermentation of whole crop maize after these have been chopped, heavily compacted and covered with plastic. Plant enzymes and bacteria quickly consume the available oxygen lowering the concentration to a few percent while CO_2 concentration rises above 20%. The lactic acid bacteria (LAB) then begin fermenting sugars to primarily lactic and acetic acid thus lowering the pH to below 4 (*8*). The combination of low pH, low oxygen concentration, high carbon dioxide concentration and large amounts of LAB preserves the silage so it can be stored for up to 14 months. Spoilage bacteria can not grow in such a harsh environment and filamentous fungi only to a very limited extend.

Due to improper ensiling procedures, insufficient wrapping, damaging of the plastic and other factors spoilage of the silage may occur. Some of the most common filamentous fungi that occur in maize silage are *Penicillium roqueforti*, *Penicillium paneum*, *Monascus ruber* and *Byssochlamys nivea* (*2-4;9*). Together they are capable of producing more than 70 mycotoxins and other secondary metabolites e.g. patulin, citrinin, PR-toxin, mycophenolic acid, roquefortine C (*10*).

Fungal spoilage may be visible as colored "hot spots" or layers in the silage. But with a daily use of several tons of silage it is not possible to sort out all spoiled lumps. Besides, even healthy looking silage may contain up to 10^6 viable fungal units per gram (unpublished results), *P. roqueforti* seems to present in most silage (Unpublished results). The isolation and identification of fungi from hot-spots can also be problematic by classical microbial techniques. Species, like *P. roqueforti* with a large production of spores are easily over-represented and may conceal less sporulating species, like *Monascus* and *P. paneum*.

The e-nose is a cheap and easy method of analysis which potentially can be brought to problematic silage stacks in the field. With this study we therefore wish to explore the capabilities of the e-nose to identify silage spoiled by filamentous fungi since it has already been shown that the e-nose is capable of detecting fungal spoilage in a high background matrix such as apples used for apple juice production (*11*).

The first aim of this study was to use e-nose analysis to classify spoiled "hotspot" like samples from non-spoiled samples, as well as classifying the spoilage by fungal species responsible for the deterioration, thus creating a classification model. Secondly it was aimed to correlate the e-nose response with the mycotoxin level in a given sample, thus creating a prediction model for

mycotoxin content based on e-nose analysis. It was not the aim of this study to predict biomass. The choice of model system in this study was silage, and on the major problem of storage spoilage by e.g. *M. ruber*, *P. paneum* and *P. roqueforti*. The study was conducted as a "worst case" study by analysis of spoiled or non-spoiled samples. A total of 16 different silages, the main one from Research Center Foulum and the remainder from various farms in Jutland, Denmark were included to investigate the influence on different silages on the e-nose analysis.

MATERIALS AND METHODS

Fungi and Media. Two isolates of the following fungal species were used *Monascus ruber*, *Penicillium paneum* and *Penicillium roqueforti,* all available from the IBT culture collection author address. The fungi used were originally isolated from silage. The main silage used in the incubation experiment was obtained from a silo at the Faculty of Agricultural Sciences, Research Center Foulum, Aarhus University. The silage was sampled in December 2004, vacuum packed and stored at -20°C. The 15 other silage types were acquired at different farms located in Jutland, Denmark.

Every isolate, as well as a mixture of one isolate from all three species, was 2 point inoculated with 2 x 100 µL spore suspension ≈1-5 x 10^7 spores / mL for the pure spore suspensions and 2.5 x 10^5 spores / mL for each species in the mixed spore suspension) in 6 replicates on maize silage, 6g silage per replicate. The silage was incubated at 25 °C in the dark in petri dishes packed in microperforated bags. A total of 21 control samples were used. Six of these samples were from the maize silage used for inoculation and the remaining were from 15 different maize silages.

