Modern Methods in Cereal Grain Mycology

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Abstract

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A simple rapid DNA extraction method, equally suitable for spores and mycelia is proposed. Heating samples in NaOH and SDS provides DNA of high purity, suitable for Polymerase Chain Reaction (PCR) analysis. For *Penicillium roqueforti* the detection limit was 6×10^3 conidia and 1 mg (fresh weight) mycelium in the extraction liquid. The method proved efficient with *Aspergillus flavus*, *Fusarium graminearum*, *Rhizopus stolonifer*, *Eurotium herbariorum*, and *Cladosporium herbarum*, as well.

An optimised competitive PCR (cPCR) method for quantifying fungal growth in cereal grain was developed using experimental design. DNA extraction efficiency was quantified by cPCR using primers specific for the internal transcribed spacers (ITS) of the ribosomal DNA of *P. roqueforti*. The proposed method can detect *P. roqueforti* at levels as low as 10^1 CFU/g grain and at levels higher than 10^2 CFU/g grain. Quantification is consistent (CV < 8%) and highly correlated with results from traditional dilution plating.

The possibilities of using an electronic nose or gas chromatography combined with mass spectrometry (GC-MS) to quantify ergosterol, colony forming units (CFU), ochratoxin A, and deoxynivalenol (DON) in naturally contaminated barley samples was investigated. The main volatile compounds of grain with normal odour were 2-hexenal, benzaldehyde and nonanal, while 3-octanone, methylheptanone and trimethylbenzene were the main volatile compounds of grain with off-odours. Both CFU and ergosterol levels could be predicted from data from either GC-MS or electronic nose measurement. It was also possible to classify the ochratoxin A level as either <5 or >5 µg/kg cereal grain, and estimate the DON level. Samples with ochratoxin A levels below 5 µg/kg had higher concentration of aldehydes (nonanal, 2-hexenal) and alcohols (1-penten-3-ol, 1 octanol). Samples with ochratoxin A levels above 5 µg/kg had higher concentration of ketones (2-hexanone, 3-octanone). Pentane, methylpyrazine, 3-pentanone, 3-octene-2-ol and isooctylacetate were positively correlated with DON, while ethylhexanol, pentadecane, toluene, 1-octanol, 1-nonanol, and 1-heptanol were negatively correlated with DON.

Keywords: electronic nose, GC-MS, quantification, mould, fungi, grain-kernels, polymerase chain reaction, competitive PCR, DON, ochratoxin A, CFU, ergosterol, fungal volatile metabolites

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Contents

Appendix

The present thesis is based on the following papers, which will be referred to by their Roman numerals.

I Olsson, J., Schnürer J., Pedersen, L.H. and Rossen, L. (1999). A rapid and efficient method for DNA extraction from fungal spores and mycelium for PCR-based detection. *Journal of Food Mycology*. 2: 251-260.

II Boysen, M.E., Pedersen, L.H., Olsson, J. and Schnürer J. Quantification of *Penicillium roqueforti* in cereal grain with competitive PCR. (Submitted)

III Olsson, J., Börjesson, T., Lundstedt, T. and Schnürer J. (2000). Volatiles for mycological quality grading of barley grains - determinations using gas chromatography-mass spectrometry and electronic nose. *International Journal of Food Microbiology*. 59: 167-178.

IV Olsson, J., Börjesson, T., Lundstedt, T. and Schnürer J. Detection and quantification of ochratoxin A and deoxynivalenol in barley grains by GC-MS and electronic nose. (Submitted)

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Aims

Man has cultivated cereal grains in order to feed himself and his livestock for several thousand years (Reed, 1992). Global production of wheat, the most important food crop, currently totals about 550 million metrics tons (MMT) annually, of which 100 MMT is traded each year on the international market (Morris & Rose, 1996). While the importance of grain moisture content for safe storage was recognised early in the last century, 10-20% of harvested cereal grain crop is still damaged by microbial growth; the two major causes being improper drying and storage procedures (Chelkowski, 1991). Fungi, the leading post harvest pathogens, are responsible for both general spoilage, characterised by the loss of nutrients and technical quality, and health problems due to the formation of mycotoxins and allergenic spores. Grain quality in both international markets and the majority of local markets should be analysed. For the most part this is done by simply smelling the grain odour.

The main goal of this thesis has been to develop and evaluate new methods for the identification, detection, and quantification of fungi and mycotoxins in cereal grains.

Specific objectives have been to:

- develop a fungal DNA extraction protocol useful with both fungal mycelium and spores,
- evaluate extraction methods suitable for a quantitative PCR method of detecting fungal growth,
- identify volatile compounds that correlate to either a grain odour class, CFU or ergosterol contents,
- investigate if an electronic nose can be used to quantify mould growth in naturally contaminated grain,
- evaluate the ability of the electronic nose and GC-MS to quantify ochratoxin A and DON in natural grain samples,

and,

- identify the volatile compounds associated with the mycotoxin contents of naturally contaminated cereal grain.

Growth and metabolism of grain spoilage fungi

Fungal growth

Cereal grain is mainly spoiled by moulds within the genera *Aspergillus*, *Eurotium*, *Penicillium*, and *Fusarium*, i.e. filamentous fungi (Filtenborg *et al.*, 1996). These have an unique mode of growth in that only the hyphal tip (apex) is extended. Through apical extension filamentous fungi can grow towards nutrients and penetrate solid substrates (Sietsma *et al.*, 1995; Wessels, 1993; Wessels, 1994). Enzymes excreted at the hyphal tip degrade substrate polymers such as starch and proteins (Fig. 1). The uptake of organic nutrients is an energy-dependent process that occurs close to the apex. Fungal mycelium can be thought of as a "tube" with a rigid wall consisting of polysaccharides (Heath, 1995; Sietsma, *et al.*, 1995), small amounts of proteins, minor amounts of lipids, and a cytoplasm that is rich in proteins, lipids, and nucleic acids (Bartnicki-Garcia & Lippman, 1982). The hyphal tip is initially highly plastic, but matures into a rigid wall less than 1 mm behind the tip (Sietsma, *et al.*, 1995; Wessels, 1986).

Filamentous fungi are more motile than unicellular bacteria and yeast. The effects of this motility are further enhanced by the ability of the organism to translocate cytoplasm, water and nutrients from older parts of the mycelia, leaving empty hyphae behind (Schnürer & Paustian, 1986).

The most important genera as regards to grain spoilage fungi are found among both ascomycetes and deuteromycetes, the latter producing only asexual spores (conidia).

Fungal metabolism

Primary metabolites are produced from metabolic pathways involved in the essential life processes of fungi (Campbell, 1984). These metabolic pathways e.g. glycolysis and the citric acid cycle are found in all eukaryotes. Fungal secondary metabolites have a more restricted distribution and are often specific for individual genera, species, or even strains (Campbell, 1984; Larsen, 1994). Secondary metabolism is not directly involved in normal growth and is, thereby, regarded as non-essential for the survival of the fungus (Campbell, 1984). Examples of fungal secondary metabolites are antibiotics commonly used in medicine, such as penicillin and griseofulvin, as well as mycotoxins (Bennett, 1995). Mycotoxins constitute a diverse range of compounds from different precursors and pathways that are grouped together based on their toxicity to higher animals and humans. Some mycotoxins are produced by only a few fungal species, while others are produced by a large range of species from several genera (Smith *et al.*, 1984).

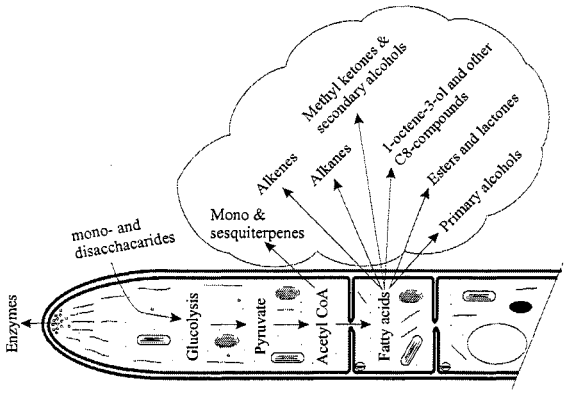

Figure 1. Schematic representation of a fungal hyphae tip, showing the release of enzymes, amylases in this case, at the tip (apical region) and the uptake of small soluble nutrients such as mono- and disaccharides further back from the tip. Glucose is then used as substrate for production of both primary and secondary metabolites from which some volatile fungal metabolites are formed.

Volatile fungal metabolites

Fungal volatile metabolites can be products of both primary and secondary metabolism. These volatiles can be used as spoilage indicators, as well as they are important flavour compounds in many fermented foods (Janssens *et al.*, 1992; Kinderlerer, 1989).

A simplified overview of the biosynthesis of some important volatile fungal metabolites is presented in Figure 1. Fungi take up glucose and metabolise it to pyrovate and then further to Acetyl-coenzyme A. Acetyl-CoA is the main precursor in the metabolism of fatty acids and mono- and sesquiterpenes (Fig. 1), and addition to being the most important precursor in biosynthesis of volatile fungal metabolites (Larsen, 1994). Schnürer *et al.* (1999) listed 2-methyl-1-propanol, 3-methyl-butanol, 1-octene-3-ol, 3-octanone, ethyl acetate, 3-methyl furan, 2-methyl-isoborneol, and geosmin as the most commonly reported fungal volatile compounds. Nevertheless, compounds such as 3-methyl-1-butanol, 1 octen-3-ol and 3-octanone can even be found in grain with normal odour. The concentrations are generally below the odour threshold and, therefore, do not influence the odour profile of sound grain (Jelen & Wasowicz, 1998).

Although the profile of volatile metabolites remains unchanged, the relative abundance of different fungal metabolites has been found to vary with the growth stage of the fungus (Börjesson, 1993). Substrate composition and environmental factors such as water activity (a_w), pH, atmospheric composition, and temperature can have a great influence on both the qualitative and quantitative production of

volatile metabolites. Conditions that favour growth generally also favour the production of volatile metabolites (Börjesson, 1993).

Larsen and Frisvad (1995) investigated the volatile compounds produced by 132 isolates of 25 terverticillate *Penicillium* for species classification. The relative amounts of the 131 detected volatiles were analysed by average linking clustering (UPGMA) and were clearly separated for practically all species investigated. The results agree with previous classifications of *Penicillium*, which have been based on the chemotaxonomy of biosynthetic families of non-volatile secondary metabolites. Larsen (1997) has also showed that volatile metabolites profiles can be used to identify fungi in mixed communities, at least on agar plates.

