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Use of an electronic tongue to analyze mold growth in liquid media

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Abstract

The feasibility of employing an electronic tongue to measure the growth of mold in a liquid medium was studied. We used the electronic tongue developed at Linköping University, which is based on pulsed voltammetry and consists of an array of different metal electrodes. Instead of focusing on a single parameter, this device provides information about the condition or quality of a sample or process. Accordingly, the data obtained are complex, and multivariate methods such as principal component analysis (PCA) or projection to latent structures (PLS) are required to extract relevant information. A gas chromatographic technique was developed to measure ergosterol content in mold biomass and was subsequently used as a reference method to investigate the ability of the electronic tongue to measure the growth of mold in liquid media. The result shows that the electronic tongue can monitor mold growth in liquids. In PLS analysis, the electronic tongue signals correlate well with the amount of ergosterol in the mold biomass as well as the microbially induced changes in the pH of the medium.

Keywords: Electronic tongue; Mold growth; Liquid media

1. Introduction

Measuring microbial activity is important in many industrial applications, including the food industry. In as much as foods are rich in easily accessible nutrients, they are an ideal substrate for many different types of microorganisms. Obviously, whenever microorganisms, wanted or unwanted, are involved in the processes, it is very important to be able to monitor their activities. Several comprehensive and more specific methods are available to detect microbial activity, many of which are slow, unreliable, costly, labor intensive, and/or require extensive knowledge about the processes to be assayed (e.g. production of metabolites and enzyme activity or genetics). Culturing techniques and direct examination of samples are traditional microbiological techniques to detect and identify microbes. Newer and faster analyses include ATP determination, procedures based on immunology, impedance-based methods, and DNA/RNA methods (de Boer and Beumer, 1999). Other techniques used to detect certain compounds that are related to microbial activity include gas chromatography (GC), high performance liquid chromatography (HPLC), and capillary electrophoresis (CE).

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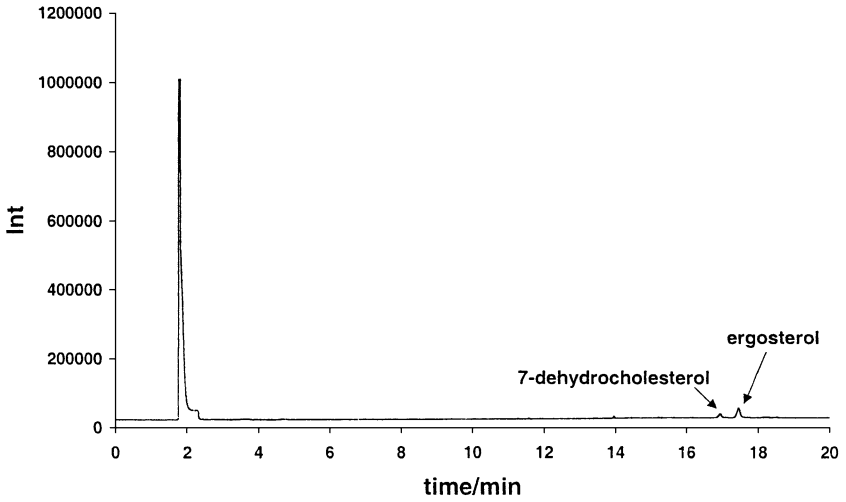

Fig. 1. Chromatogram of sample 70a showing the retention times of the internal standard (16.954 min) and ergosterol (17.470 min).

An electronic tongue is an electrochemical device that analyzes attributes of solutions (Winquist et al., 1997, 1998, 2000), in a manner similar to devices called electronic noses, which are used to examine such features in gas phases (Gardner and Barlett, 1994; Schnürer et al., 1999; Winquist et al., 1993). The electronic tongue is composed of an array of metal sensors with different selectivity patterns that are assumed to provide an output pattern that represents the combination of the components in the liquid. A useful tool for studying complex data is multivariate data analysis like principal component analysis (PCA) and projection to latent structures (PLS) (Wold et al., 1986, 1987, Wold, 1995).