Electronic nose measurements. On each sample day pre-packed frozen control samples of 16 different silages were thawed and analyzed as control standards. All samples were analyzed on days 3, 6 and 13. Prior to e-nose analysis 2 g of each sample was transferred to 20 ml headspace flasks (75.5 × 22.5 mm, La-Pha-Pack, Langerwehe, Germany) and sealed with crimp caps (3.0 mm membrane thickness silicone/PTFE caps La-Pha-Pack). The samples were left at room temperature (20 °C) for an hour to equilibrate. The headspace of the silage sample was analyzed on an electronic nose by manual injection of 1.5 mL of the headspace from 20 mL headspace flasks using a gastight Hamilton syringe. The sample was injected in the sensor chamber and flushed over the sensors at a rate of 150 mL/min. All e-nose samples were analyzed on an αFox-3000 (Alpha M.O.S., Toulouse, France) electronic nose (e-nose). The sensor array system consisted of 12 metal oxide

semiconductor (MOS) sensors. MOS sensors are known to be among the least moisture sensitive sensors available (*12*).

Data collection was performed every half second for 2 min after which the sensors were flushed with dry air (maximum air humidity 0.5%) for 2 min; furthermore, there was a 2 min delay before the next sample was analyzed.

Collection and Analysis of Volatile Metabolites. Volatile metabolites were collected overnight after e-nose analysis on days 3, 6 and 13. The volatiles were collected by diffusive sampling onto Tenax TA adsorption material placed in Perkin Elmer tubes. Volatiles were thermally desorbed (300 °, 30 min) on a Perkin Elmer ATD 400 coupled to a Hewlett Packard 5890 gas chromatograph further coupled to a HP 5972 mass spectrometer. Separation of the volatiles was done on a 60 m, 0.25 mm i.d., 1.0 µm VB-5 ValcoBond capillary column (SIS, Ringoes NJ, USA) using He as carrier gas. Initial pressure was 13 psi, and the He flow was 1 mL/min. The system was run at a 75:1 split, and the injection temperature was set to 250 °C. Chromatographic conditions were as follows: initial temperature, 35 °C for 1 min, raised at 4 °C/min to 175 °C and then at 10 °C/min to 260 °C. Separated compounds were characterized (tentatively identified) by their mass spectra generated by electron ionization (EI) at 70 eV at a scan range from *m*/*z* 33 to 330 (2.53 scans / sec).

Analysis of Secondary Metabolites and Mycotoxins.

Analysis pending.

Data Analysis.

Electronic nose data. The features, maximum value for resistance change, extracted from the e-nose system responses were evaluated by principal component analysis (PCA) as well as by classification using soft independent modeling of class analogy (SIMCA) with each spoilage condition, i.e. each fungal species as well as the control samples (class) analyzed as a PCA group (*13*). Prediction models for patulin levels were made by PLS regression analysis between the e-nose data and patulin “concentrations” of the given samples by semi-quantitative HPLC analysis (*13*). The data were standardized by multiplication with the inverse of the standard deviation and analyzed using full cross validation. For the analysis the software package The Unscrambler version 9.1 (CAMO, Norway) was used.

Volatile metabolites. All peaks that were significant enough to be integrated, using default parameters, with the ChemStation software (Agilent Technologies) were selected. Mass spectra

from compounds with identical retention indices were compared to account for similarity. The identity of the compounds was tentatively established by comparison of mass spectra and volatile metabolite profiles with data from the NIST mass spectra library (NIST92 library). The significance of difference in the amount of each volatile metabolite between the six sample groups (control, *M. ruber* infected, *P. paneum* infected, *P. roqueforti* infected and the fungal mixture infected) was determined by using Duncan's Multiple Range Test (*14*) keeping the areas logarithmized.

Secondary Metabolites and Mycotoxins. Pending.

RESULTS AND DISCUSSION

Sixteen different silages were chosen for this study to investigate the influence on silage on spoilage prediction. One of the silages was inoculated to emulate fungal "hotspots" commonly found in silage stacks. The inoculation methods were deliberately strong as absolute chance of infection was desired.