It should be remembered that yeast and bacteria, both of which are commonly found on cereal grain, also produce volatile compounds. Bacteria and fungi share many metabolic pathways and, consequently, they are expected to produce similar volatile metabolites (Garraway & Evans, 1984; Gottschalk, 1986).

Mycotoxin production in cereal grain

The fungi commonly associated with grain are commonly divided into field and storage fungi (Lacey & Magan, 1991; Pitt & Hocking, 1997). The most important species under field conditions are *Alternaria alternata*, *Aspergillus flavus*, *Fusarium*- and *Cladosporium*-species, while *Penicillium aurantiogriseum*, *P. viridicatum*, *P. verrucosum*, *P. hordei*, *A. candidus*, *A. flavus*, and *Eurotium* species are important storage moulds (Frisvad, 1994; Pitt & Hocking, 1997; Samson *et al.*, 1996). Factors that influence growth, such as substrate composition, temperature, a_w, pH, atmosphere, redox potential, and microbial competition, also influence mycotoxin production (Frisvad & Samson, 1991). For many fungi the conditions that are required for mycotoxin production are more limiting than the range over which growth can occur (Frisvad & Samson, 1991; Lacey & Magan, 1991).

Penicillium-species are more common in temperate climate zones, such as that of Scandinavia, while *Aspergillus*-species prefers tropical climates (Frisvad & Samson, 1991; Pitt & Hocking, 1997). *A. flavus*, *A. parasiticus*, and possibly *A. nomius* are capable of producing aflatoxins, cyclopiazonic acid, and, eventually even maltoryzin and 3-nitropropionic acid (Frisvad, 1994). In temperate climates ochratoxin A from *P. verrucosum* and the trichothecenes from varius *Fusarium* species are the most important mycotoxins (Frisvad, 1994).

Ochratoxin A has been detected both in grain samples and in swine and human blood (Breitholtz *et al.*, 1991; Holmberg *et al.*, 1991; Holmberg *et al.*, 1990; Olsen *et al.*, 1993). Both seasonal and geographical variation in ochratoxin A contamination of grain have been observed (Breitholtz, *et al.*, 1991; Holmberg, *et al.*, 1991; Holmberg, *et al.*, 1990). The same contamination is known to occur

naturally in barley, rye, wheat, oats, rice, and corn in countries with temperate and hot climate such as Europe, Canada, Bulgaria, Turkey, USA, Japan, and Australien (reviewed by Frisvad, 1994). Ochratoxin A has immunotoxic, nephrotoxic, teratogenic, and carcinogenic properties (Krogh, 1987; Kupier-Goodman & Scott, 1989). The fungi usually associated with ochratoxin A are *P. verrucosum* and *A. ochraceus* (Frisvad, 1994; Pitt & Hocking, 1997; Samson, *et al.*, 1996).

The trichothecene mycotoxin DON is produced by several fungal genera, but the genus *Fusarium* being the most significant (Smith, *et al.*, 1984). Eriksen and Alexander (1998) reported the field fungi *F. culmorum* and *F. graminearum* to be the most prominent DON producing *Fusarium* species with the relative dominance of these two species dependent on the temperature. The trichothecenes are cytotoxic, phytotoxic, antifungal, in addition to their insecticidal properties (Ciegler, 1979).

Mycotoxin formation has been correlated with the presence of certain fungal volatiles. Zeringue *et al.* (1993) studied headspace volatiles from aflatoxigenic strains and nonaflatoxigenic strains of *A. flavus* and found that the aflatoxigenic strains produced several $C_{15}H_{24}$ compounds that the nonaflatoxigenic strains did not. The synthesis of trichothecenes and trichodiene, and other volatile sesquiterpenes, was correlated in both wheat kernels inoculated with *Fusarium* species (Jelen *et al.*, 1997; Jelen *et al.*, 1995) and in incubated grain spikes with natural *Fusarium* head blight infestation (Jelen *et al.*, 1997). Pasanen *et al.* (1996) used toxigenic and nontoxigenic strain of *P. verrucosum* and found that ketones consisted of more than half of the microbial volatiles produced by the toxigenic *P. verrucosum* strain, whereas more alcohols were formed by the nontoxigenic strain.

Detection and quantification of spoilage fungi

In Sweden, as well as in many other countries, grains are checked for off-odours at granaries upon delivery (Börjesson *et al.*, 1996; Magan, 1993; Stetter *et al.*, 1993). The rational being that off-odours often indicate past or ongoing microbial deterioration, and that off-odours can make the grain and products less palatable (Börjesson, *et al.*, 1996). Grain inspectors smell the grain and classify the odour of the grain as either "normal", "musty", "mouldy", "acid", "sour", "burnt", or "foreign", while the intensity of the off-odour is graded as being weak, pronounced, or strong (Börjesson, *et al.*, 1996; Jonsson *et al.*, 1997). In many countries this odour check is the primary criterion for determining fitness for consumption (Jonsson, *et al.*, 1997). Nevertheless, the odour check lacks

objectivity since there are individual differences in the perception of intensities and types of odours (Seitz & Sauer, 1991; Smith *et al.*, 1994). Furthermore, inhalation of fungal spores can induce allergic reactions and exposure to fungal volatile metabolites can cause various disease symptoms (Larsen *et al.*, 1998; Rylander, 1986; Walinder *et al.*, 1998). Although smelling grain samples directly does not agree with current working environment regulations, it is still used as it is the only "standardised" method that can determine odour and the occurrence of fungi in a couple of minutes.

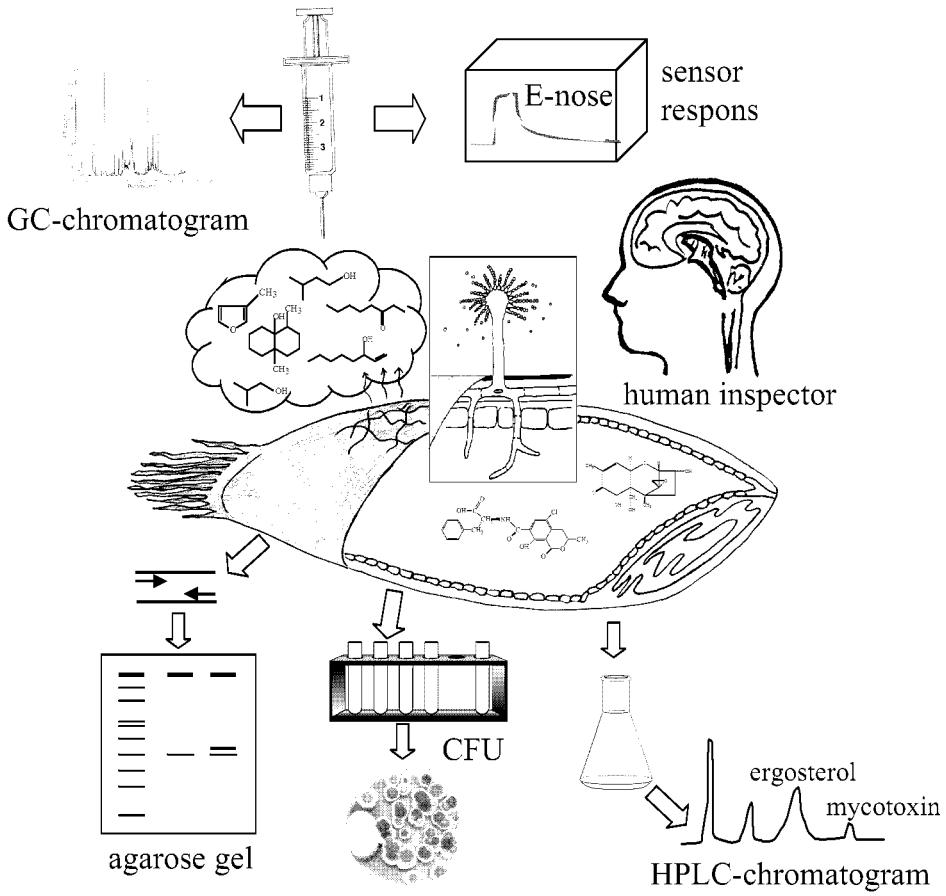

Figure 2. Overview of methods to detect fungal growth in grain (wheat) kernels. Volatile fungal compounds can be used to detect and quantify fungal infection and amount of produced mycotoxins. These volatiles can be detected by gas chromatography, an electronic nose or a human grain inspector. The DNA from the kernel can be purified and the PCR technique can be used to amplify fungal specific sequences. By homogenizing grain kernels in a diluent and spreading on agar, the number of fungal colony forming units from mycelium fragments and conidia can be determined. Fungal specific markers such as ergosterol and mycotoxins can be chemically extracted, separated, and quantified using an HPLC.

The need for alternative methods to evaluate the mycological quality of grain is obvious and has been the driving force behind the work presented here. Figure 2 summarises the different techniques that have been used in this thesis work.

Dilution plating (CFU) and direct plating

The dilution plating or colony forming unit (CFU) method is the most commonly used technique for the examination of food and feedstuffs (Jarvis *et al.*, 1985). It is performed by homogenising the sample, making 10-fold dilutions and surface spreading on agar plates. The agar plates are incubated at 25°C for 5-7 days (Thrane, 1996; Åkerstrand, 1995). The choice of mycological media is very important for the final result. There are only a few widely accepted media, among them DG18 (Hocking & Pitt, 1980) and DRBC (King *et al.*, 1979), although there are several more selective media for certain specific types of fungi such as acidophilic, xerophilic, proteinophilic etc. (Frisvad, 1983; Pitt *et al.*, 1983; Samson, *et al.*, 1996). Counting the number of CFU remains the most common method for monitoring fungal infection in grain. Nonetheless, it provides a poor estimate of fungal biomass (Pitt, 1984), as CFU results depend more on whether the fungi have sporulated than on actual biomass production (Schnürer, 1993).

For mycological examination of particulate hard foods such as grains and nuts, direct plating is considered to be a more effective technique than dilution plating. For enumeration of fungi actually invading the hard food, surface sterilisation before direct plating is considered essential. This is performed by shaking the sample in 0.4% chlorine, rinsing it with sterile water, and transfering individual particles to agar plates (Thrane, 1996; Åkerstrand, 1995).

