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An electronic nose to evaluate fungal contamination

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Abstract. Following an introduction on the characteristics and potentialities of electronic noses, results obtained using an EOS^{853} instrument on maize samples inoculated with *Fusarium verticillioides* strains are illustrated. The technique appears suitable for the rapid separation of samples colonized by either fumonisin B1 producing or non producing cultures.

Key-words. Electronic nose, fumonisins, maize, mycotoxins, odorants, sensors.

Olfactory is probably one of the most undervalued of the five senses, despite the fact that smell is responsible for the perception of 75-80% of everything we taste (Bear et al. 1996). Ageing people worry about losing their eyesight or hearing, but give little thought to being unable to appreciate the aroma, say of food, as they did when younger; usually the food is blamed ('its not the same as years ago!'). Still smell is important even in practical terms, it tells us when it is time to empty the fridge or when something is burning. It works as a danger signal if there is a gas leak in the house. At home it is the common way of detecting if food has deteriorated.

Man however is not at the apex of creation, at least in olfactory terms. For example 65 sq.cm of a dog's brain are devoted to olfaction *vs.* less than 5 in humans. Notwithstanding this, we possess about 40 million olfactory nerves and can detect some odorants at concentrations of one part per trillion. Still from a scientific point of view, there are difficulties in discriminating, and quantifying odour perceptions. People have problems even in finding words to describe sensations of smell. Generic terms like sour, sweet, mouldy, etc., are hardly of much use. We do exploit animals'

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superior capabilities, but have difficulty in making objective use of their ability (*i.e.*, translating this potential into physical data).

Chemical sensors – the electronic nose. It was essentially the demand for an instrument that could mimic the olfactory process and, more important still, also provide objective sensory information that has lead, over the years, to the development of the electronic nose (ENose).

To start with, any substance that has odour, evaporates small quantities of molecules (called odorants) that produce the smell. Anything capable of detecting the molecules is defined as a chemical sensor.

Basics of ENoses. Traditionally gas analysis is carried out by gas chromatography (GC) and mass spectrometry (MS). These analytical systems are bulky, time-consuming, rather expensive and require experienced operators. Alternatively ENoses are fast, reliable and user-friendly (Gouma & Sberveglieri 2004).

ENoses, first commercialize in the early '90s, typically consist (Gardner & Bartlett, 1999) of an array of chemical sensors, an air flow system, capable of switching from a reference air to the one to be tested, a signal analysis technique and a presentation unit. The main components are the *sensing system* and the *pattern recognition system*.

The task of the *sensing system* is to catch the odour. Each odorant produces a characteristic signature and a database is built up by presenting different odorants to the sensing system. The latter is represented either by a single sensing device (such as GC), an array of chemical sensors (with each sensor measuring a different property of the chemical involved) or a hybrid of both. There are different categories of sensors available. Choice and number of sensors depend on the purpose for which the ENose is to be used.

Conductive sensors are of two types, metal oxide and polymer. They show changes in resistance when exposed to volatile organic compounds. They are not very sensitive for specific odorants and reseach is under way to improve their performance.

In *piezoelectric sensors* a gas sample is adsorbed at the surface, thus increasing the mass of the disk-polymer and consequently reducing the resonance frequency. The reduction is inversely proportional to the odorant mass adsorbed.

Metal-oxide-silicon field-effect-transistors (MOSFET) operate on the principle that volatile components react in contact with a catalytic metal and the reaction products diffuse to change the electrical properties of the device. Selectivity can be optimized operating the device at different temperatures and/or varying type and thickness of the metal oxide.

Optical fiber sensors consist of glass fibers with a metal coating at their ends. A light source responds to the presence of the odorant with a change of colour, which can be detected and measured.

Spectrometry-based sensors use the differential infrared spectrum of the

molecule. These devices are usually quite large and expensive.

The *pattern recognition system* is capable of producing a classification or clustering of each odorant and give an automated identification. The signals generated by the array of odour sensors need to be processed. Different parametric and non-parametric pattern analysis techniques employed. These include the use of linear and non-linear techniques, such as discriminant function analysis, cluster analysis, fuzzy logic, adaptatative models, etc. At this stage pattern recognition can be applied to differentiate various substances and train a system to provide a classification based on a collection of known responses.