It was noted that the silage used for the inoculation experiment was biased towards *M. ruber* spoilage. Hence *M. ruber* germinated and grew faster than the *Penicillium* species. It was also noted that in the mixed spore inoculated silage *M. ruber* completely dominated spoilage by visual inspection though it germinated and grew slower than in the pure *M. ruber* samples. On day 3 only very small colonies of *M. ruber* were visible in 5 of the 10 samples analyzed and no growth was visible in the remaining samples. On day 6 the growth of *M. ruber* was visible in all *M. ruber* samples as well as the fungal mixture samples, 8 of 10 *P. paneum* samples had visible growth though with some impurities of *M. ruber* whereas there was no visible growth in the *P. roqueforti* samples. On Day 13 growth was visible on all inoculated samples except for 2 *P. roqueforti* samples, for both *P. paneum* and *P. roqueforti* samples as well as the control samples *M. ruber* impurities were spotted on roughly 1/3 of the samples.

SIMCA analysis of e-nose signals of samples from day 3 did not lead to any differentiation of control and inoculated samples. On day 6 it was possible to classify *M. ruber*, *P. paneum* and the mixed spores spoiled samples (as one group) from control samples whereas no differentiation of *P. roqueforti* samples was possible. SIMCA analysis of the 13 day samples proved it possible to classify spoilage 100% as well as classify the spoilage types at a high degree of certainty; *M. ruber* (90%, the remaining 10% classified as *P. roqueforti* infected), *P. paneum* (100%) and *P. roqueforti* (100%), the mixed spore samples were all classified as *M. ruber* infected (**Figure 1**). The silage samples seemed to cluster in 3 distinct groups.

