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Early Detection of Fungal Growth in Bakery Products by Use of an Electronic Nose Based on Mass Spectrometry

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This paper presents the design, optimization, and evaluation of a mass spectrometry-based electronic nose (MS e-nose) for early detection of unwanted fungal growth in bakery products. Seven fungal species (*Aspergillus flavus*, *Aspergillus niger*, *Eurotium amstelodami*, *Eurotium herbariorum*, *Eurotium rubrum*, *Eurotium repens*, and *Penicillium corylophillum*) were isolated from bakery products and used for the study. Two sampling headspace techniques were tested: static headspace (SH) and solid-phase microextraction (SPME). Cross-validated models based on principal component analysis (PCA), coupled to discriminant function analysis (DFA) and fuzzy ARTMAP, were used as data treatment. When attempting to discriminate between inoculated and blank control vials or between genera or species of in vitro growing cultures, sampling based on SPME showed better results than those based on static headspace. The SPME−MS-based e-nose was able to predict fungal growth with 88% success after 24 h of inoculation and 98% success after 48 h when changes were monitored in the headspace of fungal cultures growing on bakery product analogues. Prediction of the right fungal genus reached 78% and 88% after 24 and 96 h, respectively.

KEYWORDS: Electronic nose; mass spectrometry; fungal growth; bakery products; fuzzy ARTMAP; ANN; LDA; PCA

INTRODUCTION

Microbial spoilage is a major problem in bakery products since it can induce nutritional losses, off-flavors, and formation of mycotoxins or potentially allergenic spores. This situation can lead to an organoleptic deterioration of already marketed bakery products, which indeed threatens consumers' confidence and, therefore, results in important economical losses. This is the reason for a growing need to find a method to conveniently assess the degree of fungal growth in bakery products at a very early stage and before it becomes visible (*1*).

Classical techniques based on microbiological methods such as CFU (colony-forming units) determination are time-consuming and they cannot give on-line responses. Specific chemical markers such as ergosterol have now become commonly used as a method for the quantification of fungal biomass in food. However, they are nonspecific, they do not provide any information on the species present, and they require a laborious sample preparation (*2*).

On the other hand, it is known that fungi produce volatile compounds during both primary and secondary metabolism that can be used as markers to detect food spoilage, unwanted fungal growth, or even as taxonomic identifiers that can determine the presence of a given species. This idea was initially exploited in the field of cereals (*3*, *4*). Research studies correlated fungal activity with the production of volatile metabolites, CO_2, and CFU. Schnürer et al. (*2*) and Magan and Evans (*5*) reviewed some studies where GC−MS had been used to characterize and analyze volatile profiles of fungal cultures, listing volatiles identified in different growing substrates. More recently, GC−MS intensity peaks from key volatiles have been used by Olsson et al. (*6*, *7*) to evaluate the mycological quality of barley grains and to predict levels of ochratoxin A and deoxynivalenol.

Since the volatile headspace is complex and should be evaluated as a whole, techniques that mimic the human olfactory system (the so-called electronic noses) have already been proposed. Electronic noses based on different types of nonspecific sensors (i.e., metal oxide, conducting polymer, or quartz microbalance sensors) have been evaluated in fungal, bacterial, and yeast monitoring in food such as bakery products (*1*), cereal grains (*5*−*8*), cheese (*9*), water (*10*), bread (*11*), meat (*12*), and milk (*13*). Despite the efforts, e-noses based on nonspecific semiconductor sensors still suffer from serious drawbacks such as poor sensitivity, poor selectivity, and long-term drift. Novel electronic olfactory systems based on mass spectrometry seem to improve drift problems with respect to other classical e-nose technologies. Even so, MS e-nose suffers from low temporal

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drift. Nevertheless, this paper does not consider drift as an issue since measurements were done in a short period of time. Preconcentration and extraction techniques such as solid-phase microextraction (SPME) can increase the sensitivity and reproducibility of MS e-noses (*14*, *15*). Since Nilsson et al. (*16*) used SPME as a solvent-free extraction method for analysis of volatile metabolites emitted by *Penicillium* species, many researchers have used SPME as a tool for sampling volatile fungal metabolites (*17*, *18*).

This paper presents a study on the design, optimization, and evaluation of a MS-based electronic nose for early fungal growth detection in bakery products. Two different experiments were carried out. The first one was designed to choose the best sampling technique (SH or SPME) to be coupled to a MS-based e-nose in order to monitor fungal growth. The second study was designed to evaluate the performance of the MS e-nose optimal configuration in early detection of in vitro and in situ growing fungal cultures.

