Italian Journal of Animal Science

ISSN: (Print) 1828-051X (Online) Journal homepage: http://www.tandfonline.com/loi/tjas20

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To cite this article: Laura Rizzi, Roberta Galuppi, Marco Simioli, Giorgia Canestrari, Cristina Bonoli & Maria Paola Tampieri (2009) Grains colonised by moulds: fungal identification and headspace analysis of produced volatile metabolites, Italian Journal of Animal Science, 8:sup2, 334-336

To link to this article: http://dx.doi.org/10.4081/ijas.2009.s2.334

Grains colonised by moulds: fungal identification and headspace analysis of produced volatile metabolites

Laura Rizzi[1], Roberta Galuppi[2], Marco Simioli[1], Giorgia Canestrari[1], Cristina Bonoli[2], Maria Paola Tampieri[2]

[1]Dipartimento di Morfofisiologia Veterinaria e Produzioni Animali, *Alma Mater Studiorum*, Università di Bologna, Italy

[2]Dipartimento di Sanità Pubblica Veterinaria e Patologia Animale, *Alma Mater Studiorum*, Università di Bologna, Italy

Corresponding author: Laura Rizzi, Dipartimento DIMORFIPA, Università di Bologna. Via Tolara di sopra, 50-40064 Ozzano Emilia, Italy - Tel. +39 051 2097392 - Fax: +39 051 2097373 - Email: laura.rizzi@unibo.it

ABSTRACT - The aim of this work was to verify if the headspace analysis of fungal volatile compounds produced by some species of *Fusarium* can be used as a marker of mould presence on maize. Eight samples of maize (four yellow maize from North Italy and four white maize from Hungary), naturally contaminated by *Fusarium* and positive for the presence of fumonisins, were analyzed to detect moisture content, Aw, volatile metabolites and an enumeration of viable moulds was performed by means of a colony count technique. Headspace samples were analysed using a gas-chromatograph equipped with a capillary column TR-WAX to detect volatile metabolites of moulds. Furthermore macro- and microscopic examination of the colonies was performed in order to distinguish, according to their morphology, the genera of the prevalent present moulds. Prevalent mould of eight samples was *Fusarium*, but other fungi, like *Aspergillus*, *Penicillum* and Mucoraceae, were observed. The metabolites produced by *F. graminearum* and *F. moniliforme* were Isobutyl-acetate, 3-Methyl-1-butanol and, only at 8 days, 3-Octanone. The incubation time can affect off flavour production in consequence of the presence of other moulds. Further studies on maize samples under different conditions are needed in order to establish the presence of moulds using the count technique and through the identification of volatile compounds.

Key words: Maize, Mould, Volatile metabolites, Headspace analysis.

Introduction - Maize crops are subjected to fungal attacks resulting in contamination by mycotoxins that can cause a hazard to animal and human health deriving from consumption of cereal, maize based feed and food. Early detection of mould contamination is crucial to prevent risks and diseases. Several studies suggested that the monitoring of the appearance of volatiles might be a good early indicator of presence of moulds and quality loss due to mycotoxin formation in cereals (Magan and Evans, 2000). Fungal species can produce a multitude of volatile compounds, some of which are common to many moulds, while others seem to be unique for one species. The aim of this work was to verify if the headspace analysis of fungal volatile compounds produced by some species of *Fusarium* can be used as a marker of mould presence on maize.

Material and methods - In order to evaluate some volatile compounds produced by *Fusarium graminearum* and *Fusarium moniliforme* (Catalog No. 200362 and 96574™, ATCC, Manassas, VA, USA), the moulds were cultivated on potato sucrose agar (PSA) and potato dextrose agar (PDA) medium respectively and on maize, previously sterilized by ultraviolet rays, in Erlenmeyer flasks closed with a Whatman bug stopper. Four samples for every substrate and mould were incubated for 21 days at

