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Response evaluation of an E-nose towards contaminated wheat by *Fusarium poae* fungi

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Abstract

Cereals contaminated by toxigenic fungi can be analysed by detecting and quantifying the related mycotoxins by using complex extraction procedures and analytical techniques. In this work, we studied the complex headspace of wheat samples contaminated by *Fusarium poae* fungi by SPME-GC-MS, searching the presence of non-grain volatile metabolites due to fungi contamination and their temporal evolution after inoculation. The chemical information on the headspace composition of fungi-contaminated wheat samples were used for the analysis by an electronic nose, evaluating its discrimination ability among differently contaminated samples.

Keywords: E-nose; Wheat; Toxigenic fungi

1. Introduction

There is significant interest in methods for the early detection of quality changes in cereal grains. The development of electronic nose (EN) technology in recent years has stimulated interest in the use of characteristic volatiles and odours as a rapid, early indication of deterioration in grain quality [1]. Traditionally, toxigenic fungi and relative mycotoxins contamination of cereal grains can be detected and quantified using complex extraction procedures and analytical techniques. Furthermore analyses of fungal contamination of grain and other raw materials for the food industry have depended on culture methods which require long periods of time for enumeration and quantification. Other methods which have been examinated as indicators of the activity of spoilage fungi include effects on degradation of grain components [2], fungal enzyme activity [3,4], biochemical markers such as ergosterol and ATP [5,6] and the respiratory activity of moulds [7]. Normally, the grain odour, i.e. the presence of non-grain volatile metabolites, is used for quality classification of grain [8]. Thus it is very important to detect fungal deterioration in stored grain at an early stage. This would facilitate and improve existing management of grain storage. It would also allow remedial measures to be more effectively implemented, allowing significant losses and grain downgrading to be avoided.

In this work we have investigated the possibility of using volatile metabolites produced by *Fusarium poae* toxigenic strains as indicators of fungal deterioration and possible occurrence of mycotoxins in wheat. An electronic nose, a chemoresistive gas sensor array based on In_2O_3 and pure, Os, Pt, Pd-, Rh-doped SnO_2 sol–gel thin films [9], was used for the discrimination of different wheat samples [17–18]. Gas sensing tests were carried out on inoculated wheat samples and not contaminated samples during the time.

Classical analytical technique like static headspace-solid phase micro extraction-gas chromatography-mass spectrometry (SHS-SPME-GC-MS) analysis was performed to relate results with the sensor array responses.

Principal component analysis (PCA) was applied to sensor array responses and GC-MS data in order to assess the capability of the electronic nose to discriminate among wheat samples with different fungal growth.

2. Experimental

2.1. Inoculation

The fungal strain used first in the present study was *F. poae* ITEM 3258. The strain was obtained from the culture collection

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of the Institute of Sciences of Food Production and reported to produce trichothecenes [10,11].

ITEM 3258 was grown on YPD medium (1% yeast extract, 2% peptone, 2% glucose, 2% agar) in Petri dishes for 5–7 days at 25 °C at dark. Agar squares (2 mm^2) were then cut from the agar plate containing the mycelium of the fungus in order to inoculate the grain samples.

Samples were obtained by weighting 5 g of durum wheat (*Triticum durum*, var. *dicoccoides*) seeds which were transferred into glass vials of 20 ml capacity in presence of 45% of distilled water. The vials were covered with crimped polytetrafluoroethylene-line silicone-rubber closures and held at 23 °C at dark overnight. The day after the samples were autoclaved at 120 °C for 20 min. After cooling down at room temperature, 30 samples were inoculated with the isolate ITEM 3258. The vials were then sealed and held at 23 °C at dark. Controls were treated in the same way, except that they were not inoculated.

2.2. Electronic nose

Headspace analysis were carried out (in triplicate trials) at the same day of inoculation (T_0) and after 1, 2, 5 and 7 days (T_1, T_2, T_5 and T_7) by either electronic nose and HS-SPME-GC-MS.

Moreover, at the same time, as reference sample a not contaminated headspace sample was measured.