Fungal specific chemical markers

Fungal biomass can be determined by chemical extraction of ergosterol or chitin, the specific fungal marker substances. The fungal cell wall consist mainly of polysaccharides (Cabib *et al.*, 1982), chitin (β-1,4-linked *N*-acetylglucosamine) and β-1,3-/β-1,6-glucan of the major wall polysaccharides (Gooday, 1995). Chitin occurs in all true fungi (Bartnicki-Garcia & Lippman, 1982), as well as in other groups of organisms such as insects, nematodes, crustaceans, protozoa, and diatoms (Gooday, 1990).

Ergosterol on the other hand, is a fungal-specific membrane lipid (Weete, 1980). Seitz *et al.* (1977) pioneered a HPLC method for quantification of ergosterol that has become widely used for estimating the degree of fungal infection in grain and other plant materials.

A correlation between ergosterol content and hyphal length and CFU for nonsporulating fungi on synthetic agar substrate (Schnürer, 1993), as well as

between ergosterol and CFU in grain has been reported (Schnürer & Jonsson, 1992). One drawback to this method is that ergosterol content does not increase in relation to sporulation, whereas the number of CFU does (Schnürer, 1993). Furthermore, ergosterol and chitin can not be used to identify species. Neither does it indicate mycotoxin production nor can it distinguish between pre- and post-harvest invasion.

An electronic nose was able to predict ergosterol and CFU contents both in wheat kernels infected with *P. roqueforti* (Schnürer, *et al.*, 1999), and in naturally infected barley samples as well (**III**).

Molecular techniques

Polymerase chain reaction (PCR)

Fungi have been identified on the basis of morphological characteristics which is often difficult and requires highly skilled personnel. This method is also time-consuming since fungal cultures have to be grown for one to two weeks before identification is feasible. The food industry often demands answers in minutes, an impossible request for the traditional mycological techniques. It is often important to determine whether a certain fungus might have been growing in a raw material before it was killed by processing, e.g. heat treatment. Since the traditional CFU method requires culturing of the fungi, fungal contents from dead material can not be estimated. PCR technique offers much faster alternatives to standard identification procedures since fungal DNA can be extracted from food samples without incubation. Killed fungi can also be detected which might be an additional advantage (see above). The technique offers effective detection of fungal genus or species if a suitable set of primers is used. Some common amplification targets have been 18S rDNA (Fletcher *et al.*, 1998; Kappe *et al.*, 1998), the internal transcribed spacer (ITS) region of the ribosomal genes (Pedersen *et al.*, 1997)and mitochondrial DNA (Smith *et al.*, 1996), as well as genes coding for specific proteins (Niessen & Vogel, 1997).

DNA amplification efficiency depends heavily on sample preparation since various components can inhibit the rate or extent of the reaction. Food containing high levels of fat and protein has been reported to inhibit PCR (Lantz *et al.*, 1994; Rossen *et al.*, 1992) as well as factors from plant tissue, soil and sediments (Wilson, 1997). Rossen *et al.* (1992) tested various concentrations of substances used for DNA extraction such as detergents, NaOH, and alcohols, and found that these compounds could have an inhibitory effect on the PCR, the size of which depends on the amount used. The DNA extraction methods used should render

DNA of high purity, while retaining insignificant amounts of PCR-interfering compounds. Most of the published methods for fungal DNA extraction have been developed using mycelium grown in liquid culture. This is very different from the natural environment with a solid substrate like cereal grain, where spores also are produced.

Paper I describe a fast and efficient method for DNA extraction from both fungal spores and mycelia from various food- and feed-born fungi. Since the number of amplified gene copies per genome equivalent of conidia or mycelium mass can differ, it is diffucult to compare the efficiency of different DNA extraction protocols. Notwithstanding, the NaOH/SDS method seemed more efficient than the methods described by Ferreira and Glass (1996) and Xu and Hamer (1995). The detection limit was found to be less than 15 conidia in the PCR reaction or 5×10^3 conidia in the original 500 µl sample (Figure 5; paper I). For mycelium the detection limit was found to be 0.25 µg fresh weight per PCR reaction (Fig. 6, **I**).

Quantitative polymerase chain reaction

It is often of interest to quantify the degree of fungal contamination. PCR can become quantitative by either comparing the amount of product with an external standard (Cross, 1995), or by using an internal standard (competitor DNA; (Wang *et al.*, 1989). Use of an external standard involves comparing the intensity of a PCR band against standard curve. The use of external standard has the drawback that small variations during sample preparation will multiply during the amplification process. To overcome sample variation an internal standard that is competitive to the sample DNA can be used. The competitive DNA and target DNA share primer-binding sites and is distinguished from the target by size. It is, however, important that the competitor DNA is properly constructed so the amplification efficiency of both the target and competitor is equal through the entire PCR process (Zimmermann & Mannhalter, 1996). This makes the construction and validation of the competitive PCR assay time-consuming. Quantitative measurements are also most accurate when the ratio of competitor and target templates is equal, and when the PCR process is in the exponential phase (Arnold *et al.*, 1992).

During the last years, new techniques called real-time methods have been developed for clinical use. The use of fluorophores is common to most of these methods and is described in detail by Boysen *et al.* (2000). These methods rely on fluorescence detection to monitor and quantify in real time. The instrument runs the cycle until the emission is strong enough for detection. The number of cycles that necessary for detection is used to calculate the initial amount of target DNA.

A protocol for quantitative extraction of fungal DNA from grain was proposed in **II**. An internal standard with primer-binding sites identical to the *Penicillium roqueforti* specific PCR-primers described by Pedersen *et al.* (2000) was used. DNA extraction efficiency was optimised using statistical design of experiments (Lundstedt *et al.*, 1998). Ten factors were varied in a screening design (Fractional Factorial Resolution III) and the numbers of rDNA copies were used as response (**II**). In design of experiments, the invested factors are varied simultaneously around the center-point experiment. The objective in screening designs is to find important factors, while it in optimisation is to optimise the response (Lundstedt, *et al.*, 1998). Among the ten factors, only SDS concentration, sonication time, and freeze drying had significant effects on the extraction efficiency (Fig. 3, **II**). A central composite face-centered (CCF) design was adopted that allowed the SDS concentration and sonication time to vary, while the rest of the factors were fixed. Based on the results from the CCF design, an optimised quantitative DNA extraction protocol is proposed (**II**).

Analyse of volatile compounds

Headspace analysis

There are several sample preparation techniques for volatile analysis such as steam and vacuum distillation, dynamic headspace, static headspace, and direct injection (Snyder, 1995). In static headspace, the sample is placed in a vessel and the opening is sealed (Fig. 3). The volatile compounds from the sample will then form an equilibrium with the headspace. The headspace can then be withdrawn with a gas tight syringe, and sample is injected into a Gas chromatograph (GC) or an electronic nose. Since only small amounts of gas can be injected into a GC, volatile compounds are often concentrated in a cold trap, on an adsorbent or in liquid before injection. This concentration step is not common in electronic nose measurements, but at least one manufacturer of electronic noses uses an adsorbent technique to concentrate volatiles before injection onto the gas sensor array. Tenax TA and Chromosorb 102 are the most commonly used adsorbents for sampling and quantitative analysis of fungal volatiles (Sunesson *et al.*, 1995).

We used technical air (80% N_2, 20% O_2) to obtain a stabile reference atmosphere for measurements with both the electronic nose and when adsorbing volatile compounds from grain to adsorbents (**III** and **IV**) for GC-MS analysis. Figure 2 in **III** shows how the volatile compounds from a grain sample were adsorbed onto an adsorbent. A detailed description of the electronic nose measurements can be found in **III** and Börjesson, *et al.* (1996).

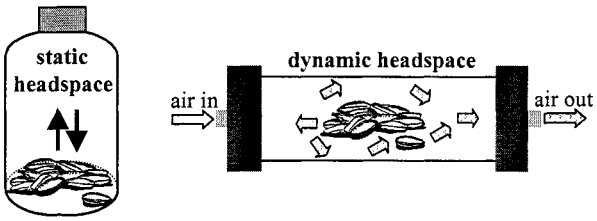

Figure 3. The principle of static headspace (left) and dynamic headspace (right) from grain kernels that have been placed in different types of vessels to generate of headspace.

In the dynamic headspace technique volatiles are swept away from the sample with an inert gas. As opposed to static headspace, dynamic headspace will never reach equilibrium since the volatiles are steadily forced from the sample chamber to the adsorbent as described above. Dynamic headspace is the method of choice when compounds of low concentration or low volatility are to be analysed (Przybylaki & Eskin, 1995).

In **III**, each grain sample was split into three subsamples, which were then analysed three times each with the electronic nose. The idea was to obtain large amounts of data for evaluation, model derivation, and interpretation and validation of these models. Nevertheless, when a PCA model was developed for the sensor signals from the measured grain samples, we found that the scores for t_1 decreased for each sample measurement (Fig. 4). Seitz (1995) reported that the amounts of total volatiles from five consecutive purges of a wheat sample were nearly uniform in size. The composition of the volatiles was slightly changed, however, and the first purge contained the largest amounts of low molecular weight compounds. It is most likely that the electronic nose easier detect low molecular weight compounds. That is why only the first measurement (average of the three subsamples) was used for further modelling.

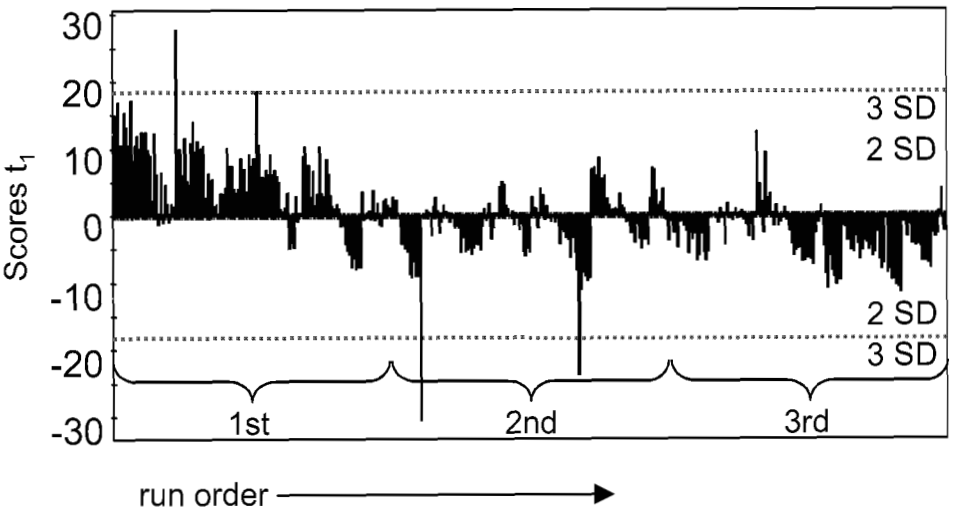

Figure 4. The scores of the PC 1 (t_1) plotted against measurement order. The measurements obtained with the electronic nose in paper **III** were sorted by measurement order and a PCA were performed.

