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ARE VOCS AN EFFICIENT WAY TO DETECT FUNGAL DEVELOPMENT IN MAIZE GRAINS?

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Abstract

The objective of grain storage control is to ensure a secure supply of food industries both in quantity and quality between two harvests. Despite efforts to monitor and control the environment, there are still risks of material and/or quality loss that can reach 50% of the total value of the harvest in developing countries, and between 5 and 10% in industrialized countries. In industrialized countries, economic losses are more associated with deterioration of the sanitary and technological quality of grain than with losses of material. Control of this quality is a real challenge, especially during the storage phase. Among other factors, fungal contamination and subsequent mycotoxins production present serious problems in terms of diminishing grain quality and health risks for consumers.

It has been shown that moulds emit Volatile Organic Compounds (VOCs) when they colonize a substrate, these compounds being detectable before the infestations are visible. The fungal contamination Index (FCI) was initially developed by CSTB to monitor early fungal developments in different indoor environments. This study aims to test the feasibility to use VOCs emission and especially FCI to detect fungal growth on maize grains during storage.

First results allowed the characterization of specific VOC profile of maize, with more than 100 different VOC emitted during grain storage, in laboratory conditions. Furthermore, results clearly demonstrated that the development of fungi following artificial contamination or development of mycoflora initially present on grains led to the emission of a different pattern of VOCs.

Key words: grain infestation, grain storage, monitoring tool, fungal contamination, early detection, VOC profile

1. INTRODUCTION

There is an increasing demand for safe and high-quality food worldwide (Weinberg et al. 2008). Cereals represent one of the most important sources of food (FAO 2002), and cereal-based foods are a staple food, representing the major source of energy, B vitamins and minerals in many countries (McKevith 2004). However, the nutritional value of stored cereals may vary significantly due to interactions with biological, chemical and physical factors. Moreover, fungal contamination (moulds) can lead to the accumulation of mycotoxins in cereals and the presence of these toxic compounds constitutes a major health problem for both human and animals (Krnjaja et al. 2013).

Controlling the conditions during grain storage is therefore essential. Despite efforts to monitor and control the environment, there are still risks of loss of material and / or quality that can reach 50% of the crop in developing countries, and 5 and 10 % of the total value of the harvests in industrialized countries (Yigezu et al. 2010). In general, in industrialized countries, losses are primarily economic and associated

with deterioration of the sanitary and technological quality of the grains: loss of germination capacity, abnormal flour, appearance changes and nutritional degradation which are mainly due to fungal and insects infestation.

Wheat and maize are the main cereal crops with 725 and 989 million tons respectively produced worldwide in 2014 (Statista 2015; Statista 2014). They represent 69% of world cereal production. In the EU, maize accounts for 23.4% of total cereal production (Passion Céréales 2016). Moreover, the demand for maize is increasing (Suleiman et al. 2013; Moreno- Martinez et al. 2011) for its multipurpose, as human food, animal feed, raw material for starch and ethanol production (Weinberg et al. 2008; FAO 2011; Suleiman et al. 2013).

When grains are ensiled after harvest, they are not sterile and already carry a certain number of microorganisms, such as bacteria, yeasts, moulds. Their development during the storage will depend on environmental conditions and more specially moisture and temperature. When grains are stored at a relative humidity lower than 70% (a water content of about 14%), microorganisms do not grow.

Maize grains are hygroscopic by nature and tend to absorb the moisture (Suleiman et al. 2013). Together with their relatively high starch contents they are favorable material for mould growth (Weinberg et al. 2008). Moreover, insects increase moisture content and temperature providing better conditions for mould development, and they damage the grain which facilitates availability of nutrients to the mould (Ullah et al. 2010). The relative humidity can be controlled by drying of grains before storage, and the temperature is lowered thanks to the cooling ventilation. However, the heterogeneity of the moisture content of the grains at harvest, the presence of impurities that are wetter than the grains, insufficient drying or rehumidification of the grains due to condensation phenomenon during storage, can create, locally, a favorable environment for fungal and/or insects development (Magan and Aldred 2007). Fungal development can result in mycotoxin production. Mycotoxins are secondary metabolites produced during fungal growth and pose a serious health risk to both humans and animals (Bennett and Klich 2003; Jarvis and Miller 2005; Reboux 2006).

Nowadays, monitoring of stored grains is ensured by thermometry. The first purpose of this measure is to control the cooling ventilation. Moreover temperature monitoring is an indirect indicator of a recovery of biological activity. However, due to the low heat conduction of the grain, quality monitoring through this process requires a very tight mesh of the sensors: the temperature should be measured every 50 cm (Neethirajan and Jayas 2007). Storage silos are not equipped for surveillance of this nature. Also, when heating is detected, the deterioration of the quality is already important, limiting the effectiveness of the corrective measures. Therefore, an essential control point to limit the contamination of raw materials is early detection. The development of "real-time" detection systems for mould contamination of grains during storage could thus improve safety and quality of the product, while minimizing losses.