MATERIALS AND METHODS

Samples. Seven fungal species (*Aspergillus flavus*, AF; *Aspergillus niger*, AN; *Eurotium amstelodami*, EA; *Eurotium herbariorum*, EH; *Eurotium rubrum*, EU; *Eurotium repens*, ER; and *Penicillium corylophillum*, PE) were isolated from bakery products. One isolate from each species was used for the present study.

For in vitro studies, 0.90 aw slants of 2% wheat flour agar medium were prepared in 20-mL headspace vials and needle-inoculated with 10^{-6} spores mL^{-1} suspensions (adjusted by use of a Thoma chamber) of the fungal cultures mentioned above. Uninoculated vials with agar medium were used as control blanks (BL).

In situ studies were performed on bakery product analogues prepared as described by Abellana et al. (*19*), adjusted to a water activity of 0.95. Pieces measuring 8 × 8 × 20 mm were introduced in 20-mL headspace vials. Analogues were needle-inoculated at random with the seven cultures mentioned above and, again, uninoculated analogues were used as control blanks. Once sealed, all vials were incubated at 25 °C until their measurement. Incubation periods ranged from 24 h to 7 days depending on the experiments carried out.

MS E-nose Configuration. A Shimadzu QP 5000 GC/MS (Shimadzu Corp., Tokyo, Japan) was used to implement a MS-based e-nose. The instrument was equipped with a deactivated PR-100052 5 m × 0.25 mm ID precolumn (Teknokroma, St Cugat Del Vallès, Barcelona, Spain) that only acted as a transfer line from the injector port to the mass detector. The column was kept isothermal at 250 °C to coelute all volatile components in one single peak. This implies that the components in the headspace of the vials passed directly to the mass detector without any chromatographic separation. In this manner, for any given measurement, the resulting mass spectrum gives a fingerprint that is characteristic of the volatiles present in the headspace of samples. Helium flow was set to 1.4 mL/min. The mass spectrometer operated in the electron impact ionization mode (70 eV) and acquired in a scan range from *m*/*z* 35 to 120 at 0.5 scan/s. Ion source temperature was set at 250 °C.

Two different sampling techniques (SH and SPME) were evaluated as the best candidates to be coupled to the MS-e-nose.

Choosing the Best Sampling Technique. (A) Static Headspace Optimization. Sampling based on a static headspace autosampler was done by coupling an HP-7694 (Agilent Technologies) to the MS-based e-nose. All experiments dealing with MS e-nose optimization were carried out on in vitro growing cultures for 10 days. The main sampling parameters that influence sensitivity when working with the SH technique, namely, temperature of equilibration and vial equilibration time, were modified to improve fungal culture classification and fungal growth detection.

To select the optimal temperature, three oven temperatures, 50, 80, and 100 °C, were tested. For each temperature, a batch of 16 measurements corresponding to two replicates for each of the seven species plus two additional control blanks incubated for 10 days was performed. Equilibration time was fixed at 5 min in the three batches of measurements. The temperature of the loop and the transfer line were always kept at 5 °C above oven temperature to avoid condensation. Each vial was pressurized with helium (i.e., the carrier gas) for 12 s. Then, the 3-mL internal loop of the headspace autosampler was filled with volatiles coming from headspace of the fungal cultures, and finally volatiles were injected into the gas chromatograph. The goal was to determine whether the instrument could distinguish between inoculated and blank vials. A secondary goal was to observe whether the system was able to classify samples according to fungal genera.

In a second experiment, equilibration temperature was kept constant at 50 °C and equilibration time was increased to 50 min. The remaining parameters were kept as described above. A total of 32 samples (four replicates of eight different types of vials) were measured.

(B) SPME−MS E-nose Measurements. Sampling based on SPME was performed with a 75-μm Carboxen/PDMS fiber purchased from Supelco (Supelco Park, Bellefonte, PA). Jelen (*18*) compared four SPME fibers used to perform an extraction of volatile metabolites from fungal cultures. This study showed that the highest amount of isolated volatiles expressed as total peak area was observed for fibers based on Carboxen (CAR/PDMS and CAR/DVB/PDMS). Fibers based on Carboxen are the best choice in terms of sensitivity to extract low molecular weight analytes such as low-chain alcohols, ketones, and aldehydes. The main volatile metabolites involved in early stages of fungal growth that have been cited in the scientific literature belong to this type of molecules. As the goal was to detect the production of these volatiles as early as possible, priority was given to sensitivity and that is why a CAR/PDMS-based fiber was chosen. Prior to any extraction, the fiber was conditioned following the manufacturer's recommendations. In each measurement, the fiber was introduced into the vial and exposed to the headspace of fungal cultures for 20 min at room temperature. Thermal desorption of volatiles trapped on the fiber was conducted for 3 min in the chromatograph injection port at 300 °C. The split valve was closed during desorption. The fiber was always left five additional minutes to ensure its complete cleaning.