Gas chromatography with mass spectrometer (GC-MS)

Several techniques are available for the analysis of volatile compounds such as spectrometry, gas chromatography, and gas sensor arrays. Spectrometry methods such as FT-IR and NIR can be used to analyse complex gas mixtures such as smoke gases (Pottel, 1996). They can also be used to detect kernels of corn infected with mycotoxigenic fungi (Gordon *et al.*, 1998) or to predict scab, vomitoxin, and ergosterol (Dowell *et al.*, 1999). Recently, mass spectrometric (MS) technology has also been used to analyse volatile compounds since this technique can accommodate both qualitative and quantitative determination.

The GC consists of an injector, a column located in an oven, and a detector. A carrier gas (inert gas) flows through the column. When a mixture of volatile compounds is injected into a GC the different compounds will be separated in the column and, given that the mixture is completely resolved, detected one after another. Several factors affect the degree of separation such as column parameters (length, diameter, material, etc.), oven temperature, and carrier gas (flow, gas). Flame ionization detection (FID) is probably the most commonly used detector for GC, although it does not give any structural information about the detected compounds. The most important detection method for identification purposes is mass spectrometry (MS) (Larsen, 1994).

Electronic nose

Over the last twenty years there has been a rapid expansion in the development of gas sensor arrays. Gas sensors are chemical sensors that transduce a chemical state into an electrical signal. The signal is used as input in pattern recognition system in order to recognise different volatiles and odours. The integrated system of gas sensor array and pattern recognition is often called "electronic nose" (Gardner & Bartlett, 1994).

The first electronic nose, developed by Persaud and Dodd (1982), was based on three chemoelectronic sensors and the same architecture that most systems use today. The most important part of an electronic nose is the sensor array, which can differ in composition between the different manufacturing companies.

The usefulness of electronic noses has been investigated in numerous products such as food, beverages, packaging, cosmetics, cars etc. and for broad range of applications: quality control of raw and manufactered products, shelf life investigations, microbial pathogen detection, and environmental studies (reviewed by Schaller *et al.*, 1998).

Gas sensors

A particular odour have a highly complex composition and consist of several hundred different molecules. Electronic noses similar to the human nose, consist of arrays of sensors with broad overlapping sensitivity towards a range of simple volatile organic molecules (Pearce, 1997; Pearce, 1997). This is maintained by combining several types of sensors into a sensor array.

The electronic nose used in **III** and **IV** was developed and manufactured by S-SENCE, Linköping, Sweden. It contained 5 MOSFET sensors in two different capsules held at 140°C and 175°C, 6 metal oxide sensors (Taguchi; Figaro Inc., Japan) and one optical CO_2 sensor. These three sensor types are described in more detail below, while some other common sensor types are described only briefly.

Metal Oxide Semiconductor Field Effect Transistor (MOSFET) sensors

The ability of palladium to react to small amounts of hydrogen was discovered over 20 years ago by Lundström *et al.* (1975) which led to its use as a gate metal in MOSFET sensors (Fig. 5).. A MOSFET sensor consists of three layers; a doped silicon as a substrate, a metal insulator (~ 100 nm SiO_2) and a catalytic gate metal layer (e. g. Ir, Pt, Pd). The catalytic metal layer can be either dense (thick) or discontinuous (thin), meaning that the layer will contain holes and cracks. By using different catalytical metals (Ir, Pt, Pd), varying the thickness,

and operating temperature of the device, a variety of molecules can be detected (Lundström *et al.*, 1990).

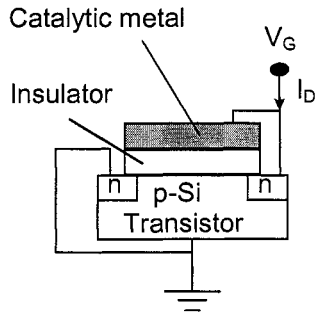

Figure 5. Schematic MOSFET structure with a thick (dense) catalytic metal layer (modified from Eklöv (1999).

The basic principle for MOSFET sensors with a thick (dense) catalytic metal layer is that when volatile compounds containing hydrogen reach the surface of the catalytic metal layer they react (dehydrogenate), and hydrogen atoms are released. The hydrogen atoms diffuse through the metal layer to the metal-oxide interface, as shown in Figure 6 (Lundström *et al.*, 1989). The adsorption of hydrogen atoms at the interface give rise to a dipole layer. This is in equilibrium with the outer layer of adsorbed hydrogen and, hence, with the concentration of hydrogen containing volatile compounds. The raised dipole layer will introduce a voltage drop in the characteristic current-voltage (I-V) curve of the sensor. This voltage drop is measured as a sensor signal. When using a discontinuous layer it is believed that other electrical polarisation phenomena can also contribute to the voltage shift (Eklöv, 1999).

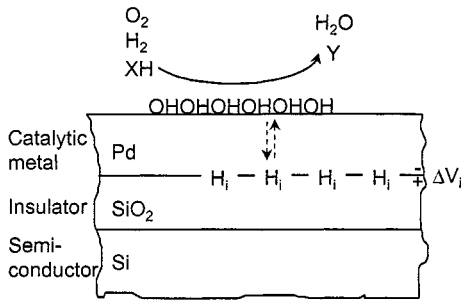

Figure 6. Detection principle for a MOSFET sensor with a thick film of the catalytic metal, in this case Pd. (from Eklöv (1999) with permission from T. Eklöv).

Metal Oxide Sensors (MOS)

MOS are based on different kinds of semiconducting metal oxides (Göpel & Schierbaum, 1995) and are the most common sensors used in electronic nose systems. Both oxidising and reducing molecules, such as CO_2, O_2, CH_n, and H_2 can be monitored by MOS sensors (Göpel, 1996).

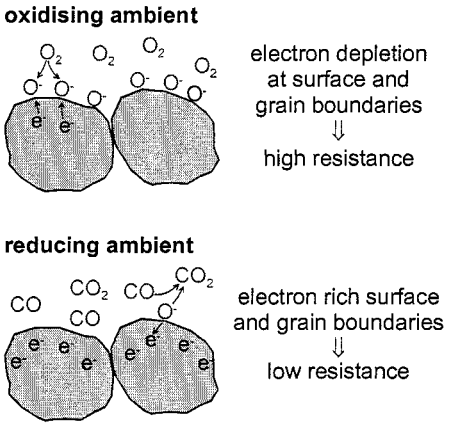

Figure 7. Principle for MOS sensor operation. (With permission from P. Mårtensson, NST, Linköping).

When oxygen reaches the surface of the grain boundaries oxygen ions are formed as the electrons close to the surface of the grains react with O_2 (Fig. 7). This leads to a decreased conductivity since these electrons contribute to the conductivity of the grains. In a reducing ambient, the oxygen ions react with the reducing compounds, which in turn causes a decrease of the conductivity since the concentration of electrons at the surface of the grains increases. The sensitivity profile of these sensors can be modified by changing the oxide microstructure, by addition of different dopants/catalysts to the oxide or by exchanging the oxide. The sensor's operating temperature is also important, since it may affect different gas sensing properties (Göpel, 1996).

Optical sensors

Optical sensors can detect a large number of compounds. In most sensors a light source excites the molecule, and the resulting signals are registered in a number of ways; as reflectance, absorbance, optical thickness, flourescence, or chemiluminiscence (Wolfbeis *et al.*, 1991). The Gascard CO_2 monitor used in **III** and **IV** is based on optical absorption at a CO_2 specific wavelength. This device is particularly useful in biological systems where CO_2 is an important indicator of biological activity, especially as a first indication of microbiological growth and activity.

Other common gas sensors

Conducting polymer sensors are derived from aromatic or hetero-aromatic polymers which have been doped with ions into the polymer matrix. By changing the structure of the polymer, the organic materials, and the dopants sensitivity towards many organic ocmpounds is obtained (Eastman *et al.*, 1999).

Two types of oscillating sensors have been used in electronic noses. By varying the sensing layer sensitivity profiles for different volatile compounds can be obtained. Quartz micro balance (QMB) sensors consist of a single quartz crystal with a gold layer evaporated on the top of both sides of the crystal. The sensing layer is then placed on the gold layer and can consist of materials similar to those used in surface acoustic wave (SAW) sensors (see below). Adsorption of molecules onto the sensing layer will in both SAW and QMB sensors results in increased mass and changed viscosity of the sensing layer (Persaud & Travers, 1997). The SAW sensor consist of a strip of piezoelectric material with the adsorbing layer in the middle. SAW sensors are usually operated as delay lines by having both input and output transducers. The delay between launching and receiving the wave depends on wave velocity (Eklöv, 1999). The sensing layer can be made up of polymers, biomolecules, metals, or supramolecular structures (Göpel & Schierbaum, 1997).

Feature extraction

Sample preparation for measurement with electronic noses is commonly performed as headspace analysis, which is accomplished by placing the sample in a flask and sealing it with a septum. To eliminate variation due to changes in the air composition and humidity in the lab, the air in the flask is replaced with a reference gas such as technical air, which is sometimes humidified to a specific level. In on-line applications the set-up of the electronic nose sampling interface to the reactor can differ slightly (Bachinger *et al.*, 1998; Bachinger *et al.*, 2000).

A measurement cycle starts and ends with exposure of the gas sensor array to the reference gas. The purpose of using the reference air is twofold; to remove volatile compounds from the test gas in the system, and to provide a stable reference for the sensor signals. The baseline value (Fig. 1, **III**) for MOSFET sensors varies considerably. Since the sensor signals extracted from the sensor curve are related to the baseline, drift can be compensated nevertheless (Eklöv, 1999).

An example of a typical sensor response curve from a MOSFET sensor is shown in Figure 1, **III**. From the curve five signals, response height, on- and off-derivatives, and on- and off-integrals, are calculated. The electronic nose model used in (Schnürer, *et al.*, 1999) was only able to extract the response sensor signal (Figure 8). Even so, we were able to predict ergosterol and CFU in wheat grain inoculated with *Penicillium roqueforti*. Eklöv *et al.*, (1997) showed that the

response signals were approximately similar when a Pt MOSFET sensor was exposed to either 50 ppm hydrogen or 50 ppm ethanol, while the shape of the response curves were highly different (Fig. 8). Consequently, it might be important to extract several sensor signals from each response curve.