It has been shown that moulds emit volatile organic compounds (VOCs) during colonization of a substrate, these compounds being detectable before the infestation become visible (Fleurat-Lessard 2017). These VOCs are linked to both primary and secondary metabolism since formed from a wide variety of starting compounds, e.g., acetate, amino acids, fatty acids, and keto acids (Schnürer et al. 1999). These molecules can be used for detection and identification after characterization by gas chromatography, mass spectrometry, and sensory analysis. They could represent a fingerprint for rapid and early detection of mould growth on stored grains.

In order to detect the presence of moulds in indoors, Fungal Contamination Index (FCI) was developed by the French Scientific and Technical Centre for Building (CSTB) in previous works (Stephane Moularat et al. 2008; Stéphane Moularat et al. 2008a; Stéphane Moularat et al. 2008b). This index determines the presence or the absence of fungal development thanks to the detection of specific VOCs. This FCI was tested and validated in different indoor environments like dwellings, libraries, archives, the Louvre Museum... (Hulin et al. 2013; Joblin et al. 2010; Moularat et al. 2011). This technology was implemented into a beacon, dedicated to the surveillance of closed spaces sensitive to the fungal infestation.

The aim of this study was to evaluate the possible extension of the application of developed surveillance system to cereal industry in order to early detect fungal development during grain storage.

2. MATERIALS AND METHODS

2.1. Materials

Fungal strains. Two strains of *Penicillium verrucosum* were used for this study. *Penicillium verrucosum* NRRL 965 was isolated from cereal grains (Frisvad et al. 2005). *Penicillium verrucosum* MRI 555 strain (Max Rubner Institut, Germany) was chosen as capable of producing ochratoxin A. *Aspergillus brasiliensis* IHEM 05077 (Institute of Hygiene and Epidemiology, Brussels) was used as a reference strain for FCI (Anton et al. 2016). All strains were maintained in the laboratory on MEA (Vegi and Wolf-Hall 2013) at 4°C and were regularly checked for viability by culturing on MEA.

Maize grains. Maize for animal feed “Gasco” (lot25616, 5kg, Ref. 810122, 32300 Mirande, France) was used in experiments. This is Non-GMO maize produced in South West of France; it has been sorted, brushed and dusted.

2.2. Methods

2.2.1. Artificial fungal contamination of maize

Before fungal contamination, maize grains were firstly sterilized (121 °C, 20 min, moist heat) in order to control precise fungal contamination and then humidified by addition of deionized water 1: 1 (30 mL for 30 g of grains, per flask) to maintain the moisture level at saturation throughout the test. Sterile and humidified maize was placed into flasks especially designed for VOC measurements. Two strains of *P. verrucosum* were grown on MEA for 14 days at 25°C to obtain highly sporulating cultures. Spores were harvested by covering the plate with 10 mL of Tween 80 (0.05%). Spore concentration was measured by direct counting on Malassez cell (Micrograve precis, Briare, France). Spore suspensions were then diluted in order to obtain the needed concentration of 10⁸ spores/mL, and contamination was achieved by applying dropwise 3 mL of latter suspensions on previously prepared maize. For each strain, two samples (sterilized and then contaminated maize) and one control (sterilized maize) were prepared. Contaminated flasks were incubated for 14 days at 25°C in darkness. After incubation, fungal growth was confirmed by visual examination of samples.

2.2.2. Fungal contamination of maize by intrinsic mycoflora

Non-sterilized maize was left in the environment with moisture level at saturation during 14 days, at room temperature in darkness, to spontaneously take the water. Final moisture content of maize was 11%. During the incubation time contamination occurred by intrinsic fungal microflora initially present on grains. After incubation period VOC measurements were performed. In parallel, one control, consisted of non-sterilized and non-humidified maize (and without visible contamination), was analyzed.

2.2.3. Experimental system

The experimental system was developed in CSTB during previous studies (Joblin et al. 2010; Stephane Moularat et al. 2008; Stéphane Moularat et al. 2008a) (Figure 1). System is consisted of a glass flask (made by measure, Belleville-France), 300 ml volume, in which sample was placed. Before use, flasks were sterilized by moist heat and completely dried. During incubation time gaseous emissions are liberated in the flask space and then captured during air sampling. In order to eliminate other possible sources of VOC emission during air sampling, a purification line composed of HEPA filters (Polycap 75–0.2 lm, Whatman, Versailles- France), active charcoal (Europe Environment, Mulhouse-France) and tenax tube (Tenax TA) was sited on the input of the flask to ensure renewal of the air with air free of VOCs. VOC emissions in each flask were collected on an absorbent cartridge (Tenax TA), situated at the output of this flask. Sampling was provided by a pump (Vacuum/Pressure station 7059-60, Air Cadet, USA) coupled to a flowmeter (R2-15-AA, Brooks), in order to maintain flow rate of 100 mL/min, during 30 min. All connections (pipes, connectors) were made of VOC non-emitting PTFE. These sampling conditions made it possible to collect all the compounds emitted during the successive phases of mould development (germination, mycelial development and spore formation) and during grain alternation caused by fungal development, as well as VOCs emitted by grains themselves. A qualitative analysis of all VOCs present in the headspace gas above a sample in the flask was performed.