Three in vitro replicate vials of each fungal species plus control blanks were prepared and incubated for 10 days. Each replicate was measured three times by the SPME−MS e-nose. Therefore, a total of 72 measurements were performed. The aim was to discriminate inoculated samples from blank vials and to evaluate whether the instrument could classify samples according to their genera and species.

In Vitro Fungal Growth Monitoring. The next goal was to evaluate the performance of the MS-based e-nose to monitor early stages of fungal growth. SPME was used since it was determined that it was the best sampling method. Two replicates of each of the seven fungi plus two control blanks were grown on 2% wheat flour agar. Samples were kept under incubation and extractions were made once a day, obtaining a total of 16 experimental points at 48, 72, 96, and 168 h after inoculation.

In Situ Fungal Growth Monitoring. A final experiment designed to simulate a real application was performed. The aim was to use the final prototype in order to discriminate between spoiled and safe bakery products. Eight blank control vials containing cake analogues, eight replicate vials containing cake analogues inoculated with EA, and four replicates inoculated with ER, EU, EH, AN, AF, and PE were measured. SPME extractions and MS e-nose measurements were performed in every vial 24, 48, 72, 96, and 168 h after inoculation. Overall, 40 experimental points were obtained for each sampling time (1, 2, 3, 4, and 7 days after inoculation). The system was also evaluated as a tool to discriminate among fungal species.

Multivariate and Pattern Recognition Analysis. Data generated by the e-nose device (in any of its different configurations) were collected and processed by use of written-in-house software based on MATLAB 6.5 (The Mathworks, Natick, MA). An unresolved single peak was obtained for each measurement. Averaging mass spectra along the detected peak generated a response spectrum. Since measurements were performed in scan mode from *m*/*z* 35 to 120, the average intensity of each mass could be used as a variable (or sensor). In this manner, an experimental data matrix was built. The number of rows was the number of samples measured in each experimental batch, while the number of columns was 86, corresponding to each *m*/*z* scanned. A

principal component analysis (PCA) was applied to each response matrix, achieving a reduction in dimensionality. By use of the first 10 principal components, 100% of the total data variance was gathered. A reduced response matrix having 10 columns corresponding to scores of the first 10 principal components was obtained. Then, a discriminant function analysis (DFA) was performed on the reduced matrix. Eigenvalues obtained from the DFA were used as input variables to a fuzzy ARTMAP neural network that gave a categorization of fungal cultures according to genera or species depending on the application.

The performance of the model was evaluated by the leave-one-out cross-validation approach. In this method, a different row (measurement) from the original matrix is left out at each iteration. The remaining rows conform the training matrix, which is reduced by a PCA projection, processed by a DFA, and fed to the fuzzy ARTMAP training algorithm after scaling coordinates between 0 and 1. The procedure is then validated with the vector that had been left out. The validation vector (not used for training) is then projected against the PCA model. Then, the PCA scores are projected onto space of the canonical variables of the trained DFA. Finally, the DFA projection coordinates of the validation vector are fed to the neural network model, which produces a classification result. The whole process is repeated N times, N being the number of measurements included in the data matrix, so that each measurement is used in one iteration for evaluation purposes and in $N - 1$ iterations for training. The fact that for each iteration the validation vector is not used in the training process ensures that the vector is completely new to the processing system.

RESULTS AND DISCUSSION

Reduction of *m/z* Variable Dimensionality by Use of PCA. All the results cited bellow were obtained by applcation of multivariate analysis to the response matrix. This matrix was formed by as many rows as experimental measurements made in each study and as many columns as *m/z* variables scanned. According to Dittmann and Nitz (*20*), these *m/z* variables can be used as an array of sensors to emulate a classical electronic nose. In their paper, they claim that, in most cases, it is not useful to work with such a great number of sensors and only a very small number of ion fragments are suitable for setting up a sensor array since meaningless fragments introduce noise into the system. They also consider that there is no way to correctly select *m/z* fragments, unless there is a previous full chromatographic run. Finally, they discuss reliable strategies for selecting the optimal array configuration, based on previous knowledge of the analytes that are important for the application. This previous knowledge is normally based on time-resolved analysis to identify (and quantify) the volatiles present in the headspace of the samples to be studied. This leads, unavoidably, to more traditional analytical techniques such as GC−MS.

On the other hand, applying a PCA analysis leads to a linear combination of *m/z* variables that gathers the highest amount of variance and compresses information by eliminating redundancy and collinearity. In this manner, the best combination of *m/z* variables can be chosen without the need to perform a costly and lengthy initial study to determine the most relevant ion fragments to be monitored. This means that previous fully resolved chromatographic runs can be avoided since no previous knowledge from the samples is required.