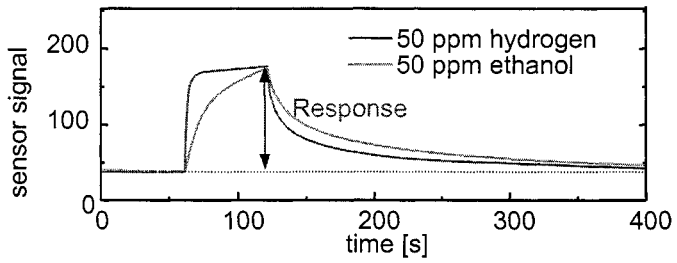

Figure 8. An example for a Pt MOSFET sensor exposed to pulses of ethanol or hydrogen in technical air. (after Eklöv, *et al.* (1997).

The results published in **III** show that all five types of calculated sensor signals, response, on and off derivatives, and on- and off-integrals, were important to get a maximum separation between grain samples with normal and off-odours (Fig. 5c-d, **III**). When only the response signals from the 16 sensors were used, the two classes (normal and off-odours) again could not be separated.

Data evaluation

A standard statistical analysis of data using the univariate procedure, is to calculate an average for each variable and a standard deviation for each class of observation. Subsequently, some kind of test for the class separation for that variable is made. The procedure is repeated until all the measured variables have been checked. This kind of statistic analysis works well on "long and lean" data matrices where many observations have been measured for few variables. Standard assumptions that have to be made are that the measured variables are independent of each other, are exact (contain little noise) and that the noise is randomly distributed. This is seldom the case since instruments such as GC, NIR, and electronic nose provide the user with 100-1000 variables or more, and these variables are often correlated. The costs of the reference analysis can at the same time be so high that only a few samples are actually analysed. A data matrix containing few observations (N) and several variables (K) necessitates the use of

multivariate methods such as Principal Component Analysis (PCA) and Partial Least Squares (PLS). The advantages of these techniques is that they can deal with data matrixes of almost any size (N>>K, N<<K, and N~K), multi-collinearity, noise they are robust to noise in both X and Y data, extract the information and save the noise in a separate matrix, and can handle missing data as long as the data are not missing in some systematic pattern). Additionally, PCA and PLS provide many informative diagnostic and graphical tools.

Multivariate analysis

Principal Component Analysis (PCA)

In PCA, the measured variables are transformed into a new set of variables, called Principal Components (PC) (Wold *et al.*, 1984; Wold *et al.*, 1987). Since the different PC's are not correlated to each other, PCA can handle multi-collinearity, and dimensionality is reduced at the same time. The advantages to this is that every measured object (sample) and variable can be studied simultaneously and that the important information is extracted in only a few PC's (usually 2-4).

The geometric principle of PCA can be described as follows. Assume that interest is on studying how the first twenty samples (ten normal and ten with off-odours) described in table 1 in **III** are correlated to each other. The variables CFU, ergosterol, and water content (K = 3) of these twenty barley samples are used. Each variable represents one co-ordinate axis, together forming a multidimensional space (here three dimensional, Fig. 9). The samples are then plotted onto the variable space according to the results from the analysis.

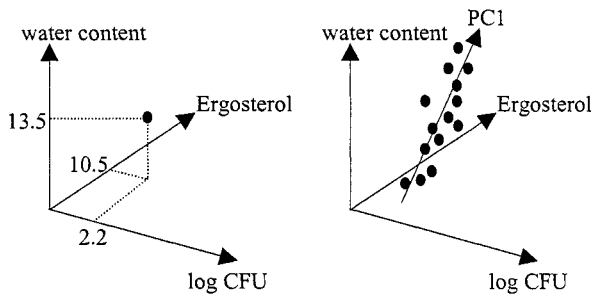

Figure 9. A K dimensional (K = 3) variable space were the first sample from table 1 paper III has been plotted (left figure). Each sample is represented by one point in a three dimensional space. PC1 is calculated to capture the maximum variance direction in the data (right figure).

The first PC is then computed in the variable space so that the distance for each sample to PC 1 will be minimised. PC 1 is subsequently the line that best approximates the data in a "least squares sense". PC 2 is then computed orthogonally to PC1 and improves the approximation of the data as much as possible; describes the second largest variance direction in the data set. PC1 and PC2 together define a 2D space into the variable space. The observations can be projected down into this reduced dimension sub-space and be visualised. The score plot (Fig. 10 left figure) shows how the samples are related to each other, while the loading plot (Fig. 10 right figure) shows how the variables relate to each other.

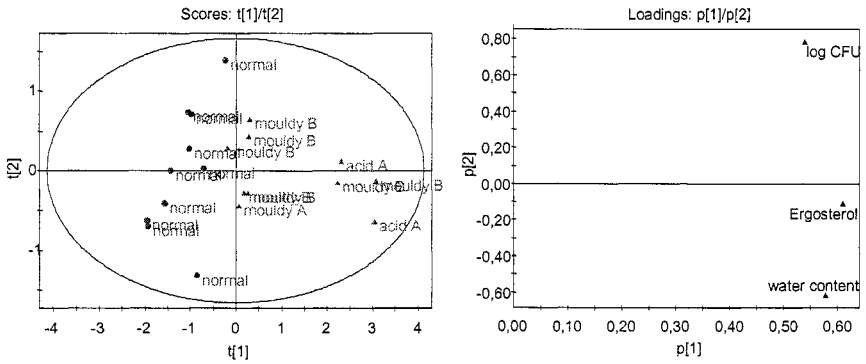

Figure 10. Example of a score plot (t_2 vs t_1; left) and loading plot (p_2 vs p_1; right) based on the first 20 samples and the three variables shown in table 1 of paper III.

For each PC, R^2- and Q^2-values are computed. R^2 is a measure of the amount of variation explained by the respective PC. R^2 can take values between 0 and 1. Q^2 is a measure of the predicted variation after cross-validation for that PC. R^2_{Cum} and Q^2_{Cum} are the cumulative value for R2 and Q2 values, respectively, summed over all PC's.

For a given model (set of variables), R^2_{cum} and Q^2_{cum} values close to 1 are desirable since it means that the particular set of variables adequately explains the variation in the data. For a satisfactory model, both R^2_{cum} and Q^2_{cum} should be above 0.8 and not separated from each other by more than 0.2 or 0.3. Since biological data often contain a lot of noise, models with Q^2_{Cum} values > 0.4 are considered satisfactory and Q^2_{Cum} values > 0.6 are regarded as excellent.

Multivariate methods like PCA and PLS are sensitive to scaling. Scaling is an integral part of data pre-processing. The most common transformation is to unit variance and no scaling. In unit variance each variable is first divided with the standard deviation for that variable and then mean-centred. This will give all

variables equal opportunities of influencing the data analysis. If the variables with large variation are known to be important, it is often better to use no scaling since it will allows variables with large variation to influence the result more than variables with small variation (Eriksson *et al.*, 1999).

Classification using pattern recognition (PARC) techniques

When the data contains classes, pattern recognition (PARC) techniques can be used to classify samples. PARC is often described as a procedure for formulating rules of classification (Albano *et al.*, 1981). Asymmetric data and SIMCA are two examples of situations where PARC can be used.

SIMCA classification (Soft Independent Modelling of Class Analogy) can be used to visualise the distance between the classes (Albano, *et al.*, 1981; Coomans *et al.*, 1983; Sjöström & Wold, 1980). In SIMCA classification a PC model is made for every class. For each sample, the distances to the two models is computed and plotted along with the class membership limit (critical distances). The result can be visualised in a Coomans plot (see Fig. 4a-b, **III**). In **III** one of the goals was to investigate if the volatile compounds detected by a GC-MS and the electronic nose could be used to classify grain samples as having either normal or off-odour. For both GC-MS and the electronic nose one PC model was developed for normal odour and one for off-odours. Coomans plots were used to visualise if the samples were classified as normal, off-odour, none of these classes, or both. The results showed that the electronic nose misclassified three of the 40 samples, compared to six samples misclassified when using GC-MS data (Fig. 4a-b, **III**).

A special case of PARC is the asymmetric case exemplified in Figure 1, **IV**. Asymmetric data are commonly found in medical diagnosis (one disease vs. all others), chemical structure determinations (one type of structure vs. all others), biomedical applications (biological active compounds vs. inactive compounds), and quality control (good vs. all types of inferior) (Albano, *et al.*, 1981; Sjöström & Wold, 1980; Thelin *et al.*, 1995). We found in **IV** that samples with an ochratoxin A concentration < 5 μg/kg could be assigned to the same group, based on similarities in volatile metabolite profiles. The defined class is tight and forms a well-defined tolerance volume. In contrast, the grain samples with ochratoxin A > 5 μg/kg were scattered around the tight class. By performing a PC model on the tight class, and estimate the distance to the model for new samples, these can be classified as either belonging to the tight or the diffuse class (**IV**).

Partial Least Squares (PLS)

PLS is a regression extension of PCA, since PLS take into account two blocks of variables designated X and Y (Höskuldsson, 1988; Sjöström *et al.*, 1983). The Y block may consist of a single response (variable) or several. In PLS two co-

ordinate systems, one for the X block and one for the Y block are performed. This means that each sample corresponds to one point in the X space and one in the Y space. As in PCA, a vector is computed, but in PLS it is tilted so that it reflects the information in the X-block that is of relevance for prediction of the response. This means that in PLS the PC's are computed to maximise the covariance between X and Y space. This is done by finding a weight, c, that can be multiplied by the score, t, to estimate y. The geometry of PLS has been described by (Phatak & DeJong, 1997).

It is important to remember how PC 1 is computed in PCA and PLS. In PCA PC 1 is computed so it explains the largest variation in the data. Nevertheless, it is not certain that the maximum variation directions coincide with the maximum separation of classes. An example of this can be seen in Figure 3a-b (**III**). By constructing dummy variables and using a PLS based technique called PLS discriminant analysis it is possible to rotate the PC so that it focuses on class separation (Sjöström *et al.*, 1986).

Other methods

There are several other data evaluation and pattern recognition techniques in use such as cluster analysis, Nearest neighbour classification, System identification methods, Fuzzy models, Genetic algorithms. Artificial neural networks (ANN) are frequently used to handle sensor signals (Bachinger, *et al.*, 1998; Brezmes *et al.*, 1997; Börjesson, *et al.*, 1996; Eklöv *et al.*, 1998; Gibson *et al.*, 1996).

