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Volatile Compounds of *Aspergillus* Strains with Different Abilities To Produce Ochratoxin A

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Volatile compounds emitted by *Aspergillus* strains having different abilities to produce ochratoxin A were investigated. Thirteen strains of *Aspergillus ochraceus*, three belonging to the *A. ochraceus* group, and eight other species of *Aspergillus* were examined for their abilities to produce volatile compounds and ochratoxin A on a wheat grain medium. The profiles of volatile compounds, analyzed using SPME, in all *A. ochraceus* strains, regardless of their toxeginicity, were similar and comprised mainly of 1-octen-3-ol, 3-octanone, 3-octanol, 3-methyl-1-butanol, 1-octene, and limonene. The prevailing compound was always 1-octen-3-ol. Mellein, which forms part of the ochratoxin A molecule, was found in both toxigenic and nontoxigenic strains. Volatile compounds produced by other *Aspergillus* strains were similar to those of *A. ochraceus*. Incubation temperatures (20, 24, and 27 °C) and water content in the medium (20, 30, and 40%) influenced both volatile compounds formation and ochratoxin A biosynthesis efficiency, although conditions providing the maximum amount of volatiles were different from those providing the maximum amount of ochratoxin A. The pattern of volatiles produced by toxigenic *A. ochraceus* strains does not facilitate their differentiation from nontoxigenic strains.

KEYWORDS: ***Aspergillus*; volatiles; ochratoxin A; mellein**

INTRODUCTION

Recent interest in instrumental olfactory technologies that can be used for rapid methods of food quality control, especially detection of microbial spoilage based on headspace analysis, requires thorough investigation of volatiles characteristic for bacteria or fungi involved in such processes. Finding specific volatile markers or specific patterns of volatile compounds accompanying the presence of microorganisms and toxins would give a basis for the quality check of foods based on their headspace composition and might provide information necessary for selection or manufacturing of specific sensors used in electronic noses.

Volatiles that can be used for the detection of microbial spoilage of cereal grain are of special importance because grain is a staple food constituent in many cultures. Moreover, apart from off-odors that can develop in the improperly stored grain as a result of microbial activity, fungi contaminating grain can be a source of mycotoxins—mainly ochratoxins, fumonisins, and trichothecenes. For these reasons volatile compounds, which can provide information on microbial growth and activity in grains, have been of great interest to scientists since the early 1970s (*1*, *2*). In natural conditions, Abramson et al. (*3*) observed a significant increase in volatile compounds in stored barley, and the most characteristic ones were 3-octanone, 1-octanol, and 3-methyl-1-butanol. These metabolites were also the most predominant ones in stored wheat. It was observed that a high content of 3-methyl-1-butanol was correlated with the presence of *Penicillium*, *Aspergillus*, and *Alternaria alternata* fungi (*4*). Tuma and co-workers (*5*) noted that the presence of 3-methyl-1-butanol accompanied *Penicillium*, *Aspergillus*, and *Fusarium* fungi. Börjesson et al. identified volatile compounds characteristic for *Penicillium* and *Aspergillus* fungi grown under laboratory conditions (*6*, *7*). In recent years advances in electronic nose technologies have enabled their applications in grain quality assessment (*8*−*10*).

The literature on fungal volatile metabolites has been summarized in several reviews (*11*−*13*). There is still relatively little information on the relationship between the formation of volatiles and the biosynthesis of mycotoxins (*14*−*17*). Considering the importance of ochratoxin A volatiles accompanying the formation of this toxin need to be explored.

Along with aflatoxins, fumonisins, and some fusariotoxins, ochratoxin A (OTA) is one of the most important toxins of concern to the food industry. Ochratoxin A was isolated for the first time from *Aspergillus ochraceus* (*18*), which is the main fungus forming this metabolite. Ochratoxin A has been also identified as a metabolite of other *Aspergillus* species belonging to section *Circumdati* (group *A. ochraceus*): *A. alliaceus*, *A. melleus*, *A. sulphureus*, *A. ostianus*, *A. petrakii*, and *A. sclerotiorum* (*19*). There are also reports on the formation of OTA

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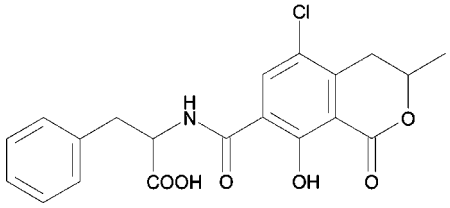

Figure 1. Structure of ochratoxin A.

by *A. niger* var. *niger* (*20*), *A. versicolor*, *A. fumigatus* (*21*), and *A. albertensis* (*22*).

Ochratoxin A [(*R*)-*N*-[(5-chloro-3,4-dihydro-8-hydroxy-3-methyl-1-oxo-1*H*-2-benzopyran-7-yl) carbonyl]-L-phenylalanine] consists of a 3,4-dihydro-3-methylisocoumarin fragment linked by a carboxyl group at C8 with L-β-phenylalanine (**Figure 1**). Ochratoxin A is the most important toxin of the ochratoxin group, which includes ochratoxin B (lacking the chlorine atom) and products of their hydrolysis devoid of the phenylalanine fragment—ochratoxins α and β. Ochratoxin A inhibits proteins biosynthesis in vitro and in vivo, accelerates lipid peroxidation, and reacts with enzymes utilizing phenylalanine as a substrate. It shows genotoxic, teratogenic, and nephrotoxic properties causing threats to humans and livestock.