The reduced response matrix obtained from the PCA analysis is then used as the input matrix to perform a DFA. DFA is a supervised model that finds a function-based projection that minimizes distances between measurements from the same category and maximizes distance between centroids of each category. Finally the two first factors resulting from the DFA were used as input variables to a fuzzy ARTMAP neural network as described before.

Static Headspace Optimization. A preliminary analysis of the results on increasing oven temperature in SH was performed by plotting PCA scores at 50, 80, and 100 °C separately. At 50 and 80 °C those plots did not show any clustering, and samples with fungal contamination and blank vials overlapped. At 100 °C, inoculated samples clustered together, clearly separated from blank vials. Setting a headspace oven temperature of 100 °C permitted the extraction of a larger quantity of volatiles, which enhanced the sensitivity of the system, allowing it to achieve a better discrimination than at 50 or 80 °C. A cross-validated fuzzy ARTMAP classification of the 16 experimental points at 100 °C achieved an 88% success rate when trying to determine whether the vial was inoculated or not. When attempting to determine the fungal genera, the success rate decreased to 56% (**Table 1**). Measuring samples at 100 °C may accelerate oxidation processes modifying the qualitative volatile pattern profiles. This may be very difficult to control and could introduce noise in our mathematical model.

Therefore we designed a second experiment where temperature of equilibration remained constant at 50 °C and equilibration time was increased. The concentration of an analyte in the headspace usually follows a linear dependence with equilibration time until it comes to a point where the concentration becomes stable. At this point volatiles reach equilibrium and achieve their maximum concentration in the headspace, while the composition of volatile patterns remains stable. That should enhance the repeatability on MS e-nose measurements. Sanz et al. (*21*) studied equilibration time in *Arabica* coffee and they concluded that when the equilibration time increased, the quantity of volatile compounds also increased but in an irregular way, depending on the chemical family considered. Similarly, every analyte from each fungal culture has a different equilibration

Table 1. Success Rate Comparison between the Two Different Sampling Techniques Studied[a]

technique	goal discrimination between	total exptl points	failures	success rate (%)
HS–MS, 100 °C	fungal growth	16	2	88
	genera	16	7	56
HS–MS, 50 min	fungal growth	32	1	97
	genera	32	13	59
SPME–MS	fungal growth	72	0	100
	genera	72	0	100
	species	72	6	92

[a] Data processing was performed with PCA–DFA–fuzzy ARTMAP models. The goal was to classify between inoculated and uninoculated vials (fungal growth) and between genera or species. All tests were performed over in vitro growing cultures.

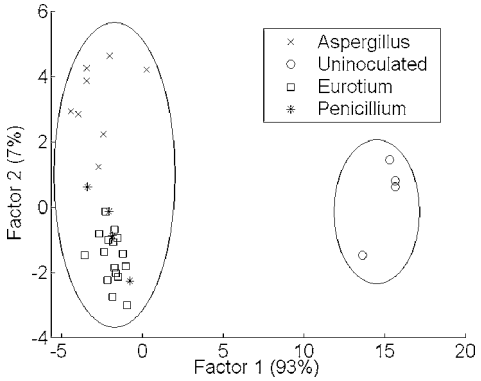

Figure 1. Two-dimensional DFA plot from 10 days in vitro growing cultures measured with the SH–MS e-nose configuration (50 min equilibration time, 50 °C temperature).

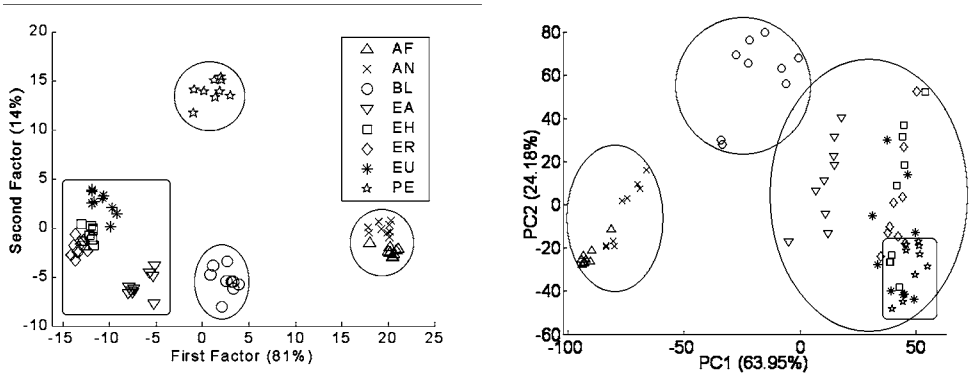

Figure 2. Two-dimensional projections from 10 days in vitro growing cultures measured with the SPME–MS e-nose configuration. (a, left) DFA; (b, right) PCA.