For the electronic nose measurements, a multisensor array consisting of six different sensors has been used for the reported research. Seven of them were tin oxide-based sol–gel thin films (pure and doped with Os, Pt, Pd and Rh), the other two were based on pure sol–gel indium oxide thin films. For the thin films preparation, different sol–gel methods have been developed by using different precursors (Sn(II)ethylexanoate or $SnCl_4$ in the case of SnO_2 thin films) and different doping materials (Pd or Rh) with or without surfactant (alkylamoniumbromide in the case of In_2O_3 thin films) [9,12]. Table 1 reports the characteristics of the prepared sol–gel thin films.

The thin films were deposited on suitable Al_2O_3 substrates pre-arranged for cutting and obtaining 2 mm × 2 mm single samples. The sensitive layers, after spinning the starting solution, were thermally annealed at 500 °C temperatures. Afterward, by using a suitable mask and photolithographic process, the Ti/Au interdigitated electrodes on the front and the Pt integrated heater on the back were performed. The final devices were mounted onto a standard TO39 socket and placed in the test chamber for headspace measurements.

During operation the sensors were heated by applying a voltage V_H across the heating meander, whose resistivity value gave the sensor's operating temperature of 300 °C. At the same time a voltage of 2 V was applied between the sensor's electrodes and the current was continuously monitored by means of an electrometer Keithley model 6517A equipped with a multiplexer module.

The whole experimental system was connected to a PC equipped with a Pentium microprocessor and a IEEE 488 board for acquiring and plotting the electrical response of each sensor in real time by using Labview National Instruments software.

Table 1
Thin films preparation parameters

Sensing material	SnO_2	SnO_2	SnO_2	SnO_2	SnO_2	SnO_2	In_2O_3	In_2O_3
Dopant	–	–	Pd	Pt	Rh	Os	–	–
Dopant content (%)	–	–	5	5	5	5	–	–
Sol–gel route	Sn(II)ethylexanoate + alkylamoniumbromide	Sn(II)ethylexanoate + alkylamoniumbromide	$SnCl_4$ + $Pd(CH_3COO)_2$	$SnCl_4$ + $PtCl_2$	$SnCl_4$ + $RhCl_3{\cdot}H_2O$	$SnCl_4$ + $OsCl_3$	$In(NO_3)_3{\cdot}5H_2O$	$In(NO_3)_3{\cdot}5H_2O$ + alkylamoniumbromide
Film thickness (Å)	1000	1000	1500	1500	1500	1500	1500	1500

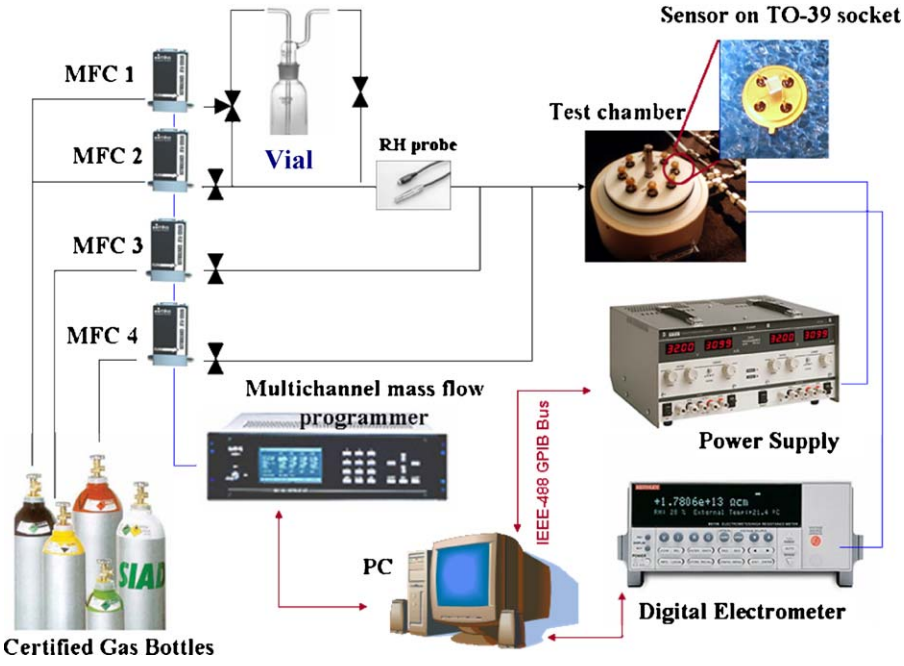

Fig. 1. Experimental apparatus for sensor array measurements.