ENose Applications. Initially the authors of this note intended to produce a table listing the fields in which the ENose can be used. On second thoughts this was discarded, the mere list of references would have occupied several pages. It is enough to point out that a simple quest on ENoses on the web, can result in something like 50.000 citations. In other words, considering the potential of a system capable of giving objective and repeatable answers, it is obvious that the field of possible uses is virtually unlimited. Here are a few examples of their applications, some just suggested, others only tested experimentally and still more already of practical use.

Drinking, waste and sewage waters can be monitored in time. The same applies to soils, whether used for plant cultivation or following recovery from chemical pollution. Air quality is of utmost interest at any level, whether it is in the home, the car one drives, a spacecraft or the factory where one works. The ENose is pertinent in this field too. Identification of perfumes and detection of drugs, explosives, etc. are also just a nose matter.

In agriculture ENoses are used as non intrusive detectors of fruit ripeness and for early determination of disease agents. The timing of harvest for several crops can be decided by ENose evaluations. In the food field the applications are too many to enumerate. To start with, judgements related to freshness, reliability of containers, spoilage of primary sources and of transformed products, are commonly based on olfactory evaluations, a parameter which is highly subjective, not reproducible, not quantifiable and completely unscientific. The ENose could be and actually is of help. Problems could be solved in relation to typicity of wines, malodours in beers, detection of secondary components in distilled spirits. ENoses can determine the effect of different temperatures in the roasting of coffee beans. In addition in all processed agricultural commodities a reliable monitoring system is needed and ENoses seem capable of doing the job.

Medicine at first appears to be the most unlikely discipline to take advantage of ENoses. We tend to laugh at the thought that, not so long ago, doctors smelt the patients' breath to detect their illnesses. Now it appears that oral malodours can easily be clas-

sified and that the analysis of breath, by ENoses, is useful in diagnosing illnesses such as type 2 diabetes, lung cancer, etc. Alltogether it is obvious that forensic medicine could gain enormously from ENose technology. And in fact it does.

To end on a lighter note, everybody knows somebody who "smells", the truth is that all human beings smell but differently. So, in addition to physical characteristics such as eye features (iris, retina), facial, hand and ear conformation, wrist/hand veins geometry, etc., why not take advantage of body smell a "biometric person authentication"? With ENoses of course! Finally it should also be mentioned that a variety of sensor materials and technologies are used in "electronic tongues", *i.e.*, sensor arrays able to operate in liquid environments.

Experimental work. As an example of the potentialities of the technique, illustrated in the previous pages, results obtained in a study on the use of an ENose for the detection of fumonisin producing fungi in maize are reported. Characteristics and relevance of fumonisins in maize cultivation in Friuli were detailed in a previous note (Locci & Gobbi 2002).

Cereal grain spoilage is frequently caused mainly by members of the animal kingdom (rodents, insects, etc.), but fungi are the principal culprits. Losses due to fungi are related both to their growth in the substrate and to the production of undesiderable metabolites (mycotoxins). In addition to mycotoxins, they may produce volatile metabolites which can be used as spoilage indicators or mycotoxin markers (Jelen et al. 1995). Olfactory grading of grains is routinely carried out by grain inspectors upon delivery of cereals to granaries (Magan 1993). The questionable reliability of human odour classification does not need to be stressed, and we must not forget the health hazards caused by repeated exposure to fungal spores and toxic volatiles.

Materials and Methods

Maize Samples. Twenty-five grams of sterile crushed kernels of a commercial hybrid of maize (*Zea mays* L.) were distributed in 250 ml glass jars. Preliminary tests showed that larger quantities did not significantly affect signal intensity and reproducibility. Two syringe filters (0.45µm) were inserted into the holes of the lid of each container to ensure the sterility of the air flow. Eight jar replicates were used for each treatment.

Fungi. Two strains of *Fusarium verticillioides* (Sacc.) Nirenberg were used in this study. Strain M3120 is a standard fumonisin B1 ($FB1^+$) producer, while M5496 is a mutant unable to produce the mycotoxin ($FB1^-$). Both cultures were obtained from the *Fusarium* Research Center, Pennsylvania State University, University Park, PA, USA. Kernels were inoculated with conidia ($1x10^7$) of each strain and incubated at 26°C. Sterile kernels were kept as controls. In addition non-autoclaved corn was also incubated in order to monitor the capability of the system.