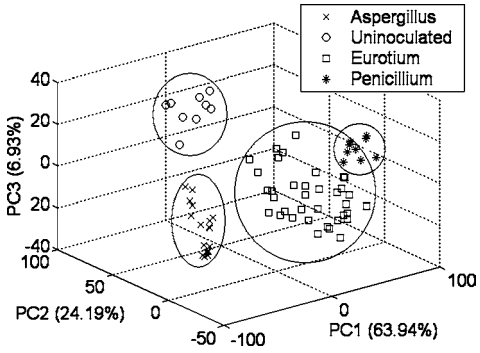

Figure 3. Three-dimensional PCA scores plot from 10 days in vitro growing cultures measured with the SPME–MS e-nose configuration.

time. An equilibration time equal to 50 min was chosen to ensure maximum concentration of volatile compounds on the headspace.

This ensured improved repeatability and sensitivity. Since four in vitro replicates for each type of sample were prepared, 32 measurements were performed. A 2D DFA plot (**Figure 1**) of the reduced response matrix shows two clearly separated clusters corresponding to inoculated and blank vials, respectively. A fuzzy ARTMAP neural network achieved a 97% success rate in the discrimination between inoculated and uninoculated vials. This success rate fell down to 59% when attempting to discriminate among fungal genera (**Table 1**).

Results of SPME Sampling. A total of 72 measurements were performed to test in vitro SPME−MS measurements. Therefore, the resulting data matrix had 72 rows and 86 columns (*m*/*z* ranged from 35 up to 120). **Figure 2**a shows a 2D DFA plot of the restricted response matrix, while **Figure 2b** shows a 2D score plot from the original data matrix. Samples belonging to *Aspergillus*, *Penicillium*, and *Eurotium* clustered together with low dispersion and without overlapping with blank controls, which were clearly separated from the rest. The first two factors from DFA accounted for 95% of the variance in the data. In the case of the PCA, the variance gathered by the two first factors decreased to 88%. In this case, *Penicillium* and *Eurotium* isolates appear to overlap. However, use of the third principal component leads to discrimination between these two genera (**Figure 3**). In both 2D DFA and PCA plots *E. amstelodami* can be distinguished from the other *Eurotium* species. Looking at *Aspergillus* samples, there is a clear separation between species *A. flavus* and *A. niger*.

Applying DFA coupled to a fuzzy ARTMAP neural network model resulted in a 100% success rate when discriminating between fungal growth and blank vials. Also a 100% classification was achieved when classifying fungal genera. The attempt to classify samples according to their fungal species reached a 92% success rate (**Table 2**). The system misclassified seven measures out of 72, confusing two EH, three ER, one AF, and one AN. All failures were mistaken between species from the same genus.

Table 2. Results Achieved by Use of a PCA–DFA–Fuzzy ARTMAP Model for Classifying Blank/Inoculated Vials (Fungal Growth) and Genera[a]

fungal growth (h)	goal differentiation between	total exptl points	failures	success rate (%)
	In Vitro Measurements			
48	fungal growth	16	0	100
	genera	16	0	100
72	fungal growth	16	0	100
	genera	16	0	100
96	fungal growth	16	0	100
	genera	16	0	100
168	fungal growth	16	0	100
	genera	16	0	100
	In Situ Measurements			
24	fungal growth	40	5	88
	genera	40	17	58
48	fungal growth	40	1	98
	genera	40	9	78
72	fungal growth	40	0	100
	genera	40	9	78
96	fungal growth	40	0	100
	genera	40	5	88
168	fungal growth	40	0	100
	genera	40	5	88

[a] Tests were performed for in vitro and in situ growing fungal cultures, and sampling was always performed for SPME.

Both PCA and DFA plots show a clear distinction between *E. amstelodami* and other *Eurotium* species. These results seem to be in good agreement with previous works where Börjesson et al. (*3*, *4*, *22*) described some differences between the volatile pattern profiles from *E. amstelodami* and other fungal species due to its lower percentage of alcohol release (50% for *E. amstelodami* vs 80% in other fungal species studied).

The SPME technique gave better results than the SH technique when applied as the sampling system coupled to our MS-based e-nose. It achieved better repeatability and it was more sensitive due to its ability to concentrate volatile analytes. SPME allows distinguishing between fungal genera or even between several species. On the basis of these results it was decided to continue the studies with the SPME−MS e-nose configuration since the instrument is meant for the fast detection of fungal growth at early stages.