As regard the experimental set-up and the protocol used for wheat headspace sampling it was arranged as follows.

The baseline of sensors was acquired in a mixed dry air–nitrogen ambient in a continuous total flow of 100 sccm (50 sccm dry air, 50 sccm nitrogen). For the measurement the previous described samples were kept at the temperature of 21 °C and the headspace was stripped by means of a deviation of the only 50 sccm nitrogen flow for 15 min, keeping constant the other 50 sccm flow of dry air to the chamber, while a 30 min recovery time was set. In this way the volatile compounds were directly transferred by the carrier into the sensors chamber. Scheme of the whole apparatus is reported in Fig. 1. Moreover, typical sensor array dynamic responses are reported in Fig. 2. Fig. 2(a) reports the responses of the sensors to inoculated wheat sample T_2 while Fig. 2(b) shows the correspondent not inoculated ones.

2.3. SHS-SPME-GC-MS analysis

Headspace analysis were carried out (in triplicate trials) at the same day of inoculation (T_0) and after 1, 2, 5 and 7 days (T_1, T_2, T_5 and T_7) by either electronic nose and SPME-GC-MS. Moreover, at the same time, as reference sample, a not contaminated headspace sample was measured.

Wheat samples were analysed for the qualitative determination of volatile compounds by SHS-SPME-GC-MS. SPME 50 μm CAR (Carboxen)–PDMS (polydimethylsiloxane) fibres were used and exposed to wheat sample in 20 ml gastight vials at 21 °C. After this, fibre was desorbed in a split/splitless injector, equipped with deactivated SPME glass inserts and analyses were carried out on a 30 m × 250 μm i.d. × 0.25 μm HP INNOVAX polyetilenglicole column. A GC system HP 6890 Series, Agilent Technologies, was coupled with HP 5973 mass selective detector, Agilent Technologies. Injection was split 5:1 with 5 min relay time. GC conditions were: 40 °C for 5 min, 150 °C at 4 °C/min, then 250 °C at 15 °C/min; injector temperature was 250 °C, helium flow was 1 ml/min. Transfer line was held at 260 °C. Spectra were produced in the electron

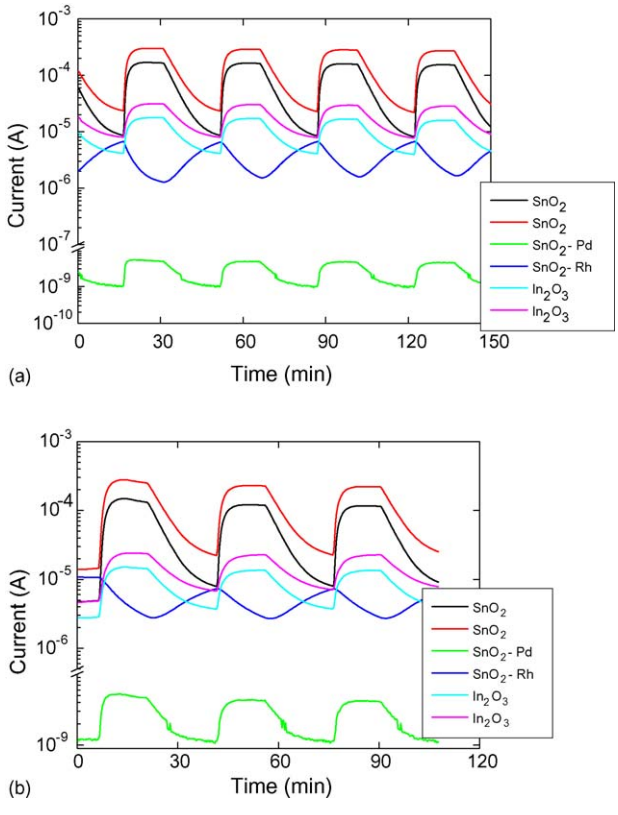

Fig. 2. Sensor array dynamic responses to (a) inoculated wheat sample and (b) not contaminated wheat samples after 2 days (T_2).