We needed a reliable reference method to investigate the efficiency of the electronic tongue in measuring the growth of a mold (*Aspergillus oryzae*). Ergosterol is the major component of the outer membrane of fungi (except Hyphochytridiomycetes and Oomycetes; Newell, 1992), and it is essentially unique to fungi (along with certain algae and protozoa; Newell, 1992). Research has shown that ergosterol concentrations increase in parallel with fungal growth (Schnürer, 1993; Seitz et al., 1979; Stahl and Parkin, 1996). Several reports indicate that measuring ergosterol is suitable for the general analysis of fungal growth, apparently better than determining levels of chitin (Ekblad et al., 1998; Seitz et al., 1977, 1979). There is also evidence that ergosterol may not be completely accurate as a fungal growth indicator (Bermingham et al., 1995).

Previous studies in our laboratory have indicated that the electronic tongue developed at Linköping University can distinguish between media that have and have not been contaminated with mold (unpublished data). The objective of the present study was to further examine the sensitivity of the electronic tongue in analyzing mold growth in liquid media. This was done in two steps. First, we developed a gas chromatograph with a flame ionization detector (GC-FID) method to prepare and measure ergosterol based on previously reported procedures (Eash et al., 1996; Gao et al., 1993; Newell et al., 1987, 1988). This method

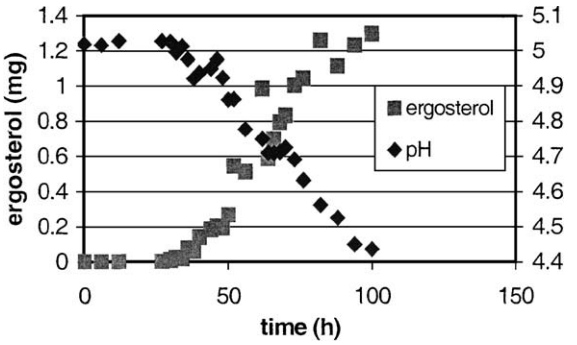

Fig. 2. Growth curves of mold cultured in liquid malt extract medium (values are means of duplicate samples). Right and left *y*-axes respectively represent amounts of ergosterol and pH values.

was then applied to determine whether the electronic tongue data can be used to predict ergosterol levels and in turn to monitor the growth of a mold.

2. Material and methods

2.1. Culturing the mold

The mold (*A. oryzae*) was originally collected from contaminated jam and was inoculated on 3% malt extract agar (Merck, Darmstadt, Germany). To produce standardised inoculas for future experiments, spores from the agar plate were suspended (2×10^6 spores/ml) in freeze medium (containing 0.18 g of KH_2PO_4, 0.82 g of K_2HPO_4, 0.59 g of sodium citrate, 0.25 g of $MgSO_4 \cdot H_2O$, and 172 ml of glycerol (87%) in 1 l of distilled water), and 1-ml aliquots of the suspension were stored at -70 °C. Each aliquot was later used to inoculate 100 ml of liquid malt extract medium (3%) in 250-ml Erlenmeyer flasks (cotton plugged). These cultures were incubated at room