In Vitro Fungal Growth Monitoring. Table 2 shows the success rates in the classification of in vitro samples between

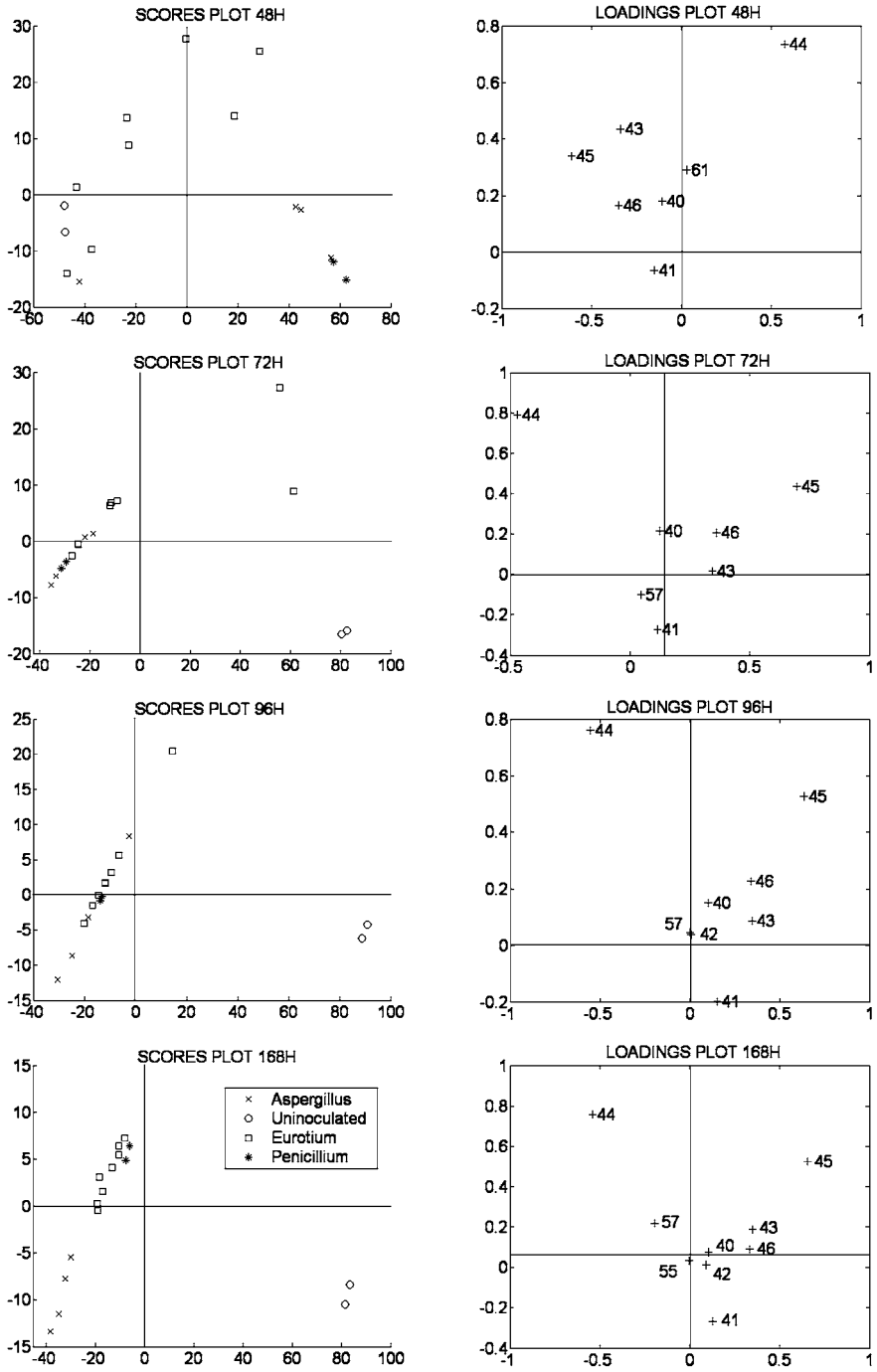

Figure 4. Two-dimensional PCA scores and loadings plots corresponding to 48, 72, 96, and 168 h of headspace monitoring of in vitro growing cultures.

two categories (inoculated and blank vials) and between four categories (corresponding to three fungal genera plus blank vials). These measurements were performed between 48 and 168 h after inoculation. By application of DFA−fuzzy ARTMAP on the reduced response matrix, the system achieved a 100% success rate after 48 h from inoculation when attempting to discriminate both between inoculated and blank samples and among fungal genera. This demonstrates that the SPME−MS-based e-nose is a suitable tool for on-line in vitro monitoring and early detection of unwanted fungal spoilage.