impact mode at 70 eV. Compounds were identified by comparison with NIST reference spectra. Mass range was 30–350 amu, solvent delay time was 4 min, threshold 150 and scan rate 4.45 scan/s.

3. Results and discussion

3.1. Electronic nose results

In the case of sensor array measurements, the electrical signals of the sensors were acquired for many times in order to evaluate the repeatability of the measurement and in order to have a sufficient amount of data useful for the subsequent analysis. The response of the sensors was defined as $I_{headspace}/I_0$, where $I_{headspace}$ is the electrical current under exposure to the wheat headspace after 15 min and I_0 is the electrical current in the reference gas flow. An evaluation of the data were performed with principal component analysis (PCA). The pre-processing of the data consisted of a linear normalization performed in order to remove as much as possible any concentration effects. Then the data set was autoscaled (zero mean and unitary variance). PCA score plot of the whole set of data is reported in Fig. 3 where closed symbol represent inoculated wheat sample (T_0, T_1, T_2, T_5 and T_7) while opened symbol represent the controls, i.e. not contaminated samples at the same day (BT_0, BT_1, BT_2, BT_5 and BT_7). It can be observed the capability of the system to discriminate among clusters. Not contaminated samples seems to be very similar excepted for the first one (BT_0). This phenomenon could be due to the small evolution of the not contaminated wheat samples during time. Clusters related to inoculated wheat samples at different days are separated. Clear trend could be find in this case. In fact, each samples is very close to its control, moreover samples of the same day of inoculation (T_0) is far from samples after 1 day (T_1) and 2 days (T_2), that are close each other and very far from T_5 and T_7. Superimposition between T_5 and T_7 cluster data can be observed. This phenomenon could be due to a high similarity between samples T_5 and T_7 due to anoxia conditions that could limit the fungal growth.

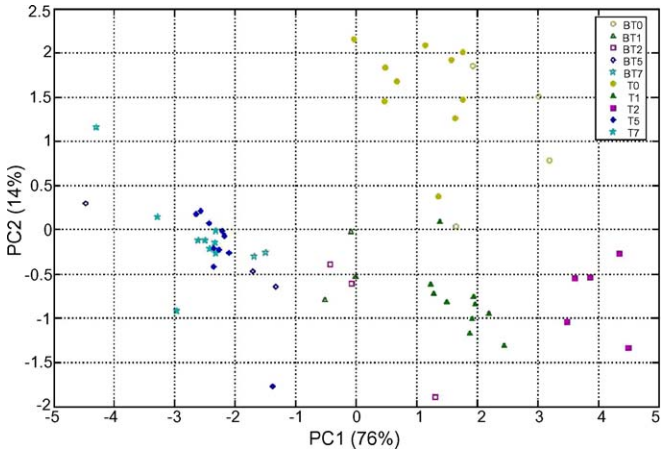

Fig. 3. PCA score plot of EN analysis of contaminated and not contaminated samples during the time. (T_0 = zero time, T_1 = after 1 day, T_2 = after 2 days, T_5 = after 5 days, T_7 = at 7 days for contaminated samples; BT_0 = zero time, BT_1 = after 1 day, BT_2 = after 2 days, BT_5 = after 5 days, BT_7 = at 7 days for not contaminated samples.)