An ANN consists of an input layer, one or several hidden layers, and an output layer (Fig. 11). The basic unit is called neuron and consists of the input weight (w_{jk}), a summation function, and a transfer function. Several transfer functions exist, but the sigmoid function is the most frequently used since it supports non-linear models as well. Data presented to the network through the input layer are transferred to the hidden layer. The signals are then propagated to the output layer, which produces an output from the model. The output is then compared with the appropriate calibration value, and weights, which are free parameters, are adjusted so the model will fit the data.

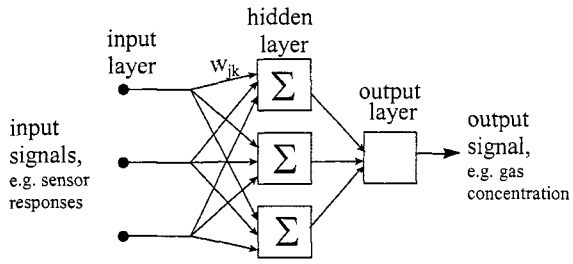

Figure 11. The architecture of a feed forward ANN with input layer, a hidden layer, and an output layer. The hidden layer and the output layer contain a summation and a transfer function (modified from (Eklöv, 1999).

The advantage of ANNs are their adaptability and non-linearity, while the disadvantage is that it requires large data sets which are often difficult to obtain. The major drawback however, is that ANN models lack a complete theoretical basis, and can conceivably be difficult to analyse.

Sensitivity, speed, and economics of grain mycology methods

Figure 12 illustrates a "guestimate" of time to detection for the methods presented here.

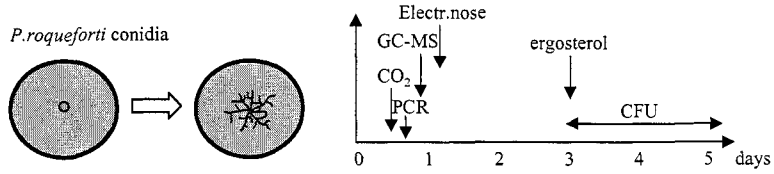

Figure 12. Estimate of the earliest time to detection for the methods described in the thesis, assuming growth of a single *Penicillium roqueforti* conidia.

Both CFU and ergosterol are used today to determine the level of fungal infection in cereal grain samples. The advantage of CFU is that this method is

established and used world-wide. Nevertheless, the method requires 5-7 day incubation, and skilled technicians for interpretation of the results. The ergosterol analysis is also time consuming and requires access to a HPLC system. The sensitivity is somewhat higher than CFU.

cPCR shows much higher sensitivity than both ergosterol and CFU. The instrument is on the other hand expensive and since the technique often requires several primer pairs to be used, the cost per analysis might be quit high. However, a large number of samples can be analysed per day. The technique also offers a possibility to detect dead mycelium or spores. The major drawback is that the result is highly depending on the DNA extraction method.

Analysis of volatiles using GC-MS the highest sensitivity, but requires sample preparation, i.e. use of adsorbents, and time for evaluation of the data. It can on the other hand detect and quantify every single compound. This can not be done with the electronic nose. The sensitivity is also lower for the electronic nose, and calibration against other methods have to be done, but no sample preparation is needed.

Concluding remarks

Detection of fungal volatile metabolites for evaluation of the mycological quality of cereal grains has been main topic of this thesis work. The investigation have proved that both an electronic nose and GC-MS systems can be used to predict ergosterol, CFU, ochratoxin A, and DON contents, as well as to differentiate between normal and off-odours in naturally infected barley samples (**III**, **IV**). Further validation of these detection techniques requires investigations of a large number of samples from all the major cereal crops. This would provide a better understanding of the natural variation in grain volatile metabolites. Although the electronic nose has proved valuable in the studies reported here, little is known about the sensitivity of the gas-sensors to different volatile molecules, in particular with regard to combinations of volatiles. The construction of a headspace system with a split injection system, enabling simultaneous injection if volatile compounds to both an electronic nose and a GC-MS system, would facilitate such studies.

Furthermore, much more knowledge about the physiology and metabolism of grain spoilage fungi is needed. Volatile metabolites produced by the most common grain associated fungi, both in single culture and in mixed communities on cereal grains, needs to be identified. The relationship between mycotoxin formation and the metabolism of volatiles has only just been touched upon. From

a practical point of view the adsorption and desorption of fungal volatiles to grains also merits further studies. If these metabolites could easily be removed by passing an airstream through the grain, active manipulations that would hide mould problems could potentially occur in the grain handling chain.

Amplification of fungal DNA with PCR provides an alternative method for estimation of fungal infection in grain kernels. We have developed a fast and simple method for extraction of DNA from both fungal spores and mycelium (**I**) and a quantitative DNA extraction protocol for cereal grain (**II**). Compared to ergosterol detection, molecular methods have the advantages that they can detect specific genera or species by using different primer sets, while the ergosterol content only estimate the level of fungal infection.

An important aspect concerning the introduction of novel detection methods is that they have to be included in government regulations in order for the industry to see the necessity of using them. Specified CFU limits for cereal grains are part of national regulations today, while a legislation establishing maximum PCR product levels, volatiles concentrations, or sensor responses is somewhat difficult to envision.

In spite of what is said above, the sensor technology is developing rapidly, as are the statistical tools for data evaluation. Through clever combinations of knowledge of biology, technology (IT, sensor, hardware), and mathematical tools the near future will see the development of user-friendly, fast, and economic instruments for determination of fungal contamination, to the benefits of farmers, the grain industry, and the society at large.

References

Åkerstrand, K. (1995). Mould and yeast. Determination in foods. UDC 582.23:633.1. Nordic Committee on Food Analysis.

Albano, C., Blomqvist, G., Coomans, D., Dunn III, W. J., Edlund, U., Eliasson, B., Hellberg, S., Johansson, E., Nordén, B., Sjöström, M., Söderström, B., Wold, H. & Wold, S. (1981). Pattern recognition by means of disjoint principal components models (SIMCA). Philosophy and methods. In *Proceedings Symposium i Anvent Statistik*, pp. 183-218. Edited by A. E. A. Höskuldsson. Copenhagen: NEUCC, RECAU and RECKU.

Arnold, B. L., Itakura, K. & Rossi, B. T. (1992). PCR-based quantitation of low levels of HIV-1 DNA by using an external standard. *Genet. Anal. Tech. Appl.* 9, 113-116.

Bachinger, T., Martensson, P. & Mandenius, C. F. (1998). Estimation of biomass and specific growth rate in a recombinant *Escherichia coli* batch cultivation process using a chemical multisensor array. *J. Biotech.* 60, 55-66.

Bachinger, T., Riese, U., Eriksson, R. & Mandenius, C. F. (2000). Monitoring cellular state transitions in a production-scale CHO-cell process using an electronic nose. *J. Biotech.* 76, 61-71.

Bartnicki-Garcia, S. & Lippman, E. (1982). Fungal cell wall composition. In *CRS Handbook of Microbiology*, pp. 229-252. Edited by A. I. Laskin & H. A. Lechevalier. Vleveland, Ohio: Boca Raton

Bennett, J. W. (1995). From molecular genetics and secondary metabolism to molecular metabolites and secondary genetics. *Can. J. Bot.* 73, S917-S924.

Boysen, M. E., Eriksson, A. R. B. & Schnürer, J. (2000). Detection of food-borne toxigenic molds using molecular probes. in press.

Breitholtz, A., Olsen, M., Dahlbäck, Å. & Hult, K. (1991). Plasma ochratoxin A levels in three Swedish populations surveyed using an ion-pair HPLC technique. *Food Addit. Contam.* 8, 183-192.

Brezmes, J., Ferreras, B., Llobet, E., Vilanova, X. & Correig, X. (1997). Neural network based electronic nose for the classification of aromatic species. *Anal. Chim. Acta* 348, 503-509.

Börjesson, T. (1993). *Volatile fungal metabolites as indicators of mould growth in stored cereals,* Dept. of Microbiology 55, Swedish University of Agricultural Sciences, Uppsala.

Börjesson, T., Eklöv, T., Jonsson, A., Sundgren, H. & Schnürer, J. (1996). Electronic nose for odor classification of grains. *Cereal Chem.* 73, 457-461.

Cabib, E., Roberts, R. & Bowere, B. (1982). Synthesis of the yeast cell wall and its regulation. *Ann. Rev. Microbiol.* 51, 763-793.

Campbell, I. M. (1984). Secondary metabolism and microbial physiology. In *Advances in Microbial Physiology*, pp. 1-60. Edited by A. H. Rose & D. W. Tempest. London: Academic Press.

Chelkowski, J. (1991). Cereal grain - Mycotoxins, fungi and quality in drying and storage. In *Developments in Food Sciences*, pp. xiii-xx. Edited by Chelkowski, J. Amsterdam: Elsevier Science.

Ciegler, A. (1979). Mycotoxins-their biosynthesis in fungal: biosynthesis of the trichothecenes. *J. Food Protect.* 42, 825-828.

Coomans, D., Bossuyt, A., Broeckaert, I., Ingels, M., Jonckheer, M., Massart, D., Musch, W. & Wold, S. (1983). SIMCA; Pattern recognition: A new approach to automated medical diagnosis. C Proc. Fourth World Conf. Med. Informat. In *MEDINFO-83*, IFIP world Conf. Ser. Med. Informat. vol 4. pp. 545-547. Edited by J. H. van Bemmel, Ball & O. Wigertz: North-Holland, Amsterdam.

Cross, N. C. P. (1995). Quantitative PCR techniques and applications. *Brit. J. Haemat.* 89, 693-697.

Dowell, F. E., Ram, M. S. & Seitz, L. M. (1999). Predicting scab, vomitoxin, and ergosterol in single wheat kernels using near-infrared spectroscopy. *Cereal Chem.* 76, 573-576.

Eastman, M. P., Hughes, R. C., Yelton, G., Ricco, A. J., Patel, S. V. & Jenkins, M. W. (1999). Application of the solubility parameter concept to the design of chemiresistor arrays. *J. El Chem. Soc.* 146, 3907-3913.

Eklöv, T. (1999). *Methods to improve the selectivity of gas sensor systems,* Dept. of Physics and Measurement Technology 573 Linköping University, Linköping.

Eklöv, T., Johansson, G., Winquist, F. & Lundström, I. (1998). Monitoring sausage fermentation using an electronic nose. *J. Sci. Food Agric.* 76, 525-532.