25°C. After 8 and 21 days from trial start, the headspace of the flasks containing the volatile metabolites was sampled with a SPME equipped with adsorbent Carbowax PDMS fibre (Supelco, Bellefonte, PA, USA). Headspace samples were analysed using a gas-chromatograph HRGC- Mega 2 Fison 8560 equipped with a split- splitless injector and a flame ionisation detector (FID). Separation of compounds of interest were done using a 30 m x 0.25 mm I.D. μm df capillary column TR-WAX with the following parameters: injection mode was splitless for 300 sec, injector temperature was 250°C, initial oven temperature 45°C, then raised at 3°C/min to 80°C, and kept for 20 min, then it was programmed at 2C/min to 176°C followed by the raise al 10°C/min to 240°C and then kept for 20 min. The detector temperature was 280°C. The carrier gas helium at velocity of 1.70 ml/min. Volatile compounds were identified by comparing their retention times to those of the pure standards. Eight samples of maize (four yellow maize from Veneto and Emilia Romagna – Y A, Y B, Y C, Y D - and four white maize from Hungary – W A, W B, W C, W D), naturally contaminated by *Fusarium* and positive for the presence of fumonisins, were analyzed to detect moisture content, Aw and volatile metabolites after 8 and 21-day incubation at 25°C according the method above described. On the samples used for the test, an enumeration of viable moulds was performed, by means of a colony count technique at 25°C, according to the ISO 7954:1987 and Dragoni *et al.* (1997). Forty grams of each sample were diluted 1:10 in peptone saline solution (1g peptone, 8.5g NaCl, 1000 ml deionized water) and homogenized with a stomacher for 2 minutes. Four further dilutions (10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}) were obtained for each sample. For each dilution, 1 ml of suspension was transferred, in double, in sterile Petri dishes. About 15 ml of yeast extract-dextrose – chloramphenicol – agar medium (Yeast extract 5g, Dextrose 20 g, Chloramphenicol 0.1g, agar 12g, water 1000 ml) maintained at 45°C in a water bath was put in each Petri dish. The inoculum was carefully mixed and the mixture let to solidify by leaving the Petri dishes to stand on a horizontal surface. The plates were placed inverted in the incubator at 25°C. After 4 days the count of the colonies was carried out and the number of Colony Forming Units of mould per gram (CFU/g) was calculated and expressed as a number between 1.0 and 9.9 multiplied by 10^{X}. Macro- and microscopic examination of the colonies was performed in order to distinguish, according to their morphology, the genera of the prevalent moulds present. At the end of the test, 4 grains of each sample were directly seeded on Sabouraud Dextrose Agar (DIFCO), incubated at 25°C and observed after four days by macro and microscopic examination.

Results and conclusions - The metabolites produced on media and on maize after 8 and 21 incubation days by *F. graminearum* and *F. moniliforme* were Isobutyl-acetate, 3-Methyl-1-butanol and 3-Octanone. At 8 incubation days, the off-flavours found in maize samples were the same of pure moulds, but at 21 days 3-Octanone was not present. Data from the present trial clearly indicate that the incubation time can affect the off flavour production and they are consistent with those reported by other authors (Börjesson, 1993; Magan and Evans, 2000). In a previous research (Rizzi *et al.*, 2007) the volatile compounds obtained from two *Aspergillus* species, cultivated on sterilized maize samples, were not influenced by incubation time. The analytical results could be explained by the presence of competitive moulds that grew during the longer incubation time and produced 3-Methyl-1-butanol and other metabolites. In the chromatograms other compounds were present, but their identification will be possible only in combination with a mass spectrometer. The results of the colony count technique and the direct seeding of the grain were displayed in table 1.Using the colony count technique, the prevalent mould isolated from the maize seeds was *Fusarium* sp., typically a "field mould" that invades the seeds before the harvest, whereas, with the direct seeding of the grain, "storage moulds" were essentially found, that probably are present on the surface of the grains and grow more rapidly. The differences observed among the moulds in Italian and Hungarian maize were probably due to used hybrids, climatic conditions of the two countries and storage. In many cases the resistance of plants was found to be connected with chemical component of plant metabolism. Further studies are needed in order to evaluate the occurrence of moulds in grains by means of volatiles produced under

different conditions and when various species are present. Developing modern, fast and easy tools for identification of *Fusarium* and other moulds is therefore one of the future challenges for researchers. Moreover, as the profile of maize contaminants seems to be related to different environmental conditions, a better evaluation of mould species should be useful for developing risk assessment models using data collected from different sites in Europe with contrasting climatic conditions.

Table 1. Moulds, moisture and water activity (Aw) of maize samples.

Colony count technique				Direct seeding of the grain	Moisture	Aw
Sample	UFC/g	Prevalent mould	Other present moulds	Present moulds	%	
Yellow maize-Y						
Y A	1.1 x 10^4	*Fusarium* sp.	*Penicillium* sp., *Aspergillus niger*	*Penicillium* sp., *Aspergillus niger*, *Aspergillus* sp., *Rhizopus* sp.	10.17	0.82
Y B	1.8 x 10^4	*Fusarium* sp.	*Penicillium* sp., *Aspergillus* sp., Mucoraceae	*Rhizopus* sp.	12.20	0.62
Y C	1.6 x 10^4	*Fusarium* sp.	*Aspergillus* sp., Mucoraceae	*Rhizopus* sp.	12.09	0.63
Y D	2.5 x 10^6	*Fusarium* sp.	*Aspergillus* sp., Mucoraceae		13.63	0.71
White maize-W						
W A	4.5 x 10^3	*Fusarium* sp.	Trichoderma viridae	*Aspergillus* sp., *Penicillium* sp., *Rhizopus* sp.	13.39	0.71
W B	1.3 x 10^5	*Fusarium* sp.	*Aspergillus* sp., Mucoraceae	*Rhizopus* sp. *Aspergillus niger*, *Aspergillus* sp., *Penicillium* sp., *Fusarium* sp.	12.63	0.68
W C	1.6 x 10^5	*Fusarium* sp	-	*Penicillium* sp., *Aspergillus* sp., *Fusarium* sp., *Aspergillus niger*., *Rhizopus* sp.	12.87	0.70
W D	5.0 x 10^4	*Fusarium* sp.	Mucoraceae	*Rhizopus* sp., *Aspergillus* sp., *Penicillium* sp.	13.35	0.71

The research carried out by 2007 founds of Alma Mater Studiorum- University of Bologna, Italy.

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