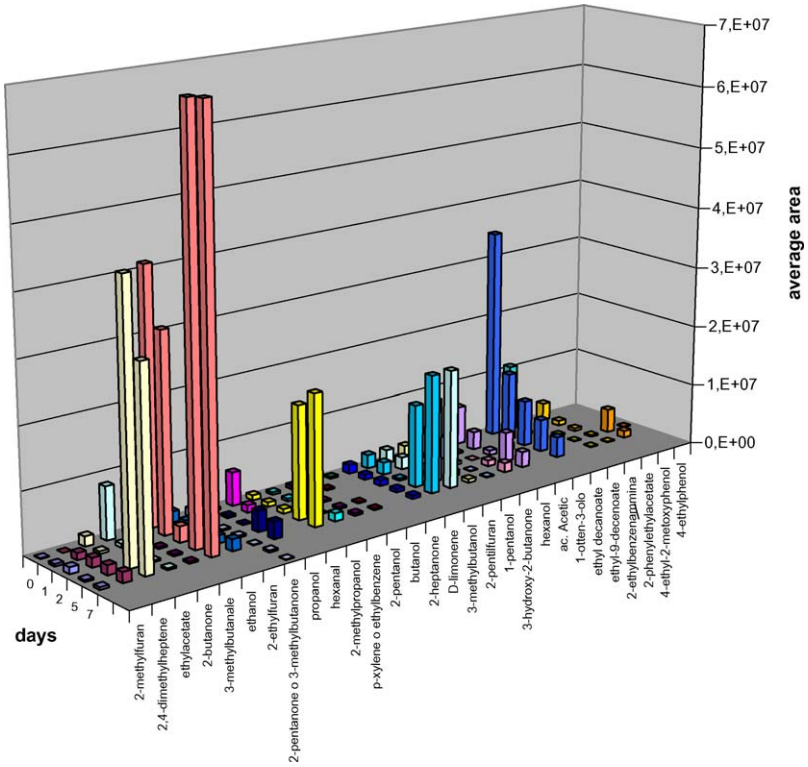

Fig. 4. Histogram of main volatile compounds in contaminated wheat samples.

3.2. GC-MS Results

In vitro studies have been carried out to determine some volatiles produced by grain spoilage fungi. *F. poae* fungi has been grown on sterilized wheat and GC-MS was used to quantify the key volatile compounds produced. A range of classes of volatile compounds including alcohols, carbonyls and hydrocarbons have been identified.

Volatile compounds has been compared between contaminated samples and no fungi inoculated samples. There were a lot of differences, in fact the concentrations of some analytes grew during days after the inoculation of fungi, others decreased. In Fig. 4 the histogram of the significant volatiles compounds has shown.

The plot of average area of chromatographic peak towards days is in Fig. 5, where can be point out the increasing of the concentration of 2-methylfuran during the second day. This compound has been also found in the case of the contamination of other fungi like *E. amstelodami*, *Aspergillus flavus* and *P. cyclopium*. [13].

In the analysed samples contaminated by *F. poae* fungi, we found an increasing of the ethyl acetate concentration as occurs in contamination by *Fusarium culmorum* fungi. Moreover, in contaminated wheat by *A. flavus* and *E. amstelodami* and *Penicilium verrucosum* one of key volatile compound is 2-methyl propanol like in *F. poae*. In Fig. 6 we can highlight the different concentration of this compound between contaminated samples and reference samples during the time.

Aromatic compounds have been also identified in *F. poae* fungi contaminated sample in higher concentration as compared to not contaminated samples like in some cereals by *F. poae* fungi contamination [13]. The increase of aromatic compounds by *E. amstelodami* contamination was particularly evident at the seventh day since the inoculation was made. All compounds belonging to the class of alcohols like ethanol, 3-methylbutanol [14,15], hesanol [16], 2-methylpropanol and propanol after 5 days, increased in contaminated samples rather than in not contaminated wheat. Pentanol and butanol were not included in this classification because they had lower area peak average in comparison with not contaminated samples.