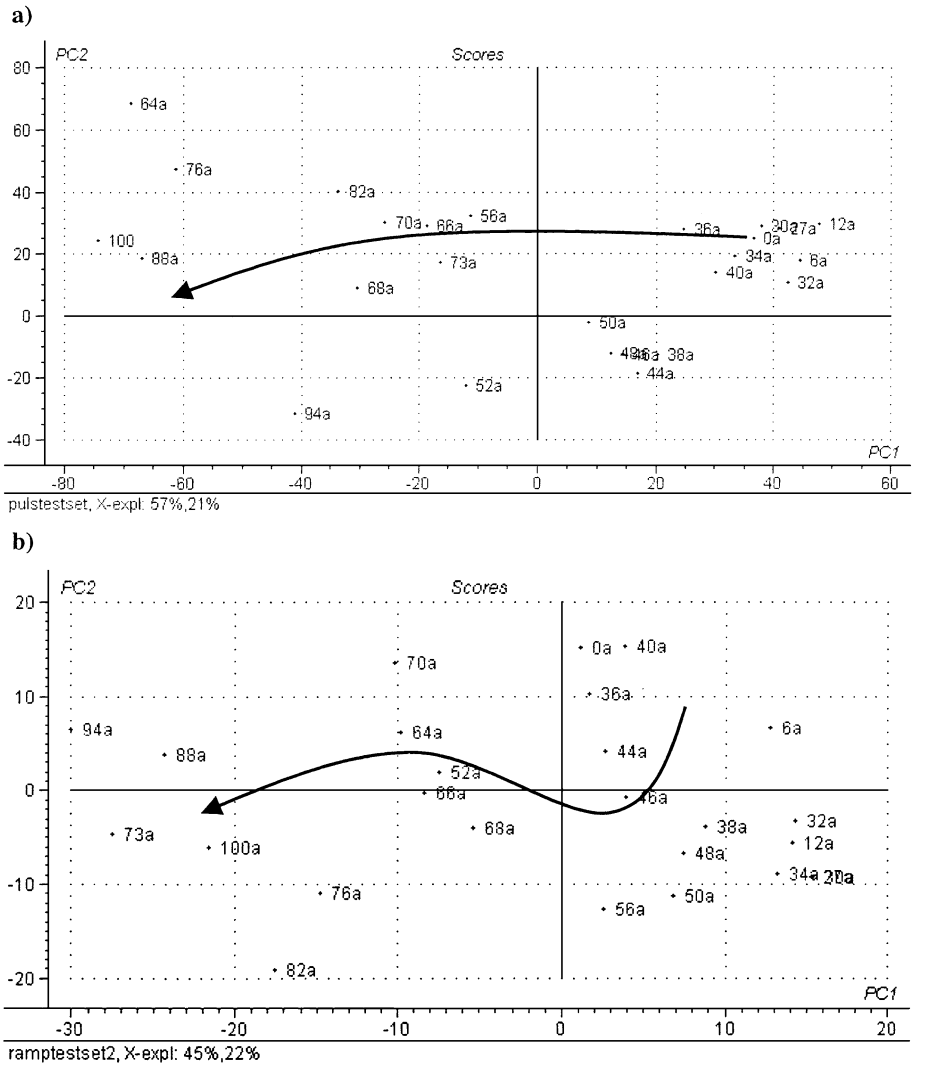

Fig. 3. Score plots of PCA values of (a) pulse voltammetry data and (b) staircase voltammetry data. The numbers represent the various samples, which are designated according to incubation time in hours (a in plot indicate one of the duplicates, only validated samples are shown).

temperature (22 °C) on a rotary shaker (80 rpm) in complete or semidarkness until subjected to measurements. Sampling was done on 27 different occasions, the first at time 0 h and the last at 100 h, with more frequent sampling during the exponential phase. All samples were collected in duplicate (designated a and b) and were inoculated and measured in random order on each occasion, giving a total of 54 samples. Uninoculated controls were measured at 30, 40, 50, and 100 h.

2.2. Experimental set-up of the electronic tongue

The electronic tongue used in this project included four working electrodes, an Ag/AgCl (3 M KCl) reference electrode (Beta Sensor, Lund, Sweden), and a stainless steel auxiliary electrode, and these were arranged in a standard three-electrode configuration. The working electrodes consisted of metal wires made of gold, iridium, platinum, and rhodium (purity 99.9%, 99.8%, 99.95%, and 99.8%, respectively; Johnson Matthey, Gothenburg, Sweden), which were mounted in dental composite material (Z250, 3M, Forssbergs Dental, Stockholm, Sweden), exposing only the edges of the metal wires in the stainless steel tube that constituted the auxiliary electrode. The reference electrode was mounted in the center of the working electrode set-up. The working electrodes were connected to a relay box, a potentiostat, and a computer for handling measurements and data storage. Signals from the potentiostat were transferred to the PC via a 1200-DAQPad (National Instruments, USA). Two measurement procedures were used: pulse voltammetry, giving 7560 variables and staircase voltammetry, giving 924 variables. The electrodes were cleaned mechanically and electrochemically before each measurement to minimize drift and memory effects.