Eklöv, T., Mårtensson, P. & Lundström, I. (1997). Enhanced selectivity of MOSFET gas sensors by systematical analysis of transient parameters. *Anal. Chim. Acta* 353, 291-300.

Eriksen, G. S. & Alexander, J. (1998). *Fusarium* toxins in cereals - a risk assessment. TemaNord 1998:502 (ISBN 92-893-0149-X). Nordic Council of ministers.

Eriksson, L., Johansson, E., Kettaneh-Wold, N. & Wold, S. (1999). *Introduction to multi- and megavariate data anlysis using projection methods (PCA & PLS).* Umeå: Umetrics AB.

Ferreira, A. V. B. & Glass, N. L. (1996). PCR from fungal spores after microwave treatment. *Fungal Genet. Newsl.* 43, 25-26.

Filtenborg, O., Frisvad, J. C. & Thrane, U. (1996). Moulds in food spoilage. *International J. Food Microbiol.* 33, 85-102.

Fletcher, H. A., Barton, R. C., Verweij, P. E. & Evans, E. G. V. (1998). Detection of *Aspergillus fumigatus* PCR products by a microtitre plate based DNA hybridisation assay. *J. Clin. Pathol.* 51, 617-620.

Frisvad, J. C. (1983). A selective and indicative medium for groups of *Penicillium viridicatum* producing different mycotoxins in cereals. *J. Appl. Microbiol.* 54, 409-416.

Frisvad, J. C. (1994). Mycotoxins and mycotoxinogenic fungi in storage. In *Stored Grain Ecosystems*, pp. 251-288. Edited by D. S. Jayas, N. D. G. White & W. E. Muir. New York: Marcel Dekker.

Frisvad, J. C. & Samson, R. A. (1991). Filamentous fungi in foods and feeds: ecology, spoilage, and mycotoxin production. In *Foods and Feeds*, pp. 31-68. Edited by D. K. Arora, K. G. Mukerji & E. H. Marth. New York: Marcel Dekker.

Gardner, J. W. & Bartlett, P. N. (1994). A brief-history of electronic noses. *Sens. Actuators B* 18, 211-220.

Garraway, M. O. & Evans, R. C. (1984). *Fungal nutrition and physiology*. New York: John Wiley & Sons.

Gibson, T., Prosser, O., Hulberg, R., Corcoran, P., Lowery, P. & Ruck-Kenne, E. (1996). Detection and simultaneous identification of microorganisms from headspace samples using an electronic nose. In *Eurosensors X*, pp. 1341-1344. Leuven, Belgien

Gooday, G. W. (1990). The ecology of chitin degradation. In *Advances in Microbial Ecology*, vol 11. pp. 387-430. Edited by K. C. Marshall. New York: Plenum Press.

Gooday, G. W. (1995). Cell walls. In *The Growing Fungus*, pp. 43-62. Edited by N. A. R. Gow & G. M. Gadd. London: Chapman and Hall.

Gordon, S. H., Wheller, B. C., Schudy, R. B., Wicklow, D. T. & Greene, R. V. (1998). Neural network pattern recognition of photoacustic FTIR spectra and knowledge-based techniques for detection of mycotoxigenic fungi in food grains. *J. Food Protect.* 61, 221-230.

Gottschalk, G. (1986). *Bacterial metabolism*, 2nd ed. New York: Springer Verlag.

Göpel, W. (1996). Ultimate limits in the miniaturization of chemical sensors. *Sens. Actuators B* 56, 82-103.

Göpel, W. & Schierbaum, K. D. (1995). SnO_2 sensors: current status and future prospects. *Sens. Actuators B* 26-27, 1-12.

Göpel, W. & Schierbaum, K. D. (1997). Molecular recognition for chemical sensing: general survey and the specific role of π-conjugated systems. In *Handbook of Organic Conductive Molecules and Polymers*, pp. 621-659. Edited by H. S. Nalwa. Chichester, England: John Wiley & Sons.

Heath, I. B. (1995). Integration and regulation of hyphal tip growth. *Can. J. Bot.* 73, S131-S139.

Hocking, A. D. & Pitt, J. I. (1980). Dichloran-glycerol medium for enumeration of xerophilic fungi from low moisture foods. *Appl. Environ. Microbiol.* 39, 488-492.

Holmberg, T., Breitholtz-Emanuelsson, A., Häggblom, P., Schwan, O. & Hult, K. (1991). *Penicillium verrucosum* in feed of ochratoxin A positive swine herds. *Mycopathologia* 116, 169-176.

Holmberg, T., Hagelberg, S., Lundheim, N., Thafvelin, B. & Hult, K. (1990). Ochratoxin A in swine blood used for evaluation of cereal handling procedures. *J. Vet. Med. Ser. B* 37, 97-105.

Höskuldsson, A. (1988). PLS regression methods. *J. Chemometrics* 2, 211-228.

Janssens, L., Depooter, H. L., Schamp, N. M. & Vandamme, E. J. (1992). Production of flavors by microorganisms. *Proc. Biochem.* 27, 195-215.

Jarvis, B., Seiler, D. A., Ould, A. J. & Williams, A. P. (1985). Observations on the enumeration of moulds in food and feedingstuffs. *J. Appl. Bacteriol.* 55, 325-336.

Jelen, H., Kaminski, E., Wiewiorowska, M., Kuta, A. & Rudzinska, M. (1997). Assessing the toxigenicity of Fusaria contaminating grain spikes on the basis of headspace analysis of trichodiene. *Cereal Res. Commun.* 25, 331-335.

Jelen, H., Latus-Zietkiewicz, D., Wasowicz, E. & Kaminski, E. (1997). Trichodiene as a volatile marker for trichothecenes biosynthesis. *J. Microbiol. Methods* 31, 45-49.

Jelen, H., Mirocha, C., Wasowicz, E. & Kaminski, E. (1995). Production of volatile sesquiterpenes by *Fusarium sambucinum* strains with different abilities to synthesize trichothecenes. *Appl. Environ. Microbiol.* 61, 3815-3820.

Jelen, H. & Wasowicz, E. (1998). Volatile fungal metabolites and their relation to the spoilage of agricultural commodities. *Food Rev. Internat.* 14, 391-426.

Jonsson, A., Winquist, F., Schnürer, J., Sundgren, H. & Lundström, I. (1997). Electronic nose for microbial quality classification of grains. *Internat. J. Food Microbiol.* 35, 187-193.

Kappe, R., Okeke, C. N., Fauser, C., Maiwald, M. & Sonntag, H. G. (1998). Molecular probes for the detection of pathogenic fungi in the presence of human tissue. *J. Med. Microbiol.* 47, 811-820.

Kinderlerer, J. L. (1989). Volatile metabolites of filamentous fungi and their role in food flavour. *J. Appl. Bacteriol.* 133S-144S.

King, A. D., Hocking, A. D. & Pitt, J. I. (1979). Dichloran-rose bengal medium for enumeration and isolation of molds from foods. *Appl. Environ. Microbiol.* 37, 959-964.

Krogh, P. (1987). Ochratoxins in food. In *Mycotoxins in Food*, pp. 97-121. Edited by P. Krogh. London: Academic Press.

Kupier-Goodman, T. & Scott, P. M. (1989). Risk assessment of the mycotoxin ochratoxin A. *Biomed. Environ. Sci.* 2, 179-248.

Lacey, J. & Magan, N. (1991). Fungi in cereal grains: their occurrence and water and temperature relationships. In *Cereal Grain Mycotoxins, Fungi and Quality in Drying and Storage*, pp. 77-118. Edited by J. Chelkowski. Amsterdam: Elsevier Science.

Lantz, P.-G., Tjerneld, F., Borch, E., Hahn-Hägerdal, B. & Rådström, P. (1994). Enhanced sensitivity in PCR detection of *Listeria monocytogenes* in soft cheese trough use of an aqueous two-phase system as a sample preparation method. *Appl. environ. microbiol.* 60, 3416-3418.

Larsen, F. O., Clementsen, P., Hansen, M., Maltbaek, N., Ostenfeldt-Larsen, T., Nielsen, K. F., Gravesen, S., Skov, P. S. & Norn, S. (1998). Volatile organic compounds from the indoor mould *Trichoderma viride* cause histamine release from human bronchoalveolar cells. *Inflammation Res.* 47, S5-S6.

Larsen, T. O. (1994). *Chemosystematics of species in genus* Penicillium *based on profiles of volatile metabolites.*, Dep. of Biotechnology, Technical University of Denmark.

Larsen, T. O. (1997). Identification of cheese-associated fungi using selected ion monitoring of volatile terpenes. *Lett. Appl.. Microbiol.* 24, 463-466.

Larsen, T. O. & Frisvad, J. C. (1995). Chemosystematics of *Penicillium* based on profiles of volatile metabolites. *Mycol. Res.* 99, 1167-1174.

Lundstedt, T., Seifert, E., Abramo, L., Thelin, B., Nyström, Å., Pettersen, J. & Bergman, R. (1998). Experimental design and optimization. *Chemom. Intell. Lab. Syst.* 42, 3-40.

Lundström, I., Armgarth, M. & Petersson, L.-G. (1989). Physics with catalytic metal gate chemical sensors. *CRC Crit. Rev. Solid State Mater Sci.* 15, 201-278.

Lundström, I., Shivaraman, M. S., Svensson, C. M. & Lundkvist, I. (1975). A hydrogen-sensitive MOS field-effect transistor. *Appl. Phys. Lett.* 26, 55-57.

Lundström, I., Spetz, A., Winquist, F., Ackelid, U. & Sundgren, H. (1990). Catalytic metals and field-effect devices - a useful combination. *Sens. Actuators B* 1, 15-20.

Magan, N. (1993). Early detection of fungi in stored grain. *Internat. Biodeterior. Biodegrad.* 32, 145-160.

Morris, C. F. & Rose, S. P. (1996). Wheat. In *Cereal Grain Quality*, pp. 3-54. Edited by R. J. Henry & P. S. Kettlewell. London: Chapman & Hall.

Niessen, M. L. & Vogel, R. F. (1997). Specific identification of *Fusarium graminearum* by PCR with *gaoA* targeted primers. *Syst. Appl. Microbiol.* 20, 111-113.

Olsen, M., Möller, T. & Åkerstrand, K. (1993). Ochratoxin A: occurrence and intake by Swedish population. In *United Kingdom Workshop on Occurrence and Significance of Mycotoxins*, pp. 96-100. Edited by K. A. Scudamore. Brunel, the university of west London: Central science laboratory, MAFF.