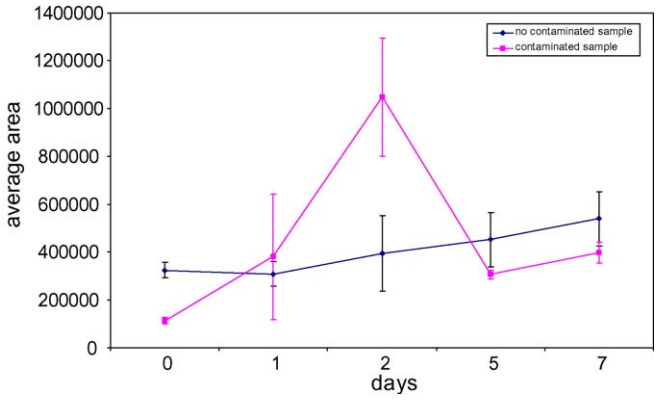

Fig. 5. Comparison between 2-methylfuran concentration in no contaminated samples and contaminated ones during days after inoculation of fungi.

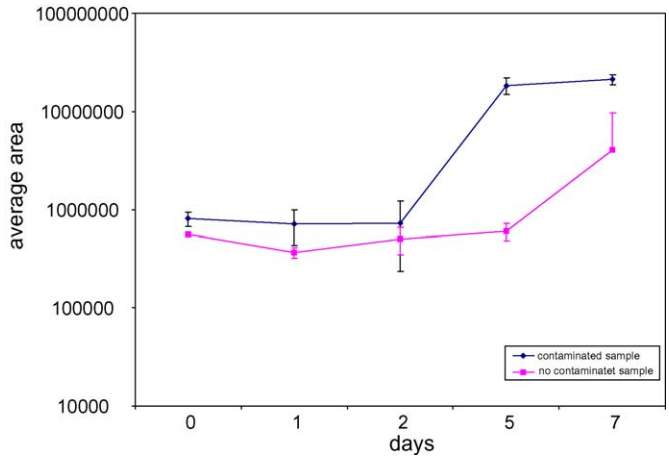

Fig. 6. Comparison between 2-methylpropanol concentration in no contaminated samples and contaminated ones during days after inoculation of fungi.

Other contaminants, 2,4-dimethylepten and 1-octen-3-ol [14,15], were found only in contaminated wheat due probably to some more favourite metabolic pathway inside fungi damaged wheat than healthy samples. 2,4-Dimethylepten has been not indicated as contaminant in other fungal activity on wheat and so this can be a key compound for the determination of *F. poae* contamination.

On the contrary, other classes of volatile compounds like carbonyls (2-butanone, 3-methylbuthanal, 3-hydroxy-butanone, hesanal and 2-heptanone) seemed to be inhibited by the presence of *F. poae* contamination.

Therefore volatile compounds have been determined by GC-MS analysis, highlighting the occurrence of contamination or not. This behaviour is clearly shown in PCA score plot where there is a separation of clusters (Fig. 7) during the time after inoculation. In this figure, contaminated sample were well dis-

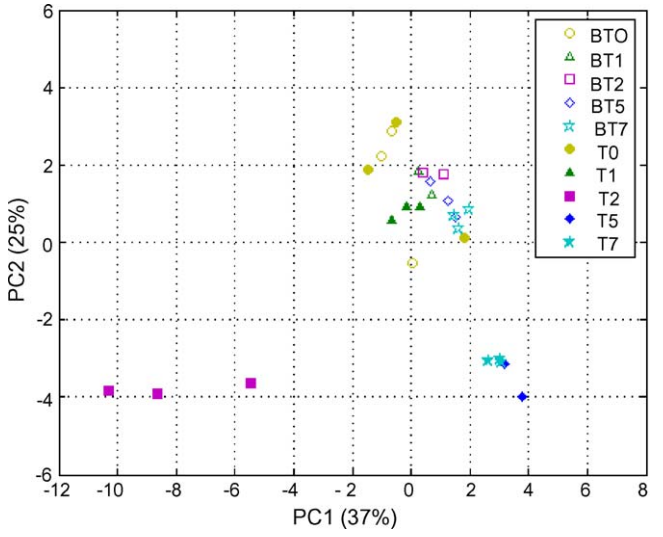

Fig. 7. PCA score plot of SPME-GC-MS analysis of contaminated samples and not contaminated samples during the time. (T_0 = zero time, T_1 = after 1 day, T_2 = after 2 days, T_5 = after 5 days, T_7 = at 7 days for contaminated samples; BT_0 = zero time, BT_1 = after 1 day, BT_2 = after 2 days, BT_5 = after 5 days, BT_7 = at 7 days for not contaminated samples).