Fumonisin. The presence of fumonisins was assessed by using the com-

mercial CD-ELISA kit (Veratox, Neogen, Lansing, USA) and following the manufacturer's instructions.

ENose: sensors, sampling and data analysis. A commercially available electronic olfactory system, EOS^{835} (Sacmi Imola scarl, Italy), was used (Figure 1). The device consists of a pump for sampling, a detector unit containing the array of sensors and software for pattern recognition.

The detector unit is equipped with an array of 6 different thin film metal oxide semiconductor (MOS) sensors (Table 1), mounted in a small chamber and kept at 250-500°C during the whole process (Falasconi et al. 2005). The sensor operates by letting an electrical current flow through the grain boundaries of the SnO_2 micro-crystal surface. At the grain boundary, oxygen is absorbed and forms a potential barrier. The size of the barrier is expressed by the value of the sensor's resistance, the higher the barrier the more resistance is met across the sensor. On the other hand, exposure to a deoxidizing gas depletes the grain boundaries, the overall resistance is reduced and the electrical charge flows more freely. Each sensor is sensitive to several deoxidizing gases.

There are different stages in odour sampling. The first is a baseline purge, where the sensors are exposed to the reference air (0.45µm filter-sterilized ambient air passed through active carbon), pumped into the sensor chamber at a constant flow rate (180 cc/min) for 60 seconds, in order to obtain a stable baseline. The second stage is the sampling cycle, when volatiles from the jars come into contact with the sensor array. The measured RH within the chamber (at 50°C) in the present study was around 28% (with a 2% mean variation) for all samples. The headspaces are pumped over the sensors for 60

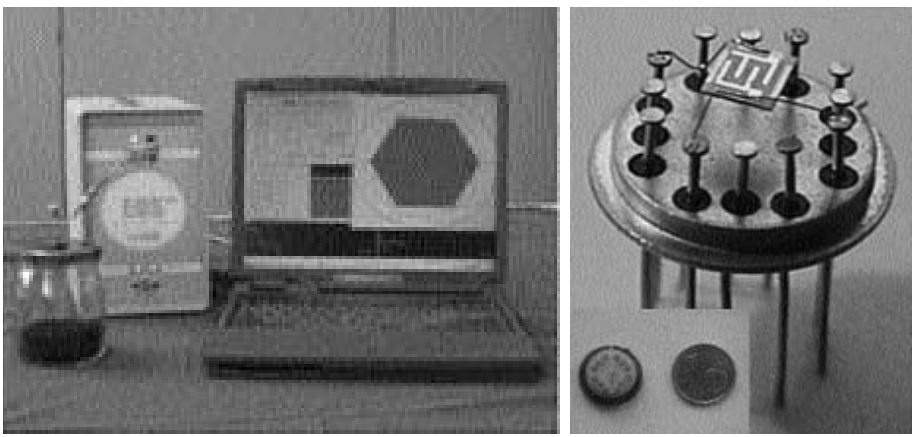

Figure 1. The electronic nose used in the present study.

Table 1. Sensor array configuration of the EOS^{835} electronic nose.

#	Sensing Layer	Catalyst (type, thickness)	Operating Temperature (°C)
1	SnO_2 RGTO	Au, 40 Å	
2	SnO_2 RGTO	Ag, 38 Å	450
3	WO_3	-	350
4	SnO_2 RGTO	Mo, 45 Å	450
5	SnO_2-In_2O_3	-	450
6	In_2O_3	-	475

seconds. Between measurements samples are incubated at 26 °C. Finally sensors are exposed to reference air for 10 minutes to recover the baseline. During the first week measurements were taken 24, 48, 72, 96 hours after inoculation and afterwards once a week for six weeks.

Exploratory analysis (or pre-processing) by PCA (Principal Component Analysis) is carried out to check data quality, detect outliers and reduce noise and anomalous readings. PCA (Wold et al. 1987) is a useful statistical technique since it allows pattern identification of large quantities of data and their expression highlighting similarities and/or differences. The other main advantage of PCA is that it involves a mathematical procedure that reduces data complexity by eliminating co-dependencies, while retaining as much information as possible, in other words it transforms a number of (possibly) correlated variables into a (smaller) number of uncorrelated variables, the so called *principal components.*