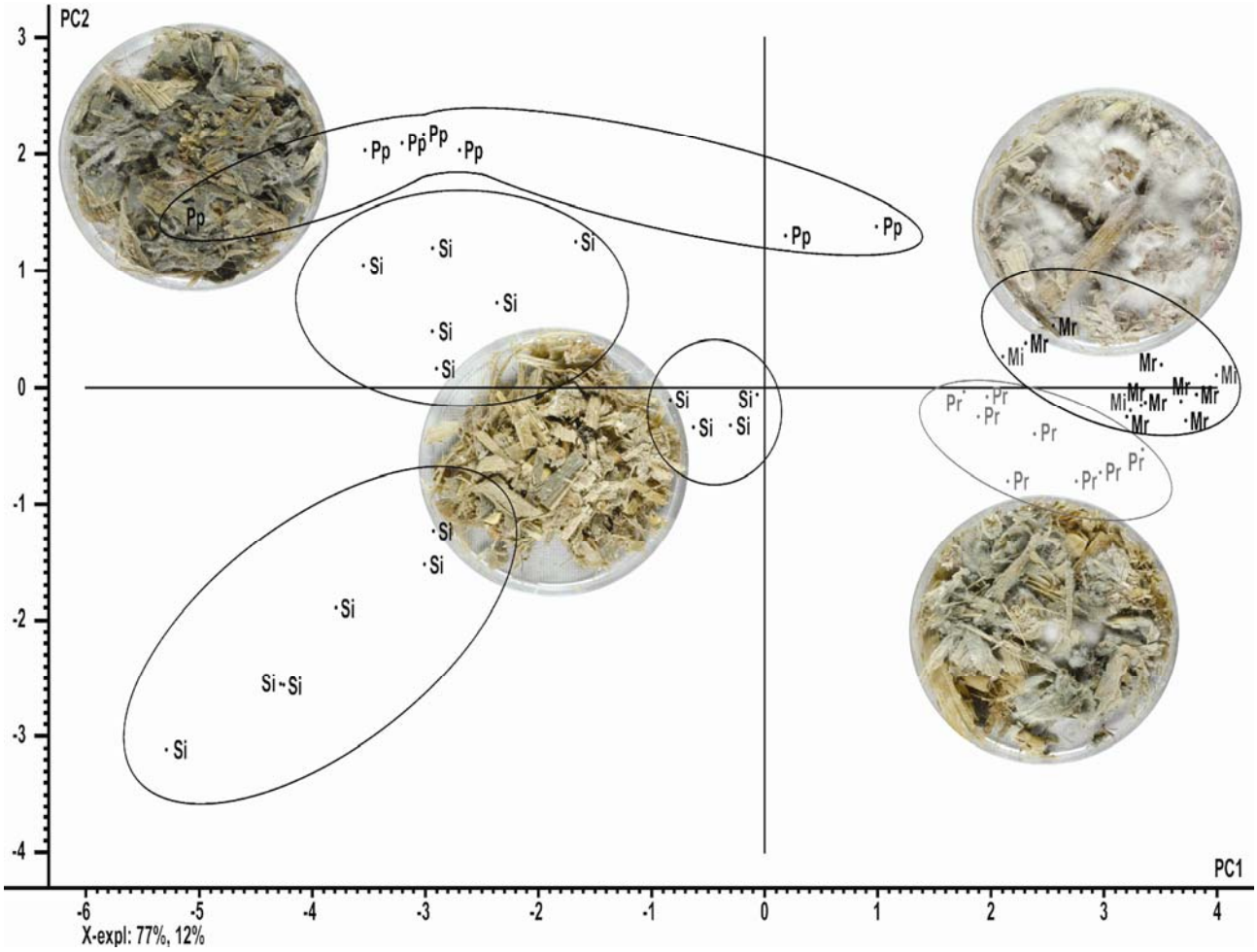


Figure 1. PCA visualization of the classes found by SIMCA analysis of the 13 day silage samples. [Si]: Silage controls; [Mr]: *M. ruber* infected samples; [Pr]: *P. roqueforti* infected samples; [Pp]: *P. paneum* infected samples and [Mi]: Fungal spore mix samples.

From the GC-MS analysis of the headspace from the 16 different silage samples 17 VOCs were detected (**Table 1**). The three compounds shown in **Figure 2**, of which 1-Propanol and Ethyl 2-hydroxypropanoate were unique to group 1 and 3, were statistically significantly different between silage group 2 and groups 1 & 3 according to Duncan's Multiple Range Test partially supporting the grouping seen in the e-nose analysis.

Table 1. The 17 Volatile Compounds Detected by Headspace Analysis of 16 Different Silage Samples Forming 3 Groups as Designated by the E-nose Analysis. Compounds are Listed by the Average Log Area for the Three Sample Groups (Sample Size Per Group Was Five For Group One, Four For Group Two and Six For Group Three. [1]: Designating Significant Difference in Log Average Area From Sample Groups Not Noted by a [1] as Per Duncan's Multiple Range Test.

RI	Compound	Silage Group 1 (log avg area)	Silage Group 2 (log avg area)	Silage Group 3 (log avg area)
473	Ethanol	6.05	nd	4.76
499	Propanal	nd	nd	1.12
552	1-Propanol	7.69[1]	nd	4.76[1]
577	2,3-Butanedione	1.46	1.85	1.24
587	2-Butanone	4.51	5.47	1.30
595	Acetic acid	7.82[1]	1.99	7.94[1]
604	Ethyl Acetate	4.83	nd	5.93
618	2-Methyl-1-propanol	1.46	3.36	1.18
648	3-Methylbutanal	1.37	nd	1.11
656	1-Butanol	nd	nd	1.13
706	2-Pentanone	2.79	5.34	1.21
708	3-Hydroxy-2-butanone	1.66	1.76	1.39
711	Propyl acetate	2.74	nd	1.17
734	3-Methyl-1-butanol	7.38	5.68	7.36
737	2-Methyl-butanol	1.42	3.40	1.16
815	Ethyl 2-hydroxypropanoate	7.09[1]	nd	4.58[1]
856	3-Hexen-1-ol	2.68	nd	3.43

nd not detected.