One of the advantages of a MS-based e-nose over e-noses based on gas sensors or other devices is the possibility to obtain structural information from the samples. Intensity on the mass detector is a function of the ion patterns in the fragmentation of each molecule present in the headspace of fungal cultures. Therefore, depending upon the molecules present in the headspace, there will be different mass intensities detected by the instrument.

Making a loadings and scores plot on the response matrix enables us to establish qualitative correlations between samples and variables (*m*/*z* fragments). We constructed PCA models for in vitro measurements at 48, 72, 96, and 168 h. **Figure 4** shows 2D PCA score plots with their corresponding loading plots for each batch performed at the different incubation times. Electron impact ionization mode (EI) causes considerable fragmentation, leading to overlapping fragments and parent ions. Because of the poor selectivity of the *m*/*z* fragments, they cannot be directly correlated with the presence or absence of a volatile. Nevertheless, mapping pattern fragmentation by means of loading plots could give some relevant information. In this case ions corre-

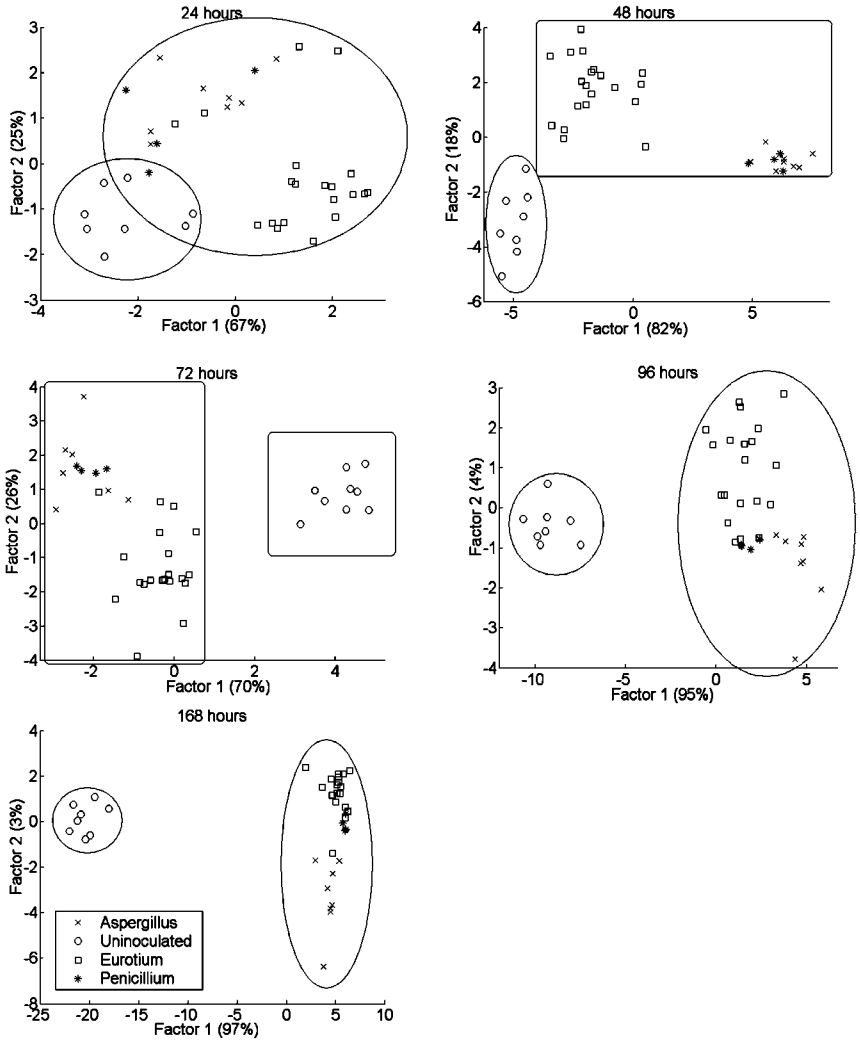

Figure 5. Two-dimensional DFA projections corresponding to 24, 48, 72, 96, and 168 h of headspace monitoring of in situ growing fungal cultures.