Graphically PCA cycles data in such a way that maximum variability is projected onto a series of axes. The first one (I principal component) "contains" (or "accounts for") the maximum amount of variation. The second axis (II pc) contains the maximum amount of variation orthogonal to the first, the third axis shows the one orthogonal to the first and to the second and so on, until the last amount of variation is plotted. R/R0 ratios (with R0 representing baseline resistance and R the minimum of the sensors response curve) are used as PCA inputs

The classification performance of the Enose is checked by the k-Nearest Neighbours (kNN) method (Cover & Hart 1967). In this supervised learning method the algorithm learns from samples with known class memberships (*training set*) and establishes a prediction rule to classify new samples (*test set*). It classifies a point by calculating the distance between that particular point and points in the training data set. In the presence of a new sample, KNN finds the nearest k neighbour from the training space, on the basis of similarity or physical distance. The number of k-nearest neighbours is user-defined. Each sample from the test set is classified by assi-

gning the point to the class that is most common among its k-nearest neighbours (where k is an integer), in other words by finding the k-nearest neighbouring training sample, on the basis of Euclidean distances.

Results. In the present study the first step was devoted to training the system to recognize colonization itself, *i.e.*, to discriminate between colonized (C, artificially inoculated) and non-colonized (NC) maize samples. The data consisted of 50 patterns of NC and 150 patterns of C colonized. The data set was randomly split into both training and test subsets. The training set had 114 patterns (86 C, 28 NC), while the test set consisted of 86 patterns (64 C, 22 NC). In addition 21 patterns, representing non-autoclaved maize samples, were also submitted to the best classifier as a further validation set.

Exploratory analysis of the data collected during the first days of fungal growth show that colonization of maize samples is detectable even 24hrs after inoculation (Figure 2). A clear division between autoclaved maize and all samples colonized by *F. verticillioides* cultures can be observed by PCA scores. The difference is typically shown by PC2, which represents almost 8% of the variance. PC2 clearly separates the sterilized maize samples from those artificially inoculated, independently of the toxigenic properties of the fungal cultures involved. PC3, expressing about 1% of the total variance, shows a partial separation of fungal strains into 2 clas-

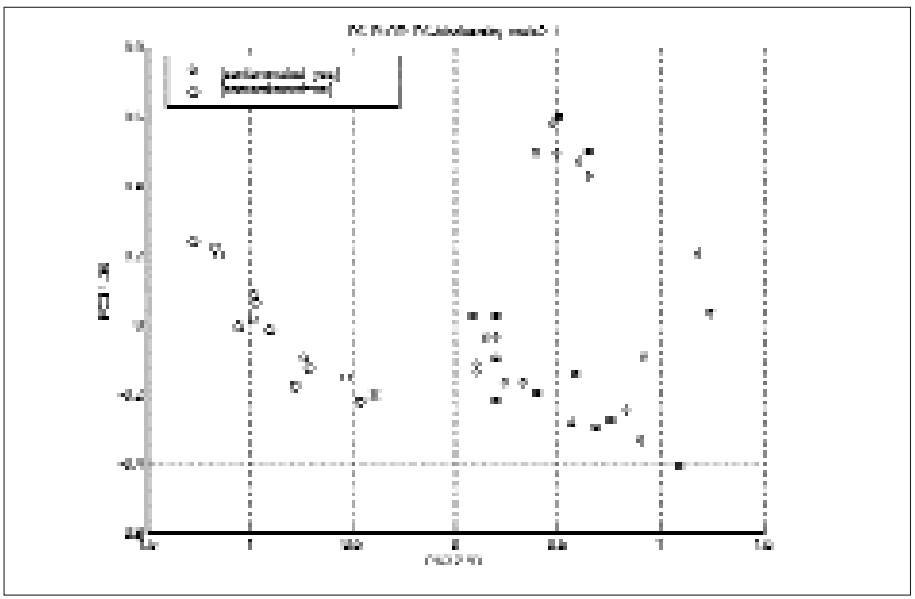

Figure 2. PCA score of data on autoclaved maize (*circles*) and maize colonized by *Fusarium verticillioides* strains M3120 and M5496 (*stars*).

ses, FB+ on the right higher part of the diagram and FB- closer to the sterile maize in the lower right sector. The separation is not yet complete due to the fact that some FB1+ strains still behave like the non-toxigenic ones.