GC-MS analysis of the headspace from control silage 1 compared to silage infected with *M. ruber*, the fungal mix, *P. paneum* and *P. roqueforti* show that, through Duncan's multiple range test, out of 12 detected compounds (**Table 2**) 9, of which one is an unknown compound, are indicators for silage (**Figure 3**), 1 is an indicator for *M. ruber* infection (**Figure 4**) and 2 are indicators for *P. paneum* and *P. roqueforti* infection (**Figure 4**). Prior to diffusive sampling of volatiles onto Tenax TA adsorption it was noted that the *P. paneum* and *P. roqueforti* samples exhibited a slight smell of

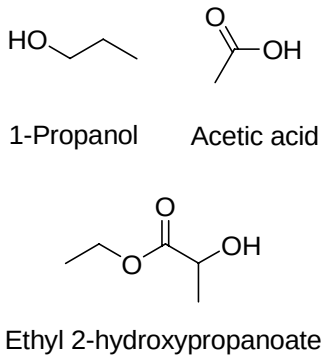


Figure 2. Chemical structures of the three volatile biomarkers differentiating the three silage groups.

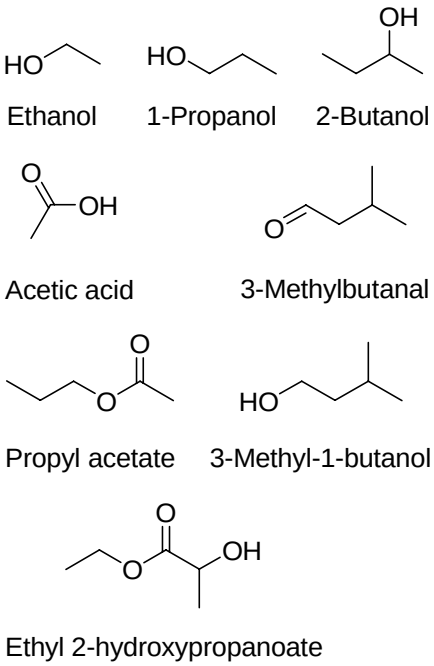


Figure 3. Chemical structures of the eight known of the nine volatile silage biomarkers.

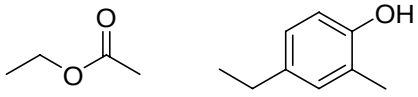
ammonia, which is not absorbed onto tenax. Also the *P. paneum* samples exhibited a slight smell of 1-Octene-3-ol which was also not detected in the GC-MS analysis. In general the fungal contaminated samples had a "weaker" odor than the pure silage samples. Of the three fungal contaminated silages the *P. paneum* contaminated silage exhibited the strongest odor and the *M. ruber* infected silage the weakest smell. It is noteworthy that fungal spoilage of silage showed to remove the silage volatile background almost completely.To our knowledge this is the first report of identification of specific spoilage organisms in a microbial complex environment such as silage by e-nose analysis.

From the results of this "worst case" study it seems very promising to use an e-nose for quality control of silage by plug analysis although additional work has to be carried out in order to produce prediction models for the various silage types. As it has been shown that it is possible to classify fungal contamination of silage to species level the next step would be to investigate whether it is possible to produce prediction models as seen in (*11*) for non-volatile biomarkers / mycotoxins produced by silage infecting fungi such as mevalonin (*M. ruber*), andrastin A (*P. paneum & P. roqueforti*), marcfortin A (*P. paneum*), mycophenolic acid (*P. roqueforti*), PR toxin (*P. roqueforti*) and roquefortin C (*P. paneum*, *P. roqueforti*)

Table 2. The 12 Volatile Compounds Detected by Headspace Analysis of Silage. Compounds are Listed by the Average Log Area for the Five Sample Groups (Sample Size Per Group Was Five For Silage and Mix Samples and Ten for the remainder), Control Silage, *M. ruber* Infected Silage, Mixed Spore Infected Silage, *P. paneum* infected Silage and *P. roqueforti* Infected Silage. [1]: Designating Significant Difference in Log Average Area From Sample Groups Not Noted by a [1] as Per Duncan's Multiple Range Test, [2]: Designating Significant Difference in Log Average Area From Sample Groups Not Noted by a [2] as Per Duncan's Multiple Range Test.