2.3. Ergosterol measurements

A gas chromatographic method was developed to measure levels of ergosterol in mold biomass. The mold culture growing in liquid malt extract medium (100 ml) was filtered, and the collected mycelia was thoroughly mixed with an internal standard (0.3 mg of 97.5% 7-dehydrocholesterol; Acros Organics, Sweden) and KOH (5 g) in methanol (50 ml). The mixture was refluxed for 20 min, and mold debris was subsequently filtered from the solution and discarded. The liquid was evaporated to 10 ml in a rotary evaporator and then mixed with 40 ml of water. Thereafter, ergosterol was extracted with hexane (2 × 10 ml), evaporated to dryness, dissolved in 1 ml of hexane, and injected on a GC-FID (Hewlett-Packard 5890). A recovery test was also carried out. In short, 0.4 mg of 98% ergosterol (Acros Organics) was mixed with internal standard (0.3 mg) and KOH

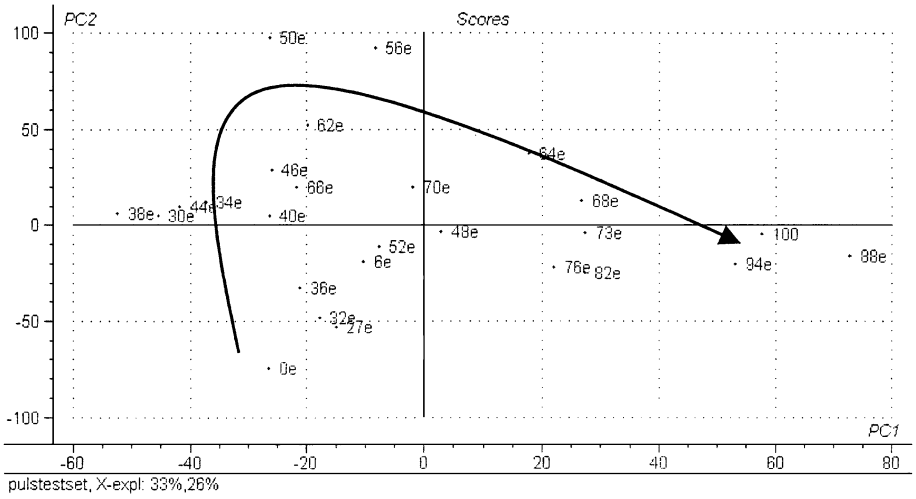

Fig. 4. PCA score plot of pulse voltammetry electronic tongue data, showing that growth can be followed also when all the samples have a similar pH (e indicate one of the duplicates, only validated samples are shown).

(5 g) in methanol (50 ml) and then treated as described above; 0.3 mg of C_{16}−Cl was added to the sample just before injection into the GC. Another sample was prepared by adding the same amounts of ergosterol, internal standard, and C_{16}−Cl as mentioned above to 1 ml of hexane, and this sample was also subjected to GC analysis. The percentage of ergosterol recovered could thereby be calculated. The amounts of ergosterol calculated from the GC measurements are based on a calibration curve, which was obtained using ergosterol that had been prepared in the same way as ergosterol that originated from mold in the malt extract medium (see above).