Once the presence or absence of fungal colonization has been established, the ENose was tested for its ability to differentiate between the cultures responsible for the colonization, *i.e.* to classify FB1+ and FB1- strains. This was carried out on the data collected during the first week of growth, in order to determine the diagnostic potential of the instrumentation at the early stages of fungal colonization.

Some indicative information can be obtained by PCA. After 24hr growth the olfactory patterns of the two classes of strains (FB1+ and FB1-) are almost identical. A distinction is possible after 48hrs and is confirmed after 72 and 96hrs. The first component takes into account about 90% of the total variance, and the second 2.5-5.0%. The first and second components separate three distinct clusters: the *Fusarium verticillioides* non-fumonisin producing (FB1-) strains are located in the right hand part of the plot; the *F. verticillioides* fumonisin producers (FB1+) in the upper central part and the sterile maize in the lower left hand part of the plot.

Separation of FB1+ and FB1- strains increases with time up to 72hrs and then remains constant. Good reproducibility of measurements is also observed; outliers due to operational errors (*e.g.*, inadequate sampling conditions) can easily be detected and removed. In the following weeks separation of the two strains is still possible. However, the level changes with time, *e.g.*, after 4 weeks it is clearer with PC2 than PC1.

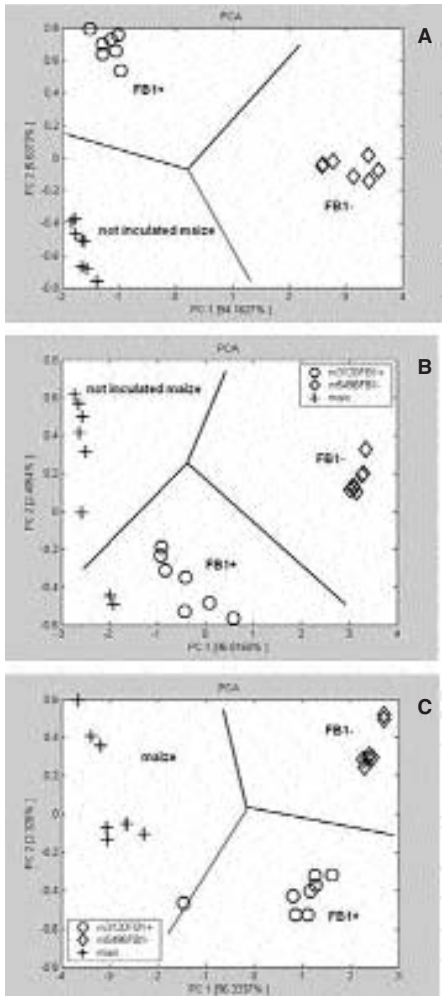

Figure 3. PCA plot of uninoculated maize samples and samples colonized by toxigenic and non-toxigenic strains of *Fusarium verticillioides* after 48 (A), 72 (B) and 96 hrs (C).

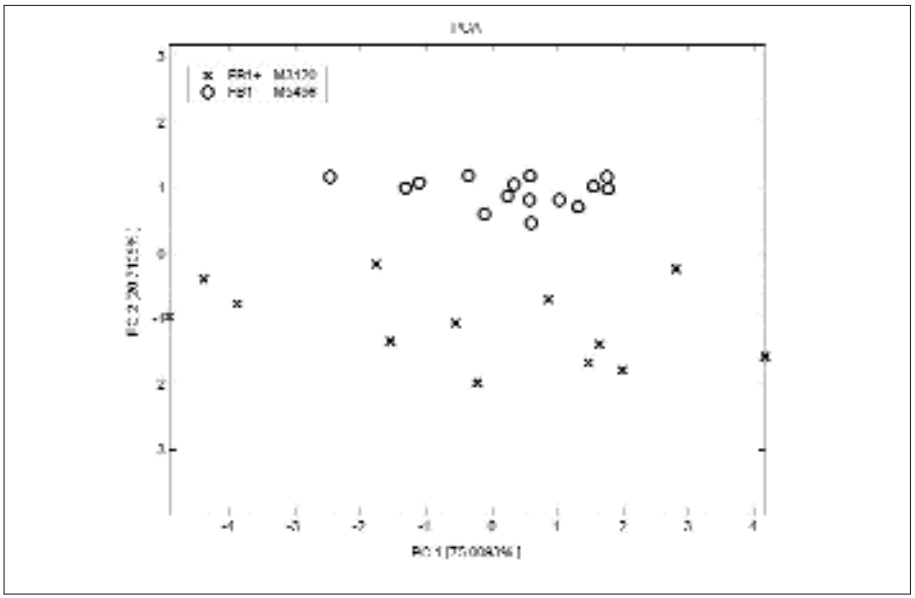

Figure 4. PCA plot of maize samples colonized by the two strains of *Fusarium verticillioides*.