RI	**Compound**	**Control Silage (log avg area)**	***M. ruber* (log avg area)**	**Mix (log avg area)**	***P. paneum* (log avg area)**	***P. roqueforti* (log avg area)**
473	Ethanol	8.09[1]	nd	nd	0.72	nd
552	1-Propanol	8.25[1]	nd	nd	1.47	nd
592	2-Butanol	7.87[1]	nd	nd	0.77	nd
595	Acetic Acid	8.52[1]	nd	nd	nd	2.45[1]
604	Ethyl Acetate	nd	6.33[1]	7.02[1]	6.70[1]	nd
648	3-Methylbutanal	5.57[1]	nd	nd	nd	0.68[2]
711	Propyl acetate	7.61[1]	nd	nd	0.66	nd
734	3-Methyl-1-butanol	7.1[1]	nd	nd	0.68	nd
815	Ethyl 2-hydroxypropanoate	7.87[1]	nd	nd	2.26	1.47
908	Unknown 1	7.18[1]	nd	nd	nd	nd
1119	4-Ethyl-2-methylphenol	nd	nd	nd	5.88[1]	5.72[1]
1302	Unknown 2	nd	nd	nd	1.56	nd

nd not detected



Ethyl acetate 4-Ethyl-2-methylphenol

Figure 4. Chemical structures of Ethyl acetate, a volatile biomarker for *M. ruber* infection of silage and 4-Ethyl-2-methylphenol, a volatile biomarker for *P. paneum* and *P. roqueforti* infection of silage.

Altogether this study has proven it possible to classify spoiled from non-spoiled silage as well as identifying the fungal species causing the spoilage. These results of course are only valid for the chosen silage type, but suggest that it ought to be possible to achieve similar prediction models with any given type of silage. In order to make industrially relevant prediction models it is required to measure many more samples, to create prediction models for all relevant mycotoxins, and to investigate whether it is possible to distinguish between all the different fungi associated with silage. We believe that this study is the first study to show classification of the spoilage organism in a highly complex system such as silage. The results of this study show a great potential for e-nose quality control of silage.

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DISCUSSION & CONCLUSIONS

In the preliminary investigation it was confirmed that even very closely related fungi, grown on synthetic media, can be distinguished by volatile metabolite profiling by GC-MS analysis. This differentiation was achieved by comparing the sesquiterpene profile of the fungi. Comparing the isolates from a given species it was also evident that there is a degree of variation in the volatile metabolite profile produced by the isolates of the species. This raised the question how much influence the sesquiterpene profiles, considering the entire VOC profile, would have on sensor responses in e-nose analyses.

Only a limited number of studies have focused on identifying spoilage organisms by e-nose analysis. Of these studies the focus has mainly been on bacterial and yeast classification (Lundström *et al.*, 1998; Dutta *et al.*, 2004; Casalinuovo *et al.*, 2005; Dutta *et al.*, 2006) with few concerning filamentous fungi (Keshri *et al.*, 1998;Vinaixa *et al.*, 2004).
Keshri *et al*. (1998) showed it possible to classify 4 of the 6 investigated bakery product associated xerophilic fungi correctly using a CP based e-nose.
Vinaixa et al. (2004) were able to identify fungal contamination of bakery product associated fungi up to 88% correctly by analysis with a MS-based e-nose collecting volatile metabolites via SPME and static headspace.