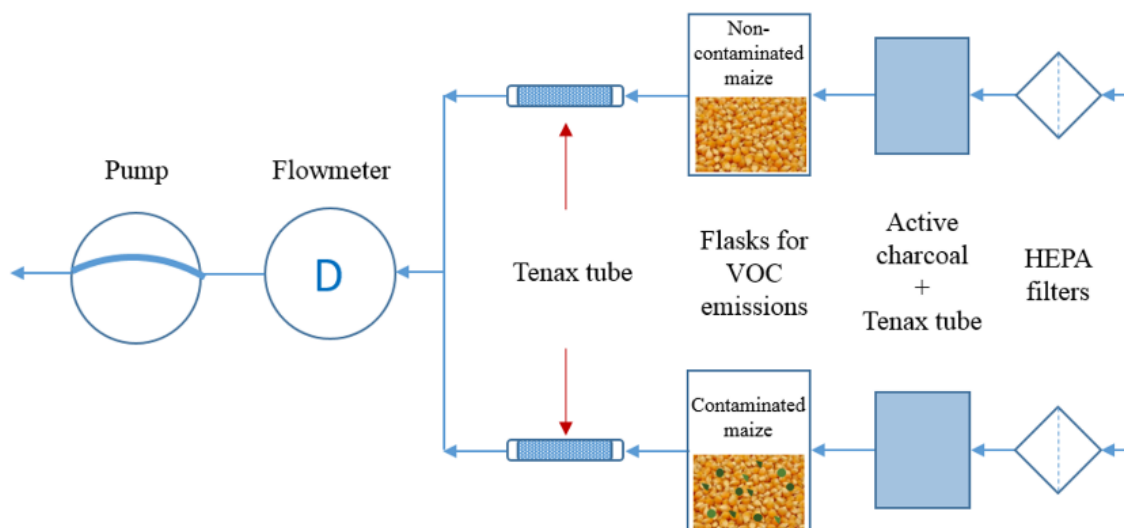


Fig 1. Scheme of the experimental assembly (VOC sampling chain)

2.2.4. Diffusion of different VOCs through maize

In order to evaluate if VOCs emitted by fungi are then adsorbed by humid maize, air rich in exactly same VOC profile (type and quantity) went simultaneously through flask filled with maize and through an identical but empty flask (control). Specific VOC profile was obtained from a flask previously contaminated with fungi commonly found in indoor environment and not found as contaminant during grain storage. Two coupons of sterilized painted fiberglass wallpaper (2x5cm) were placed in the flask on a layer of 2 cm of sterile glass beads and 5 mL of sterile water to maintain moisture level at saturation throughout the incubation period of 30 days at 25 °C. Materials were inoculated with *Aspergillus brasiliensis* IHEM 05077. VOCs were collected as previously described (2.2.3.). In order to approach conditions to the real situation moisture content of maize was adjusted. The moisture content of maize was 5.9% and in order to approach conditions to the real ones, maize was left in the environment with moisture level at saturation for 14 days, at room temperature, to spontaneously take the water. Final moisture content of maize was 11%.