We used an HP-1 fused silica GC column (30 m × 0.32 mm, film thickness 0.25 μm). Helium was used as carrier gas at a flow rate of 30 cm/s, the splitless injector valve was closed for 60 s, and 1.5 μl of sample was injected. The temperature program started at 170 °C for 30 s and was subsequently increased by 10 °C/min to a final temperature of

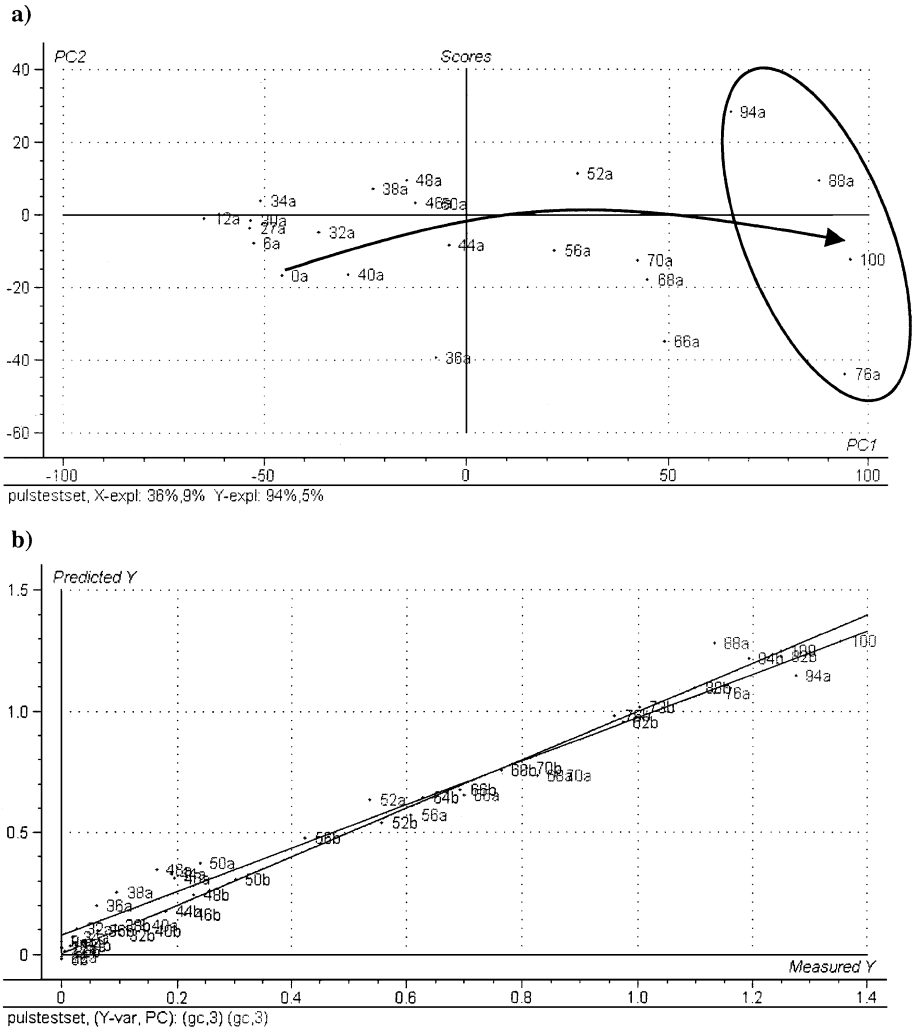

Fig. 5. Results of PLS analysis of pulse voltammetry and ergosterol data. (a) Score plot showing how growth of the mold follows PC 1 and the distribution of the late samples follows PC 2 (only validated samples are shown). (b) Plot of predicted versus measured values indicating a high correlation coefficient and little deviation between the slopes. Note: similar plots were obtained for PLS models of pulse voltammetry and pH, and of staircase voltammetry and ergosterol and pH (see values given in Table 1).

300 °C for 25 min. The retention time was about 17 min and 30 s for ergosterol and about 17 min for the internal standard (Fig. 1). Ergosterol values presented below are means for duplicate flasks.

2.4. Data analysis

The electronic tongue, pH, and ergosterol data were imported to a matrix to software designated the Unscrambler (CAMO, Oslo, Norway) and subjected to principal component analysis (PCA) or projection to latent structures (PLS) to extract relevant information. PLS models were performed with pulse or staircase voltammetric electronic tongue data in the *X* matrix and ergosterol or pH data in the *Y* matrix. All data were centered and weighted (1/SD). Test sets were used as the validation principle of the models. As there were duplicates of each sample, one was used for calibration and the other for validation. This validation technique is powerful and therefore the models can be considered as good models. Obvious outliers were removed, and variable reduction was performed where it improved the results.