The e-nose has also been applied in predicting Danish blue cheese quality (Trihaas *et al.*, 2005a) and volatile composition (Trihaas *et al.*, 2005b).
Trihaas *et al*. (2005a) showed that it is possible to construct prediction models predicting chemical changes that are strongly related to the quality parameters of Danish blue cheese using a MOS based e-nose.
Trihaas *et al*. (2005b) modelled and predicted the change in 25 identified aroma compounds in Danish blue cheese during ripening by PLS regression of data from a MOS based e-nose and GC-MS based data.

The number of studies where correlation between e-nose analysis and mycotoxin production has been attempted is even more limited to prediction of mycotoxin content in grain (Olsson *et al.*, 2002) and prediction of mycotoxins in durum wheat (Cheli *et al.*, 2005).
Olsson *et al*. (2002) showed the possibility of predicting the mycotoxin level in spoiled grain by PLS modelling using a MOSFET based e-nose. The model could not however, differentiate with sufficient accuracy between samples containing more or less than 5 μg/kg ochratoxin A, and the regression model exhibited a fairly low fit.
Cheli et al. (2005) looked at identifying mycotoxins in durum grain by e-nose analysis using a MOS based e-nose but were only able to generate a level based prediction (high vs. low) using a PCA model instead of concentration based PLS model. Neither of the two studies identified the spoilage fungi.

Classification of closely related fungi by e-nose analysis

A method was developed in order to analyze the headspace of growing filamentous fungal cultures (semi) automatically with an e-nose. This yielded a working method which proved to be adequate for the purpose though not sensitive enough to provide early detection of

spoilage since a loss of volatile metabolites was allowed in order to allow oxygen and carbon dioxide diffusion to and from the cultures. The results from analyzing four repeats of every isolate showed that the method developed was robust as the four measurements were quite identical. Working with three isolates per species showed that there is a fairly large diversity between isolates within a species in terms of volatile metabolite profiles and hence e-nose sensor response. This did not prevent differentiation of the species but added to the complexity of the task. HPLC analysis of the cultures also proved that the cultivation method did not restrict the fungi from producing the expected mycotoxins. Up to 94% of the samples were correctly identified to species level from a total of 7 fungal species, of which 3 belong to the very closely related *P. roqueforti* group. The results of this study manage to identify the contaminants to species level, whereas Vinaixa *et al*. (2004) stop at genus level and even then with a lower classification percentage. Keshri *et al*. (1998) classify 4 of 6 species, though this is only after 48 hours which suggests that Keshri *et al*. (1998) could probably attain a higher degree of correct classification, although fewer species were involved than in this study.

Detection apple spoilage and prediction of patulin concentration in apples and juice

E-nose analysis of *Penicillium expansum* infected Golden Delicious and Jonagold apples proved that it is possible to distinguish spoiled apples from non-spoiled apples, both for whole apples as well as apple pulp and apple juice despite the high VOC background from the apples. In this analysis it was also shown that the e-nose detects this change on basis of a small change in the overall headspace, as the volatile metabolites which are indicative of *P. expansum* spoilage only constituted 6% of the total amount of volatiles measured. PLS regression models, both for apple pulp and apple juice, showed a high correlation between patulin concentration and e-nose measurements, and therefore showed that it is possible to construct prediction models for patulin in apples. This experiment also showed that e-nose response is dependant on apple variety and very likely to show the same tendency as GC-MS, where analysis of various apple cultivars has shown, that apples group by peel colour (green, red or red and green). Before e-nose analysis can be applied industrially further studies have to be conducted in which it is investigated what the threshold for patulin prediction by e-nose analysis is; furthermore extensive sampling must be carried out to produce prediction models suitable for the variations in apple varieties encountered in the apple juice industry.

This study produces a PLS based regression model prediction of patulin concentration whereas, for instance, Cheli *et al*.'s (2005) study does not manage to produce a correlation model between mycotoxins and e-nose analysis data, only predicting low or high levels based on a PCA scores plot analysis.