Pasanen, A. L., Lappalainen, S. & Pasanen, P. (1996). Volatile organic metabolites associated with some toxic fungi and their mycotoxins. *Analyst* 121, 1949-1953.

Pearce, T. C. (1997). Computational parallels between the biological olfactory pathway and its analogue 'The Electronic Nose': Part I. Biological olfaction. *Biosystems* 41, 43-67.

Pearce, T. C. (1997). Computational parallels between the biological olfactory pathway and its analogue 'The Electronic Nose': Part II. Sensor-based machine olfaction. *Biosystems* 41, 69-90.

Pedersen, L. H., Skouboe, P., Boysen, M., Soule, J. & Rossen, L. (1997). Detection of *Penicillium* species in complex food samples using the polymerase chain reaction. *Internat. J. Food Microbiol.* 35, 169-177.

Pedersen, L. H., Nørskov, P. & Rossen, L. (2000). Determination of the rDNA gene copy number of Penicillium roqueforti by dot and southern blot hybridization and by quantitative competitive PCR. manuscript

Persaud, K. C. & Dodd, G. H. (1982). Analysis of discrimination mechanism in the mammalian olfactory system using a model nose. *Nature* 299, 352-355.

Persaud, K. C. & Travers, P. J. (1997). Arrays of broad specificity films for sensing volatile chemicals. In *Handbook of Biosensors and Electronic Noses: Medicine, Food, and the Environment*, pp. 563-592. Edited by E. Kress-Rogers. CRC Press Inc.

Phatak, A. & DeJong, S. (1997). The geometry of partial least squares. *J. .Chemometrics* 11, 311-338.

Pitt, J., Hocking, A. & Glenn, D. (1983). An improved medium for the detection of *Aspergillus flavus* and *A. parasiticus*. *J. Appl. Microbiol.* 54, 109-114.

Pitt, J. I. (1984). The significance of potentially toxigenic fungi in foods. *Food Technol. Aust.* 36, 218-219.

Pitt, J. I. & Hocking, A. D. (1997). *Fungi and Food Spoilage*, 2nd ed. London: Chapman & Hall.

Pottel, H. (1996). Quantitative models for prediction of toxic component concntrations in smoke gases from FTIR spectra. *Fire Mater.* 20, 273-291.

Przybylaki, R. & Eskin, N. A. M. (1995). Methods for measure volatile compounds and the flavor significance of volatile compounds. In *Methods to Assess Quality and Stability of Oils and Fat-Containing Foods*, pp. 107-133. Edited by K. Warner & N. A. M. Eskin. Champaign: AOCS Press.

Reed, C. (1992). Development of storage techniques - A historical perspective. In *Storage of Cereal Grains and their Products*, pp. 143-156. Edited by D. B. Sauer. St Paul: American association of cereal chemists Inc.

Rossen, L., Nørskov, P., Holmstrøm, K. & Rasmussen, O. F. (1992). Inhibition of PCR by components of food samples microbial diagnostic assays and DNA-extraction solutions. *Internat. J. Food Microbiol.* 17, 37-45.

Rylander, R. (1986). Lung diseases caused by organic dusts in the farm environment. *Am. J. Ind. Med.* 10, 221-227.

Samson, R., Hoekstra, E., Frisvad, J. & Filtenborg, O. (1996). *Introduction to food-born fungi*, 5th ed. Baarn, Netherlands: Centraalbureau voor Schimmelcultures.

Schaller, E., Bosset, J. O. & Escher, F. (1998). 'Electronic noses' and their application to food. *Food Sc. Technol.* 31, 305-316.

Schnürer, J. (1993). Comparison of methods for estimating the biomass of three food-borne fungi with different growth patterns. *Appl. Environ. Microbiol.* 59, 552-555.

Schnürer, J. & Jonsson, A. (1992). Ergosterol levels and mould colony forming units in Swedish grains of food and feed grade. *Acta Agric. Scand. Sec. B Soil Plant Sci.* 42, 240-245.

Schnürer, J., Olsson, J. & Börjesson, T. (1999). Fungal volatiles as indicators of food and feeds spoilage. *Fungal Genet. Biol.* 27, 209-217.

Schnürer, J. & Paustian, K. (1986). Modelling fungal gromwth in relation to nutrient limitations in soil. In *Fourth International Symposium on Microbial Ecology*, pp. 123-130. Edited by F. Megusar & M. Gantar. Ljubljana: Slovene Society for Microbiology.

Seitz, L. (1995). Volatile compounds in wheat cultivars from several locations in Kansas. In *Food Flavors: Generation, Analysis and Process Influence*, pp. 2183-2203. Edited by G. Charalambous. London: Elsevier.

Seitz, L. M., Mohr, H. E., Burroughs, R. & Sauer, D. B. (1977). Ergosterol as an indicator of fungal invasion in grains. *Cereal Chem.* 56, 1207-1217.

Seitz, L. M. & Sauer, D. B. (1991). Off-odors in grains. *Cereal Foods World* 36, 707

Sietsma, J. H., Wösten, H. A. B. & Wessels, J. G. H. (1995). Cell wall growth and protein secretion in fungi. *Can. J. Bot.* 73, S388-S395.

Sjöström, M. & Wold, S. (1980). SIMCA: A pattern recognition method based on principal component models. In *Pattern Recognition in Practice*, pp. 351-359. Edited by E. S. Gelsema & L. N. Kanal. Amsterdam: North-Holland.

Sjöström, M., Wold, S., Lindberg, W., Persson, J.-Å. & Martens, H. (1983). A multivariate calibration problem in analytical chemistry solved by partial least-squares models in latent variables. *Anal. Chim. Acta* 150, 61-70.

Sjöström, M., Wold, S. & Söderström, B. (1986). PLS discriminant plots. In *Pattern Recognition in Pratice II*, pp. 461-470. Edited by E. S. Gelsema. & L. N. Kanal. Amsterdam: Elsevier.

Smith, E. A., Chambers, E. & Colley, S. (1994). Development of vocabulary and references for describing off-odors in raw grains. *Cereal Foods World* 39, 495-499.

Smith, J. E., Lewis, C. W., Anderson, J. G. & Solomons, G. L. (1984). Mycotoxins in human nutrition and health. EUR 16048 EN. European Commission. Agro-Industrial Research Division.

Smith, O. P., Peterson, G. L., Beck, R. J., Schaad, N. W. & Bonde, M. R. (1996). Development of a PCR-based method for identification of *Tilletia indica*, causal agent of Karnal bunt of wheat. *Phytopathology* 86, 115-122.

Snyder, J. M. (1995). Historical and future development of volatile compound analyses. In *Methods to Assess Quality and Stability of Oils and Fat-Containing Foods*, pp. 134-145. Edited by K. Warner & N. A. M. Eskin. Champaign: AOCS Press.

Stetter, J. R., Findlay, M. W., Schroeder, K. M., Yue, C. & Penrose, W. R. (1993). Quality classification of grain using a sensor array and pattern recognition. *Anal. Chim. Acta* 284, 1-11.

Sunesson, A.-L., Nilsson, C.-A. & Andersson, B. (1995). Evaluation of adsorbents for sampling and quantitative analysis of microbial volatiles using thermal desorption-gas chromatography. *J. Chromatogr. A.* 699, 203-214.

Thelin, B., Lundstedt, T., Lundgren, R., Olsson, A. & Batra, S. (1995). Classification of Estradurin$^{(R)}$ batches - correlation between P-31 NMR and a biological duration test for batch approval. *Chemomet. Intell. Lab. Syst.* 27, 135-145.

Thrane, U. (1996). *Fusarium*. Determination in foods and feedstuffs. UDC 582.28:579.093. Nordic Committee on Food Analysis.

Thrane, U. (1996). Mould and yeast determination in foods. 98, 3rd edition. Nordic Committee on Food Analysis.

Walinder, R., Norback, D. & Johanson, G. (1998). Pulmonary reactions after exposure to 3-methylfuran vapour, a fungal metabolite. *Internat. J. Tuberc. Lung Dis.* 2, 1037-1039.

Wang, A. M., Doyle, M. V. & Mark, D. F. (1989). Quantitation of mRNA by the polymerase chain reaction. *Proc. Natl. Acad. Sci. USA* 86, 9717-9721.

Weete, J. D. (1980). *Lipid biochemistry of fungi and other organisms*. New York: Planum Press.

Wessels, J. G. H. (1986). Cell wall synthesis in apical hyphal growth. *Internat. Rev. Cytol.* 104, 37-79.

Wessels, J. G. H. (1993). Wall growth, protein excretion and morphogenesis in fungi. *New Phytol.* 123, 397-413.

Wessels, J. G. H. (1994). Developmental regulation of fungal cell wall formation. *Annual Rev. Phytopathol.* 32, 413-437.

Wilson, I. G. (1997). Inhibition and facilitation of nucleic acid amplification. *Appl. Environ. Microbiol.* 63, 3741-3751.

Wold, S., Albano, C., Dunn III, W. J., Edlund, U., Esbensen, K., Geladi, P., Hellberg, S., Johansson, E., Lindberg, W. & Sjöström, M. (1984). Multivariate data analysis in chemistry. (Proc. NATO ASI Chemometr. Math. Stat. Chem.). In *Chemometrics: Mathematics and Statistics in Chemistry*, NATO ASI series. Ser. C Math. Physo Sci. vol 138 pp. 1-79. Edited by B. R. Kowalski. Holland: D. Reidel Publishing Company.

Wold, S., Esbensen, K. & Geladi, P. (1987). Principal component analysis. *Chemomet. Intell. lab. syst.* 2, 37-52.

Wolfbeis, O., Boisde, G. & Gauhlitz, G. (1991). Optochemical sensors. In *Chemical and Biochemical Sensors, Part 1*, pp. 571. Edited by W. Göpel, A. Jones, M. Kleitz & I. Lundström. Weinheim, Germany: VCH.

Xu, J.-R. & Hamer, J. E. (1995). Assessment of *Magnaporthe grisea* mating type by spore PCR. *Fungal Genet. Newsl.* 42, 80.

Zeringue, H. J., Bhatnagar, D. & Cleveland, T. E. (1993). C_{15} H_{24} volatile compounds unique to aflatoxigenic strains of *Aspergillus flavus*. *Appl. Environ. microbiol.* 59, 2264-2270.

Zimmermann, K. & Mannhalter, J. W. (1996). Technical aspects of quantitative competitive PCR. *Biotechniques* 21, 268.

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