3. Results

As can be seen in the growth curve illustrated in Fig. 2, with the ergosterol preparation method, it was possible to monitor growth of the mold in liquid malt extract medium over a period of 100 h. The growth curve shows that the final amount of ergosterol in 100 ml of mold culture after 100 h was 1.3 mg (i.e. 43% of 3 mg, considering the recovery rate of the preparation method), which corresponds to about 1% of the dry weight (250 mg after 100 h). No growth was detected in the controls (data not shown). The ergosterol method first detected growth of the mold at around 30 h (Fig. 2), at which time, the *A. oryzae* in the malt extract medium was clearly visible to the naked eye. It was also possible to monitor growth by measuring the pH of the medium (Fig. 2), which was about 5.03 immediately after inoculation (0 h) and decreased to about 4.44 at 100 h; the pH values started to decrease about 30–35 h after inoculation.

PCA was performed to determine the ability of the electronic tongue to monitor the growth of mold (Fig. 3). Pulse and staircase voltammetry data were evaluated separately. The first and second principal components (PC 1 and PC 2) of pulse voltammetry, respectively, explained 57% and 21% of the variance, and these values are slightly higher than the corresponding values of 45% and 22% for staircase voltammetry. The direction of growth along PC 1 is also more obvious in the score plot of the pulse voltammetry data. In Fig. 4, the experiment has been repeated but the pH was adjusted so that it was the same in all bottles. A PCA was performed on the new data and the growth can be followed along PC 1 in the same manner as in Fig. 3.

To determine whether growth of the mold could be monitored by using the electronic tongue compared to measuring ergosterol levels or pH, we employed PLS to evaluate the data obtained with these methods. Fig. 5 shows the PLS results in the form of (a) a score plot and (b) a plot of predicted-versus-measured *Y* values (pulse data in the *X* matrix and ergosterol data in the *Y* matrix). Correlation coefficients, root mean square error of prediction (RMSEP) values, and the percent explained variance values of the first two PCs for some measurements are given in Table 1. The RMSEP values were lower and the correlation coefficients were higher for the pulse data than for the staircase data, indicating that the pulse models are better

Table 1
Results of PLS analysis of ergosterol and pH data with electronic tongue data (pulse and staircase voltammetry)

	RMSEP[a]	Correlation coefficient	*X*-explained PC 1 (%)	*Y*-explained PC 1 (%)	*X*-explained PC 2 (%)	*Y*-explained PC 2 (%)
Pulse (erg)	0.099	0.98	36	94	9	5
Staircase (erg)	0.125	0.97	40	92	16	6
Pulse (pH)	0.048	0.97	36	86	9	11
Staircase (pH)	0.054	0.96	40	83	15	12

The correlation coefficient in column two is the validated correlation coefficient.
[a] Given in original measurement units, mg ergosterol or pH units.

predictors of mold growth, related to ergosterol or pH data. The percent-explained variance of PC 1 was high in all models. The ergosterol growth curve indicates that sample number 62a was an outlier (Fig. 2). The same sample also appeared as an outlier in the PCA and PLS models, thus it was removed from the data set to improve the models. The reason for this sample to be an outlier is probably that it was the first sample to be measured and data from this measurement have been, in previous studies with the electronic tongue, shown to be unstable.