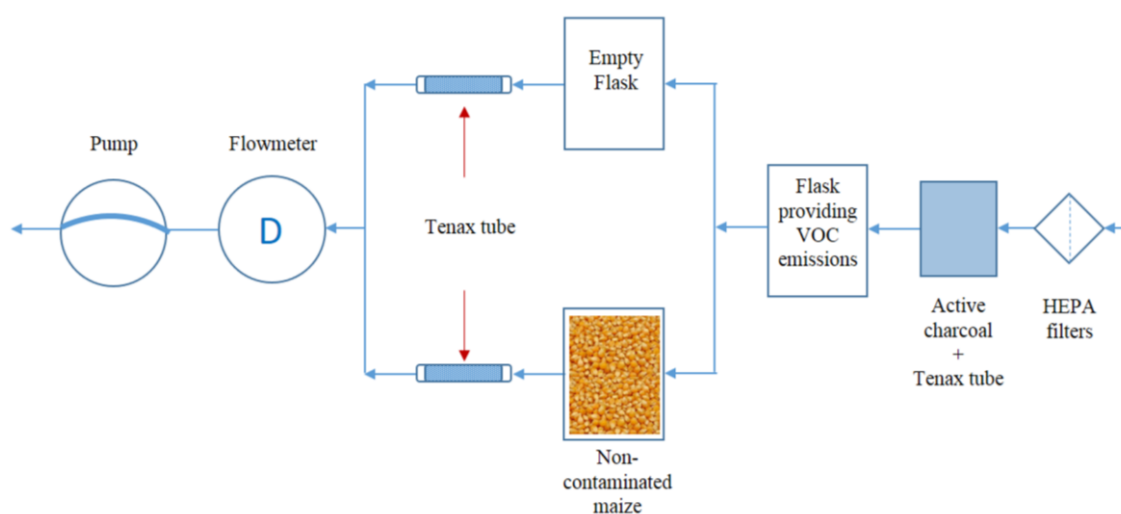


Fig 2. Scheme of the experimental assembly for evaluation of VOC diffusion

2.2.5. GC-MS Analysis of volatile organic compounds

Samples were analyzed using an analytical chain composed of a gas chromatography GC–MS (6890 Gas Chromatography (GC) equipped with a 5973 Mass Spectrometry (MS) detector, Hewlett Packard, USA). Detailed method was described elsewhere (Joblin et al. 2010; Stéphane Moularat et al. 2008; Stéphane Moularat et al. 2008a). Briefly, desorption was performed at 260 °C for 15 min (ATD650, Perkin Elmer, Courtaboeuf-France). A BPX5-SGE capillary column was used (length = 60 m, ID = 0.25 mm, film thickness = 1 μm, 5% phenyl polysilphenylene-siloxane). The carrier gas is helium (>99.995%) at a flow rate of 0.5 mL/min. The mass spectrometer, configured for electronic impact (70 eV) was used in scan mode (m/z between 35 and 425). Compounds were identified by comparison with a spectral library (National Institute of Standards and Technology – NIST 2014). The limit of detection of VOC (LD), determined on blank cartridges, was estimated at 0.67 ng of toluene equivalent (n=15). The solid absorbent used to sample VOCs (Tenax TA) limited sampling to volatile organic compounds between C4 and C20.

3. RESULTS AND DISCUSSION

3.1.1. Comparative analysis of chemical emissions of various fungal contaminations

The first step in this study was to determine specific VOCs profiles of fungal contamination. All the compounds emitted during fungal development and during grain alternation caused by fungal development, as well as VOCs emitted by grains themselves were collected. After incubation period of 14 days a qualitative analysis of all VOCs present in the headspace gas above samples was performed.

The systematic screening of VOCs emitted during different fungal growths on maize showed that the identified compounds belong to many chemical families like carboxylic acids, esters, alkenes, alkanes, aldehydes, alcohol, amino and cyclic molecules, which is in accord with already published studies (Magan and Evans 2000). More than 190 different VOCs were detected and identified during this study. The VOCs emitted exclusively from the contamination-free media have been excluded.

Table below (table 1) lists all the VOCs that were emitted by maize (sterilized and non-sterilized), during development of *Penicillium verrucosum* on maize and/or during development of intrinsic microflora already presented on maize.

N°	Voc analyte (iupac name)	Maize (non-sterilized, non-contaminated)	Maize (non-contaminated, sterilized)	Maize (sterilized) contaminated with <i>Penicillium verrucosum</i>		Maize contaminated with intrinsic microflora
				MRI 555	NRRL 965	
1	butan-2-one	+	+	+	+	+
2	3-methylbutanal	+	+	+	+	+
3	benzene	+	+	+	+	+
4	toluene	+	+	+	+	+
5	oct-1-ene	+	+	+	+	+
6	heptanal	+	+	+	+	+
7	4,6,6-trimethylbicyclo[3.1.1]hept-3-ene	+	+	+	+	+
8	2-pentylfuran	+	+	+	+	+
9	1-phenylethanone	+	+	+	+	+
10	nonanal	+	+	+	+	+
11	decanal	+	+	+	+	+
12	undecanal	+	+	+	+	+
13	dodecanal	+	+	+	+	+
14	6,10-dimethyl-5,9-undecadien-2-one	+	+	+	+	+
15	propan-2-one	-	+	+	+	+
16	pentane	-	+	+	+	+
17	butane-2,3-dione	-	+	+	+	+
18	2-methylfuran	-	+	+	+	+
19	1,4-xylene	-	+	+	+	+
20	heptan-2-one	-	+	+	+	+
21	styrene	-	+	+	+	+
22	benzoic acid	-	+	+	+	+
23	pentan-2-one	+	-	+	+	+
24	3-methylbutan-1-ol	+	-	+	+	+
25	2-methylbutan-1-ol	+	-	+	+	+
26	1,3-benzothiazole	+	-	+	+	+
27	dodecanal	+	-	+	+	+
28	tetradecane	+	-	+	+	+
29	hexane	-	-	+	+	+
30	3-methylbutan-2-one	-	-	+	+	+
31	3-methylbut-3-en-1-ol	-	-	+	+	+
32	2-methylpentan-3-one	-	-	+	+	+
33	1-chloropentane	-	-	+	+	+
34	ethyl 2-methylpropanoate	-	-	+	+	+
35	hexan-2-one	-	-	+	+	+
36	octane	-	-	+	+	+
37	octa-1,3-diene	-	-	+	+	+
38	2-methylcyclopentan-1-one	-	-	+	+	+
39	1,2-xylene	-	-	+	+	+
40	2,6-dimethylpyrazine	-	-	+	+	+
41	2,7-dimethyloxepine	-	-	+	+	+
42	3-ethylcyclopentan-1-one	-	-	+	+	+
43	oct-1-en-3-ol	-	-	+	+	+
44	5-methylheptan-3-one	-	-	+	+	+