Immunological tests (CD-ELISA) carried out at the same time confirmed that only strain M3120 produced fumonisin B1.

In the kNN method, only one sample appears to be misclassified being a false positive (*i.e.*, non- colonized maize classified as colonized). The best classification score is achieved by 1NN, both for standard and non-standard features. When the best classifier (1NN) is applied to the validation set (naturally contaminated maize), all samples are correctly classified. For classification purposes, using first 1NN as a benchmark, 48hr measurements are considered as a training set and the datasets corresponding to different growth times (72, 96hrs, 1 week) tested, then the training set is updated. Classification scores improve the longer the incubation time; however they decrease (*i.e.*, the error augments) when the time between training and test set increases, this clearly depends on the mycelial growth which causes changes in the measured pattern.

Discussion. Traditionally the mycological examination of food and feedstuff is carried out by dilution plating and colony forming unit (CFU) methods. The main advantage of CFU is that it is universally accepted. Nevertheless, it requires 1-2 weeks incubation and skilled technicians to interpret results, in addition it provides a poor estimate of fungal biomass (Pitt 1984).

The ergosterol content is used to determine the level of fungal infec-

tion. The sensitivity is somewhat higher than that of CFU and ergosterol is a good parameter for determining fungal biomass. One of the drawbacks is that the analysis is time-consuming and requires access to a HPLC system. In comparative terms ergosterol content does not increase with sporulation, whereas the number of CFU does (Schnürer 1993). Furthermore ergosterol determinations are of no use for species identification.

Fungi are identified on the basis of morphological characteristics, which are not always easy to determine. PCR and real time PCR techniques (Boysen et al. 2001) offer much faster alternatives to identification since fungal DNA can be extracted from samples without incubation. PCR shows a much higher sensitivity than either ergosterol determination or CFU countings, but the cost of a single analysis could be quite high. On the other hand, a large number of samples can be monitored each day. Another disadvantage is that the results depend to a high degree on the DNA extraction method adopted.

Analysis of volatiles, resulting from fungal growth on various substrates, using GC-MS is extremely sensitive, but the technique requires sample preparation, *i.e.*, use of adsorbents, and the time for evaluating data. However it can detect and quantify every single compound produced.

Such levels of discrimination cannot reached using ENoses. Their sensitivity is still definitely lower and calibrations with other parameters are needed. Nevertheless no sample preparation is required. The ENose tested in this study utilizes a gas sensing technology based on the chemical sensitivity of metal oxide semiconductors (MOS). MOS are the most common sensors used in ENose systems for odour measurement. The advantages of using MOS technology are their excellent sensitivity to a large spectrum of volatile compounds (in the order of 10-500 ppm), high stability and an average life span of 3-5 years, depending on the usage of the sensor.

Sensor technology has been shown to be able to determine the mycological quality of grains (Olsson et al. 2000, 2002), as well as detect some mycotoxin groups and partially quantify their level (Jelen et al., 1995). In addition the ENose enables the separation between species of bacteria (Gibson et al. 1997, Magan et al. 2001), yeasts (Magan et al. 2001) and fungi (Kershri et al. 2003).

Kershri & Magan (2000) have shown that an ENose can distinguish between toxigenic and non-toxigenic strains of *Fusarium* spp. grown on laboratory media. The present investigation is definitely more pertinent since mycotoxin production has been analysed on natural substrates.

On maize infected both by mycotoxigenic and non toxigenic cultures EOS^{835} has demonstrated an excellent classification performance even at early stages of fungal growth, being able both of detecting contaminated maize and also determining the toxigenic potential of the strains investigated. In addition the monitoring of mycelial growth on a natural substrate over a long period has demonstra-

ted the stability and reproducibility of results obtainable using the device.

The ENose is definitely a useful tool for screening fungal volatile compounds: the methodology involved is simple, rapid and does not require the isolation of the volatiles or any special treatment of the samples.

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