4. Discussion

The procedure used to extract ergosterol offered a low recovery rate (43%). However, a high recovery rate is not necessary to address the question of whether the electronic tongue can be used to monitor mold growth. The reproducibility of the GC method we used was good and is more important for this application. The limits of detection of mold growth might seem high for both ergosterol and pH measurements, but a sensitivity of around 30 h can be considered as good for ergosterol (Seitz et al., 1979). It is difficult to determine the detection limit of the electronic tongue by evaluating the score plot of the PCA results, thus it is also difficult to ascertain whether this sensor device is better than the other methods with regard to detecting microbial activity. Nonetheless, the electronic tongue does have certain advantages. Compared to the ergosterol measurements, analysis with the electronic tongue saves time and money, and it lowers the workload in the laboratory. A big advantage of measuring with the electronic tongue is that the sample is not changed after the measurement. This means that the same sample can be measured over and over again. Measuring the pH and analyzing with the electronic tongue are equally fast methods of measuring mold growth, but the advantage of the electronic tongue is that it can also be used in buffered media. Furthermore, the electronic tongue is more robust and better suited for measurements in industrial processes. In addition, the electronic tongue provides more complex data; hence, it is possible to obtain a larger amount of information about changes in the composition of the sample solution and hopefully a better understanding of the status of a process. The electronic tongue is a newly developed method that is still being perfected; thus, it has probably not yet reached its full potential to provide information. The detection limits are still good compared to other methods available, i.e. counting colony-forming units. The electronic tongue also has some advantages compared to the electronic nose. Apart from the fact that it measures directly in the liquid phase, it also does not change the medium while measuring as mentioned above. With the electronic nose, the composition of the headspace is changed after a sample has been removed for analysis. In humid environments, the electronic nose might also have problems with condensation in the tubes.

In Fig. 2, growth of the mold is illustrated by an increase in ergosterol and a decrease in pH; that is, these two parameters show a negative correlation. The electronic tongue has previously proven to be reliable for pH measurements. To prove that the electronic tongue does not only measure the pH, the device was used to measure growth while maintaining the pH at a constant value. The results of this experiment clearly show that not only pH, but also other changes in the medium, are measured by the electronic tongue since the growth can still be followed (Fig. 4).

The PCA score plots for the electronic tongue data indicate that growth of mold can be followed along PC 1 (Fig. 3). Growth is easiest to discern in the pulse voltammetry score plot (compared to staircase voltammetry). This may have been due to the design of the experiment; that is, more variables were collected in the pulse set-up, and negative voltage was also applied, increasing the range from which information was collected. The electronic tongue cannot only be used to follow the growth of a mold in liquid media. It can also be used as a method to predict the ergosterol level (Fig. 5). Compared to the extraction and measurement of ergosterol, the electronic tongue is a fast and cheap method as well as it does not include the use of harmful chemicals.

In the score plot in Fig. 5, there was a distribution of late samples along PC 2. It is possible that while the first PC explains growth of the fungi with early samples at one end and late samples at the other, the distribution of the late measurements in the second PC might be due to changes in the metabolism of the mold in a later growth phase. This hypothesis remains to be investigated.

Drift is a well-known problem for most sensors, and to be able to indicate this disturbance, we randomized the order of the measurements performed. Accordingly, the distribution along PC 1 (Figs. 3–5) is not a consequence of drift but instead reflects the true changes that occurred in the liquid as the mold was growing. This was confirmed by the fact that it was impossible to predict the measurement order (data not shown).

Although we do not know exactly what compounds are detected by the electronic tongue, we do know that ions and redox active substances are measured. In light of the intended applications of the electronic tongue, it seems to be of little interest to know the particular compound being measured in the initial stage of analysis. Instead, the electronic tongue is supposed to be used simply to get a first indication of deviations from a standard condition, for example, contamination in an industrial food production line, or to monitor a process, such as fermentation of a food product. Other more suitable methods (e.g. culturing techniques, GC or HPLC) are available to obtain additional and more specific information about the state of a process. Hopefully, further work done in the near future will increase our knowledge about what the electronic tongue actually measures and thereby render the technique even more useful for microbial applications.

5. Summary

Analysis with the electronic tongue appears to be an excellent method to follow the growth of a mold in a liquid media. As a result of that fact, it is also possible to use the electronic tongue for the prediction of the amount of ergosterol and the pH values that occur as a result of mold growth. This is supported by the low RMSEP values, high correlation coefficients, and high percentages of explained variance of the principal components in PLS.

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