sponding to the highest loading values for the first three PC were kept to perform loading plots. **Figure 4** shows an inverse correlation between uninoculated control blanks and *m*/*z* 44. This inverse correlation means that samples with low or no presence of *m*/*z* 44 correspond to blank controls and the rest share a high presence of *m*/*z* 44. Since fragment of *m*/*z* 44 corresponds to the base peak of CO_2, the relationship between the evolution of accumulated CO_2 and fungal growth was found to be significant. The MS-based e-nose differentiates between inoculated and uninoculated samples mainly on the basis of the production of CO_2 by fungi. This is in good agreement with the literature. Börjesson et al. (*3*) measured the concentration of CO_2 produced by *A. flavus*, *A. amstelodami*, *Penicillum cyclopium*, and *Fusarium culmorum* during 14 days of fungal growth and showed a continuous rise in CO_2 concentration. They also studied the volatiles released by six fungal species on grains and found that the relationship between accumulated CO_2 evolution and fungal growth was significant (*22*). The predominating presence of CO_2 in our PCA model is due to the sealing of the vials with silicon septa once they have been inoculated. The presence of other ions such as *m*/*z* 41, 42, 43, 45, 46, 55, and 57 can be considered as second-order and less relevant ions. They can be associated to other related fungal metabolites such as ethanol (*m*/*z* 45 and 46), 3-methyl-1-butanol (*m*/*z* 41, 42, and 55), 2-methyl-1-propanol (*m*/*z* 41−43), and 1-octen-3-ol (*m*/*z* 57). This is in good agreement with the review paper by Magan and Evans (*5*). In their study they reviewed the types of volatiles produced by grain spoilage fungi and listed the most common volatiles found and the fungal species involved. The major volatile compounds were found to be 3-methyl-1-butanol, 2-methyl-1-propanol, 1-octen-3-ol, and other 8-carbon ketones and alcohols.

Anyway, the origin of these fragments cannot be ensured. As **Figure 4** shows, the loading maps remained almost unchanged from 72 to 168 h of incubation. The only remarkable changes in the loading maps appear betweem the plots corresponding to 48 and 72 h of fungal growth. Finally, the presence of ion 55 close to ions 42 and 41 can indicate the raising of 3-methyl-1-butanol after 178 h of fungal growth.

In Situ Fungal Growth Monitoring. Results obtained on the in vitro preliminary experiment encouraged us to study the performance of the electronic nose on in situ growing cultures over bakery product analogues. This is a much more realistic but difficult task since analogues can produce their own volatile pattern profiles; these volatiles produce additional signals in the mass detector that introduce noise into the fungal growth predictive model.

Table 2 summarizes the results obtained when bakery products analogues were measured along the first stages of fungal spoilage. In situ monitoring was performed 24, 48, 72, 96, and 168 h after inoculation. Distinction between blank and inoculated samples reached an 88% success rate 24 h after inoculation, 98% after 48 h, and 100% after 72 h. **Figure 5** shows 2D DFA plots corresponding to fungal cultures sampled

at 24, 48, 72, 96, and 168 h after incubation. In the first plot (24 h after inoculation), blank vials overlap with inoculated samples. As the time of incubation increases, the dispersion between samples belonging to the same class decreases and the difference between classes grows. After 48 h, uninoculated and blank samples can be clearly distinguished. This is in good agreement with our prediction model that achieved a 98% success rate after 48 h. Moreover, samples belonging to the same genus appear to cluster together, a tendency that becomes more pronounced as the time of incubation increases. Once again these results are in good agreement with our predictive model because after 48 and 72 h the instrument achieved a 78% success rate and 88% after 96 h in the prediction of fungal genera

From the results obtained on the monitoring of in vitro fungal growth, it can be concluded that during the first 24 h fungi are mainly producing CO_2 and other common metabolites associated with primary fungal growth and structures formation such as 3-methyl-1-butanol, 2-methyl-1-propanol, and 1-octen-3-ol indicative from fungal presence. According to Börjesson et al. (*3*), it seems that species identification may not be possible at this early stage, since the compounds produced in the highest amounts are similar for different species. The volatile pattern profile is, therefore, very similar and does not allow discrimination between species. Characteristic volatiles that might allow species classification are mainly produced during secondary metabolism. After 48 h of incubation, the system is able to predict fungal genera with a 78% success rate, which implies that secondary metabolism has started. Sporulation happens 72−96 h after inoculation depending on fungal species, which leads to an increase in several volatile compounds generating different pattern profiles for each fungal genus or species. This allows the best discrimination results in our model.

Taking into account the results obtained with the measurements performed on in situ samples, a real quality analysis application in a bakery factory seems feasible. Since SPME sampling time was 20 min and desorption time was 5 min, each measurement took 25 min to be executed. In a real application, many SPME fibers can be used in parallel (e.g., four) and the system can get a throughput of a measurement every 5 min. Moreover, since some quality control departments already have GC−MS equipment, they can convert their units into a MS-based E-nose in a rather straightforward manner, just coupling the optimal sampling system and using additional pattern recognition software.

The next step should be focusing on tuning the prototype to work in a bakery plant, where it would be trained to monitor a certain number of samples from each batch. These measurements made at the quality control laboratory would allow detection of batches likely to be spoiled before their expiration date and thus produce a rejection decision before the batch leaves the production plant.

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