45	dec-2-en-1-ol	-	-	+	+	+
46	pentadecan-3-yl 2,2,2-trifluoroacetate	-	-	+	+	+
47	1-ethenyl-4-methoxybenzene	-	-	+	+	+
48	3-methylcyclohexan-1-ol	-	+	-	+	+
49	pentan- 3-one	-	-	-	+	+
50	methylsulfanylethene	-	+	+	-	+
51	pentadecane	-	+	+	-	+
52	methyl acetate	+	-	+	-	+
53	pentan-2-ol	+	-	+	-	+
54	1-chlorodecane	+	-	+	-	+
55	propyl acetate	-	-	+	-	+
56	anisole	-	-	+	-	+
57	2,6,10,15-tetramethylheptadecane	-	-	+	-	+
58	hentriacontane	-	-	+	-	+
59	1-decylsulfonyldecane	-	-	+	-	+
60	pentanal	+	+	-	-	+
61	hexanal	+	+	-	-	+
62	2-ethoxypropan-1-ol	+	-	-	-	+
63	4-methyloxolan-3-one	+	-	-	-	+
64	2-pentyloxirane	+	-	-	-	+
65	6-methylhept-5-en-2-one	+	-	-	-	+
66	penta-1,3-diene	-	-	-	-	+
67	dimethoxymethane	-	-	-	-	+
68	nitromethane	-	-	-	-	+
69	2-methylbuta-1,3-diene	-	-	-	-	+
70	2-methylpropanenitrile	-	-	-	-	+
71	2-bromopropane	-	-	-	-	+
72	but-2-enenitrile	-	-	-	-	+
73	4,4-dimethyloxetan-2-one	-	-	-	-	+
74	thiophene	-	-	-	-	+
75	methyl 2-methylprop-2-enoate	-	-	-	-	+
76	2-butenic acid, methyl ester, (z)-	-	-	-	-	+
77	2-methoxy-2-methylbut-3-ene	-	-	-	-	+
78	methyl but-2-enoate	-	-	-	-	+
79	pentane-2,4-dione	-	-	-	-	+
80	3-methylbut-2-enal	-	-	-	-	+
81	4,4-dimethylpent-2-ene	-	-	-	-	+
82	methyl 2-methylbut-2-enoate	-	-	-	-	+
83	methyl 3-methylbut-2-enoate	-	-	-	-	+
84	nona-1,3-diene	-	-	-	-	+
85	propylbenzene	-	-	-	-	+
86	2-phenylpropan-2-ol	-	-	-	-	+
87	2-methoxypropan-2-ylbenzene	-	-	-	-	+
88	3,7-dimethylocta-1,6-dien-3-ol	-	-	-	-	+
89	1-chloro-2-methoxybenzene	-	-	-	-	+
90	2-isopropylphenyl pentyl oxalate	-	-	-	-	+
91	hentriacontane	-	-	-	-	+
92	octylcyclohexane	-	-	-	-	+
93	unknown 1	-	-	-	-	+