Compared to Olsson *et al*.'s (2002) study, the fit of the regression model in this study is much higher which makes the correlation model of this study more reliable. It can be discussed whether the level of spoilage in the two studies was similar, but this is a quite complex issue to resolve and would require for instance comparing the dry weight ratio of fungal culture in the two sample systems.

Compared to the combined results from the studies by Trihaas *et al*. (2005a, 2005b) it seems feasible to speculate that it would be possible to

create regression models for the significant volatile metabolite biomarkers for *P. expansum* spoilage of apples.

Classification of silage spoilage fungi by e-nose analysis

SIMCA analysis of E-nose data acquired from analysis of headspace of silage spoiled with *P. roqueforti*, *P. paneum* and *Monascus ruber* proved to be usable for classification of silage as being non-spoiled, spoiled by *M. ruber*, spoiled by *P. paneum* or spoiled by *P. roqueforti*.

The headspace of silage from 15 other locations was also analyzed to investigate the influence of silage variety on e-nose analysis. SICMA analysis of e-nose data also showed that the 16 silage samples clustered in 3 separate groups. The headspace of all samples was furthermore analyzed by GC-MS to determine the volatile biomarkers for the various silage types as well as the volatile biomarkers for species specific fungal infection of silage. The grouping of silage types found by e-nose analysis was partially explained by GC-MS as one group differed significantly from the remaining two. It was also seen that fungal growth on silage caused the disappearance of the typical silage volatiles from the headspace as these were not detected in GC-MS analysis of fungal infected silage. This is not surprising as it is known that fungi growing in silage, among other things, utilize the organic acids produced by lactic acid bacteria as a carbon source.

Both compared to the previous study of cheese associated fungi, as well as Vinaixa *et al*. (2004) and Keshri *et al*.'s (1998) studies, this study exhibits the best classification of samples. This is still the case even though the volatile metabolite background from silage is likely to be the highest background.

Effect of parameterization of e-nose response on data analysis

Finally the parameters for the mathematical function describing e-nose response curves were determined and several parameters such as total curve area, curve slope and wavelet decompositions of the signal were extracted. Chemometric analysis of data generated in the previous experiments was performed on the "raw data" generated by the e-nose, specifically the maximum resistance measured (Y_{max}) and compared to chemometric analysis based on the parameters generated through mathematical modelling. The generated parameters were optimized to span the greatest possible variation with the least number of parameters. These parameters proved to be of the wavelet decomposition type. Both classification and regression modelling was performed with the two datasets, Y_{max} and wavelet parameters and it was shown that with the datasets at hand there was no advantage in parameterization of the data.

Conclusions

The classification percentages reached in this study, even from as complex samples as silage, have proven to be very reliable for predicting the spoilage organism to species level. This is promising for similar, highly complex, environments. It has also been shown that it is possible to create prediction models correlating e-nose analysis data accurately to mycotoxin concentrations in apples and apple juice.

FUTURE PROSPECTS

With this work it has been shown that prediction of mycotoxin production and spoilage source is possible by e-nose analysis in a laboratory environment using a MOS sensor based e-nose. Traditionally hand-held e-nose

systems have been equipped with CP sensors, such as the Cyranose 320 (Cyrano Science, Pasadena, California, USA) which due to their high sensitivity to changes in sample moisture have proven difficult to apply industrially. MOS based hand-held e-nose systems have started to become available such as the PEN3 (Airsense analytics GmbH, Schwerin, Germany).

This development can potentially strengthen e-nose analysis as a method which can be applied outside the lab. If the e-nose is also supplied with proper prediction models for the given task, the simplicity of operation of the instrument can potentially allow non-skilled personnel to operate it and hence analyze samples without the need for expensive laboratory equipment and highly skilled personnel. The e-nose can be applied as a tool for detecting the right samples to be analyzed further by LC-MS or GC-MS, hence improving spot sampling.

Apart from the mentioned areas, the e-nose has a great potential for use in clinical studies, for identification and detection of for instance aspergillosis. Thus the areas where e-nose technology can potentially be used are highly diverse.

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