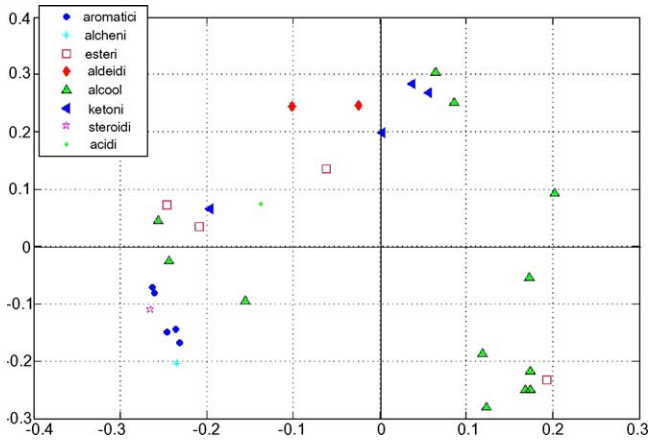

Fig. 8. Loading plot of analytical recognized substances.

criminated and, in particular, T_2 contaminated samples were sharply separated from the other contaminated and not contaminated samples. Not contaminated samples could be grouped in a big lengthened cluster. This could be due to wheat aging phenomena. Moreover, T_0 and T_1 samples were closer to not contaminated clusters and this effect seems to be related to the similarity of just contaminated and not contaminated samples. In the score plot T_5 and T_7 clusters are overlapped, the samples seems to be analogous (a good match) probably caused by fungi degradation.

4. Discussion

Fig. 8 shows the loading plot of analytical recognized substances. By comparing these results with the corresponding PCA score plot reported in Fig. 7, it is possible to identify the substances families mainly related to the T_2 sample, i.e. aromatics (2-methylfuran, 2-ethylbenzenamine, *p*-xylene (*o*-ethylbenzene)), an alkene, (2,4-dimethylepten) while in the case of T_5 and T_7 samples, the mainly related substances families are some other alcohols (2-methyl propanol, propanol, 3-methylbutanol, 4-ethyl-2-metoxiphenol) and a particular ester (ethyl acetate). Moreover, some other substances seem to be related to the possible aging effect of not contaminated wheat samples. In particular, they are alcohols and chetons (2-butanone, 2-eptanone, butanol, 1-pentanol).

By considering the overall set of data deriving from the both GC-MS analysis and electronic nose results, it is possible to find some conclusions. In fact, both PCA score plots show the same structure of data that means the same discrimination capability among clusters. The partially selective sensors array was able to cover the whole complex range of substances in the headspace samples, as analytically detected by GC-MS. Moreover, even if with preliminary, very limited set of measurements, the electronic nose reached discrimination level comparable GC-MS results.

5. Conclusions

An analytical method has been used for the determination of volatile compounds in *F. poae* contaminated wheat by SPME-GC-MS. An array of gas sensors has been suitable set for the analysis of the same samples with the aim to separate and discriminate inoculated wheat samples during fungal growth. It has been shown that both techniques allow to discriminate inoculated and reference samples during 7 days. Most differences have been highlighted between T_2 and T_5–T_7 samples, after 2 days and after 5–7 days from the inoculation. In fact, they are the crucial stages of fungal growth.

The results show a very good discrimination capability of the electronic nose, that seems to be very promising for the use of this kind of system in food safety control application.

Study is in progress for other species of toxigenic fungi (*Fusarium graminearum*, *F. avenaceum*, *A. flavus*, *A. ochraceus*, *P. verrucosum*) in order to verify the capability of the EN to discriminate different strain of fungi through the production of volatile secondary metabolites and to demonstrate the variation of the metabolic pathway due to the contamination of grain.

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