94	unknown 2	-	-	-	-	+
95	unknown 3	-	-	-	-	+
96	1-chlorohexadecane	-	-	-	-	+
97	benzaldehyde	+	+	+	+	-
98	1,1,3,3,5,5-hexamethyltrisiloxane	+	+	+	+	-
99	1-methyl-4-prop-1-en-2-ylcyclohexene	+	+	+	+	-
100	undecane	+	+	+	+	-
101	methylsulfanylmethane	-	+	+	+	-
102	(methylsulfanyl)methane	-	+	+	+	-
103	1,1,2,2-tetrachloroethene	-	+	+	+	-
104	furan-3-carbaldehyde	-	+	+	+	-
105	1,6-dimethylhepta-1,3,5-triene	-	+	+	+	-
106	4-hydroxybenzaldehyde	-	+	+	+	-
107	decylphosphonic acid	-	+	+	+	-
108	phthalic acid	-	+	+	+	-
109	pentadecan-3-yl 2,2,2-trifluoroacetate	-	+	+	+	-
110	dodecan-3-yl 2,2,2-trifluoroacetate	-	+	+	+	-
111	propan-1-ol	+	-	+	+	-
112	2,3-dimethylbutane	+	-	+	+	-
113	2,4-dimethylheptane	+	-	+	+	-
114	propan-2-ol	-	-	+	+	-
115	2-methyltetrahydrofuran	-	-	+	+	-
117	3-methylhexanal	-	-	+	+	-
118	2-propylfuran	-	-	+	+	-
119	4,4-dimethylpent-2-ene	-	-	+	+	-
120	2-methyldihydro-3(2h)-furanone	-	-	+	+	-
121	methylpyrazine	-	-	+	+	-
122	2,4-dimethylhept-1-ene	-	-	+	+	-
123	undecan-2-ol	-	-	+	+	-
124	dec-3-yn-2-ol	-	-	+	+	-
125	2-methyl-3-(methylsulfanyl)furan	-	-	+	+	-
126	6-methylheptan-2-one	-	-	+	+	-
128	1-methyl-4-propan-2-ylcyclohexa-1,3-diene	-	-	+	+	-
129	4,7,7-trimethylbicyclo[4.1.0]hept-3-en-5-ol	-	-	+	+	-
130	2,2,6-trimethylcyclohexan-1-one	-	-	+	+	-
131	2-phenylacetaldehyde	-	-	+	+	-
132	dodec-2-enal	-	-	+	+	-
133	tridecan-3-yl 2,2,2-trifluoroacetate	-	-	+	+	-
134	1-methyl-4-propan-2-ylidenecyclohexane	-	-	+	+	-
135	1,1,2-trimethyl-3-(2-methylprop-1-en-1-ylidene)cyclopropane	-	-	+	+	-
136	2-butylcyclohexan-1-ol	-	-	+	+	-
137	2-methylidene-5-prop-1-en-2-ylcyclohexan-1-ol	-	-	+	+	-
138	2,6,6-trimethylcyclohexa-1,3-diene-1-carbaldehyde	-	-	+	+	-
139	2,6,6-trimethylcyclohexene-1-carbaldehyde	-	-	+	+	-
140	1,4,6-trimethyl-1,2-dihydronaphthalene	-	-	+	+	-
141	4-(2,6,6-trimethyl-2-cyclohexen-1-yl)-3-buten-2-one	-	-	+	+	-
142	4-(2,6,6-trimethyl-1-cyclohexen-1-yl)-butan-2-one	-	-	+	+	-
143	2-[(1s,5r)-1,8-dimethylspiro[4.5]dec-8-en-4-yl]propan-2-ol	-	-	+	+	-
144	1,1,4a-trimethyl-5,6-dimethylenedecahydronaphthalene	-	-	+	+	-

145	methyl (3-oxo-2-pentylcyclopentyl)acetate	-	-	+	+	-
146	pent-2-en-1-ol	-	-	-	+	-
147	2,5-dimethylfuran	-	-	-	+	-
148	2,4-dimethylfuran	-	-	-	+	-
149	ethyl pentanoate	-	-	-	+	-
150	3,4-dimethylphenol	-	-	-	+	-
151	4-ethyloct-1-yn-3-ol	-	-	-	+	-
152	1-ethyl-4-methoxybenzene	-	-	-	+	-
153	7,11-dimethyl-3-methylidenedodeca-1,6,10-triene	-	-	-	+	-
154	(2r)-6-methyl-2-[(1r)-4-methylcyclohex-3-en-1-yl]hept-5-en-2-ol	-	-	-	+	-
155	2,6-bis(1,1-dimethylethyl)-2,5-cyclohexadiene-1,4-dione	+	+	+	-	-
156	but3-en-2-one	-	+	+	-	-
157	2,3-dimethylcyclohexan-1-ol	-	+	+	-	-
158	3,7-dimethylocta- 2,6-dienal	-	+	+	-	-
159	3-phenylfuran-2,5-dione	-	+	+	-	-
160	1,4-phenylenebis(trimethylsilane)	-	+	+	-	-
161	2,6,11-trimethyldodecane	+	-	+	-	-
162	2,6,10-trimethyltetradecane	+	-	+	-	-
163	heptacosane	+	-	+	-	-
164	1-(2-furanyl)propan-2-ol	-	-	+	-	-
165	3-methylpentan-2-one	-	-	+	-	-
166	hexan-3-one	-	-	+	-	-
167	prop-2-enyl propanoate	-	-	+	-	-
168	(3,5-dimethylphenyl) n-methylcarbamate	-	-	+	-	-
169	propan-2-yl pentanoate	-	-	+	-	-
170	2-ethylcyclopentan-1-one	-	-	+	-	-
171	2,5-dimethylhexan-3-ol	-	-	+	-	-
172	2-methyl-1-propan-2-ylsulfanylpropane	-	-	+	-	-
173	oxan-2-one	-	-	+	-	-
174	5-methyl-2-prop-1-en-2-ylhex-4-enal	-	-	+	-	-
175	3-methylbenzaldehyde	-	-	+	-	-
176	pentyl pentanoate	-	-	+	-	-
177	2,4-dimethylcyclohexanol	-	-	+	-	-
178	nonane-2,4-dione	-	-	+	-	-
179	2,2,7-trimethyl-octa-5,6-dien-3-one	-	-	+	-	-
180	4,5,7-trimethyl-1,2-dihydronaphthalene	-	-	+	-	-
181	2,2-dimethyldeca-3,5-diyne	-	-	+	-	-
182	tridecan-1-ol	-	-	+	-	-
183	n,n-dibutylformamide	-	-	+	-	-
184	1-o-hexadecyl 2-o-prop-2-enyl oxalate	-	-	+	-	-
185	dodec-2-enoic acid	-	-	+	-	-
187	4-tridecanyl valerate	-	-	+	-	-
188	4-[(1r)-2,2-dimethyl-6-methylidencyclohexyl]butan-2-one	-	-	+	-	-
189	2-methylhexadecan-1-ol	-	-	+	-	-
190	6-methyl-2-(3-methyl-1-cyclohex-3-enyl)-5-hepten-2-ol	-	-	+	-	-

Table 1. VOCs emitted by contaminated or non-contaminated maize (not identified components are labelled « unknown ») (presence of one component is noted with « + » and its absence with « - »)

Analysis of emissions revealed 30 VOCs that were emitted exclusively by both strains of *Penicillium verrucosum*. Moreover, there were 28 VOCs that were specific to the contamination of maize by *Penicillium verrucosum* MRI 555 and 9 specific to the contamination by *Penicillium verrucosum* NRRL 965. This shows that those strains, even if belonging to the same species, have certain metabolic differences. It has been already published that different fungal strains have slight difference in VOC profiles (Wilkins et al. 2003; Jelen and Grabarkiewicz-Szczęśna 2005; Polizzi et al. 2009), which was confirmed for *P. verrucosum* as well (Pasanen et al. 1996). Also there are VOCs that were 31 VOCs specific just to development of intrinsic microflora already presented on maize.

Interestingly, the analysis of emissions showed that among more than 190 detected compounds, there are 19 VOCs in common for fungal contamination, independently of its source (table 2) while absent in all control samples.

N°	VOC analyte (IUPAC name)	Chemical taxonomy
1	dec-2-en-1-ol	alcohol
2	oct-1-en-3-ol	alcohol
3	3-methylbut-3-en-1-ol	alcohol
4	octa-1,3-diene	alkadiene
5	hexane	alkane
6	octane	alkane
7	1-chloropentane	alkyl halide
8	1,2-xylene	aromatic hydrocarbon
9	ethyl 2-methylpropanoate	carboxylic acid ester
10	pentadecan-3-yl 2,2,2-trifluoroacetate	ester
11	2,7-dimethyloxepine	ether
12	1-ethenyl-4-methoxybenzene	ether
13	2,6-dimethylpyrazine	heteroaromatic compound
14	3-methylbutan-2-one	ketone
15	hexan-2-one	ketone
16	5-methylheptan-3-one	ketone
17	2-methylcyclopentan-1-one	ketone
18	3-ethylcyclopentan-1-one	ketone
19	2-methylpentan-3-one	ketone

Table 2. VOCs emitted during fungal development on maize (independently of its nature) while absent in controls

These 19 identified volatile compounds can be interesting for development of FCI specific to grain storage conditions, indicating presence of fungal contamination. Since they are absent in all control samples they can origin from fungal metabolism or from grain alterations during mould development on it.

Highlighted compounds are predominantly ketones and alcohols (table 2). It has been previously suggested that ketones can be useful markers of fungal presence, and more precisely *P. verrucosum*

presence (Pasanen et al. 1996). Additionally, alcohols 3-Methylbut-3-en-1-ol and oct-1-en-3-ol were also identified as potent indicators of *P. verrucosum* presence, and even ochratoxin A presence (Larsen and Frisvad 1995; Pasanen et al. 1996; Schnürer et al. 1999; Wilkins and Scholl 1989).

Jelen and Grabarkiewicz-Szczęśna (2005) studied the profiles of volatile compounds produced on wheat by a strains of *Aspergillus ochraceus*. The most abundant volatiles were degradation products of fatty acids-eight-carbon alcohols and ketones, in particular, oct-1-en-3-ol and 3-octanone. These compounds contribute to the aroma of *Basidiomycetes* and were also isolated from several moulds (Jeleń and Wąsowicz 1998; Jelen and Grabarkiewicz-Szczęśna 2005).

In this study some of those compounds are found on artificially contaminated grains, but not in contamination by microflora already present on grains. This can be due to the conditions like temperature and type of medium (maize), since they have important impact on VOCs emissions (Magan and Evans 2000; Matysik et al. 2008).

3.1.2. Application of FCI to determine presence/absence of fungal contamination

After analysis of VOC profiles of different contaminated maize samples, calculation of fungal contamination index (FCI) for each sample was performed.

As presented in section 3.1.1., some VOCs can be potential indicators of fungal presence in cereal grains. Nevertheless, since VOC profiles depend on several factors like temperature, cereal type, fungal species etc, and at the same time those VOCs can be produced by some external sources (for example human activities or materials) more than finding specific VOCs, finding (developing) the panel of specific volatile compounds, so-called fingerprint, for rapid and early detection of deterioration of stored grains due to the moulds should be considered.

In previous work of our team, the FCI was developed in French Scientific and Technical Centre for Building (CSTB). This index aims at determining the presence or the absence of fungal development on the basis of the detection of specific VOCs. The fungal contamination index calculates presence or absence of 19 pertinent chemical tracers.

In the table 3 are presented values for FCI, where $FCI > 0$ indicates the presence of fungal contamination, and $FCI < 0$ indicates its absence.

Sample	FCI
Maize contaminated by microflora already present on grains (sample 1)	3
Maize contaminated by microflora already present on grains (sample 2)	4
Control (maize treated exactly the same but not contaminated)	-3
Maize contaminated with <i>Penicillium verrucosum</i> MRI 555 (sample 1)	6
Maize contaminated with <i>Penicillium verrucosum</i> MRI 555 (sample 2)	7
Maize contaminated with <i>Penicillium verrucosum</i> NRRL 965 (sample 1)	6
Maize contaminated with <i>Penicillium verrucosum</i> NRRL 965 (sample 2)	6
Control (maize treated exactly the same but not contaminated)	-2

Table 3. FCI (fungal contamination index) calculated for each sample

In the laboratory conditions, use of already developed FCI showed to be suitable to determinate if fungal contamination was present in the sample or not. All controls revealed to be non-contaminated, while for all contaminated samples FCI showed clear fungal presence.

3.1.3. Analysis of different potency of VOCs to diffuse through maize layer

In order to evaluate the use of specific VOC emissions as the indicators of fungal contamination of maize, ability of those compounds to diffuse through maize was tested. In order to approach to the real conditions maize was humidified to reach the humidity of 11% m.c. Table 4 shows different % of diffusion for several VOCs of interest. For the reason of the confidentiality those compounds are cited as VOCx.

Analyte	Chemical taxonomy	Peak area		% of VOC that diffused
		Control (empty flask)	Flask with maize (m.c. 11%)	
VOC1	heterocyclic aromatic compound	160048	340549	213
VOC2	alkene	102265	105509	103
VOC3	alkadienes	461649	774261	168
VOC4	ketone	134854	57845	43
VOC5	ether	37564	11607	31
VOC6	alcohol		n.d.	
VOC7	Fatty acid ester	830050	479961	58

* VOC1-VOC7 used in the construction of FCI

Table 4. Diffusion of different VOCs through maize, calculated in % of diffused compound (Peak area of VOCs in the sample and in the control)

Six VOCs of interest were detected in the control sample, which present 100% of VOC. Those VOCs were all detected when diffused through the maize. It revealed that different molecules had different potency to be adsorbed by humid maize, but all of them were collected in at least 30%. It is already established that VOC6 is known to be easily transformed into VOC3 and some other products, which can be reason why VOC3 was found more in the sample than in the control. VOC1 was found in almost double quantity in sample than in a control, but this aromatic compound is known to be produced by several plants and it was also detected in maize control sample.

4. CONCLUSIONS

This study aimed to evaluate is it possible to use the VOCs as indicators of fungal contamination during cereal storage. Experiments were done on a laboratory level in order to determine the feasibility of such a hypothesis.

Analysis of emitted VOCs were performed on contaminated maize, while non-contaminated maize served as control. Maize was contaminated artificially with two strains of *Penicillium verrucosum* which is one of the most frequent contaminants of cereal grains and known producer of ochratoxin A. Also, VOC analysis were performed on maize when contamination occurred by intrinsic fungal microflora initially present on grains.

The analysis of VOC emissions showed that more than 190 VOCs were emitted during this study, among which 19 were present just in the contaminated samples.

Since VOCs individually can't be certain indicators, we applied fungal contamination index that is constituted of panel of specific volatile compounds. This index has been previously developed and validated by our team in various indoor environments. Results clearly show the capability of this index to be applied in grains storage environments, since it was able to distinguish all contaminated and non-

contaminated samples. Moreover, in this study nineteen VOCs were detected just when samples were contaminated and represent potential tracers of fungal presence. Therefore, future work to adapt FCI for this type of environment will be done taking into account those VOCs.

Finally, this study evaluated capacity of emitted VOCs to diffuse grains from the place of fungal contamination to the point of sampling. Analysis showed that depending on their chemical nature compounds will diffuse in different percentage, but always in at least 30%, so certain absorbance by humid grains is present, but VOCs can still be detected by this method.

In conclusion, this study showed in small volume laboratory experiments that it was possible to use the VOCs as indicators of fungal contamination in maize, and used FCI was able to determine contaminated samples, independently of nature of contamination. Further work will evaluate this method with other grains than maize, and several fungal species. Also, experiments in the field, with real silos and conditions will be performed.

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