The objectives of this study were to (i) identify volatile metabolites specific for *A. ochraceus* strains of different abilities to produce OTA, (ii) compare profiles of volatile compounds with other *Aspergillus* strains, (iii) monitor the formation of volatile compounds and OTA during fungal growth on solid medium, and (iv) investigate the influence of water content in the medium and temperature on the formation of volatile metabolites and OTA.

MATERIALS AND METHODS

Fungal Isolates, Media, and Incubation. Sixteen strains of *Aspergillus* that are potential producers of OTA were used. Eight strains of other *Aspergillus* species, known as nonproducers of OTA, were analyzed for comparison with the profiles of volatile compounds. The first group comprised the following strains: *A. ochraceus* KA-9, KA-10, KA-95, KA-100, KA-101, KA-103, KA-104, KA-156, KA-192, KA-278, KA-284, KA-297, KA-301; *A. ochraceus* group KA-7 (*A. melleus*), KA-12 (*A. ostianus*), KA-132 (*A. alliaceus*). The other group consisted of the following species: KA-30 (*A. flavus*), KA-148 (*A. glaucus*), KA-162 (*A. terreus*), KA-172 (*A. amstelodami*), KA-224 (*A. fisherii*), KA-229 (*A. repens*), KA-240 (*A. versicolor*), KA-261 (*A. niger*). All strains were from the fungal collection of the Agricultural University of Poznań. For strain propagation, potato dextrose agar (PDA) slants were used. Ten-day-old slants, after incubation at 22 °C, were washed with sterile water. Spore suspensions were used for medium inoculation. As a medium, sterilized wheat grain (121 °C, 20 min), Kobra variety, was used after adjustment of its initial water content of 13 to 40% in all but one experiment. Incubation was done in 100 mL vials, filled with 20 g of wheat kernels. During incubation, vials were capped with a cotton plug, which provided aerobic conditions. For sampling, the plugs were replaced with Teflon/silicon membranes and aluminum caps that made solid-phase microextraction (SPME) sampling possible. To investigate the influence of water content and temperature on the volatile compounds formation and OTA production, wheat kernels of 20, 30, and 40% water content were used and temperatures of 20, 24, and 27 °C were tested. Spore concentrations used for medium inoculation in strain comparison experiments ranged from 0.25×10^6 to 1.87×10^6/mL. For the experiment on the dynamics of formation of volatile compounds and other metabolites by two *A. ochraceus* strains (KA-10 and KA-192), concentrations of 5.5×10^6 and 7.4×10^6/mL were used, and the incubation was carried out at 27 °C. All samples were run in triplicate: for each strain or investigated parameter three bottles of medium were inoculated with a spore suspension from the same solution. After incubation, samples were analyzed for the volatile compounds by headspace solid-phase microextraction/gas chromatography—mass spectrometry (SPME/GC-MS) and then for the OTA by thin-layer chromatography (TLC) and for ergosterol by high-performance liquid chromatography (HPLC).

Analysis of Volatile Metabolites, Ochratoxin A, and Ergosterol. Volatile metabolites were analyzed as previously described (*23*). SPME with a PDMS fiber was utilized. Capped vials were placed in a water bath (40 °C), and volatiles were sampled for 20 min. After extraction, volatiles from the SPME fiber were desorbed at the injection port of a gas chromatograph coupled to a mass spectrometer (HP 5890II—HP 5971MSD, Hewlett-Packard). Compounds were resolved on an MDN-5 column (30 m length × 0.25 mm i.d. × 0.25 μm film, Supelco, Bellefonte, PA) and identified on the basis of comparison of their mass spectra with authentic standards when possible or with spectra from the NBS 75K and NIST 98 libraries. Also, retention indices were compared to data available in the literature. The analysis of OTA was done on the basis of previously described methods (*24*, *25*). The quantitation limit for OTA was 0.2 mg/kg. Ergosterol was determined according to the method of Seitz (*26*) by HPLC using an Adsorbosphere C18 (150 mm × 4.6 mm, Alltech) column and an HPLC system with a diode array UV—vis detector (Merck Hitachi La Chrom). Identification of ergosterol was done in the 220—400 nm range spectrum, comparing it and retention time with standards. For ergosterol quantification a maximum at $\lambda = 282$ nm was used. Standards of OTA and ergosterol were purchased from Sigma-Aldrich. Due to the toxicity of OTA it must be handled with appropriate safety measures.

RESULTS AND DISCUSSION

Toxigenicity of Investigated Strains. All strains were analyzed for their ability to produce ochratoxin A and volatiles. On the basis of this experiment they could be divided into nontoxigenic ones (KA-95, KA-101, and KA-284), which did not produce OTA after 14 days of incubation, and a group of toxigenic strains. The amount of OTA produced varied considerably depending on strain: KA-7 (0.45 mg/kg), KA-12 (0.63 mg/kg), KA-192 (0.78 mg/kg), KA-132 (6.8 mg/kg), KA-100 (7.1 mg/kg), KA-278 (9.2 mg/kg), KA-103 (12.4 mg/kg), KA-104 (42.9 mg/kg), KA-9 (48.3 mg/kg), KA-297 (51.4 mg/kg), KA-156 (80.0 mg/kg), and KA-301 (96.7 mg/kg). The highest amount of OTA (150.0 mg/kg) was noted for strain KA-10. On the basis of these results KA-10 was selected as the main toxigenic strain for subsequent experiments. No OTA was detected in *Aspergillus* strains of other species.

Identification of Volatile Compounds Produced by *A. ochraceus*. Compounds that were detected in 13 strains (10 toxigenic and 3 nontoxigenic) of *A. ochraceus* are listed in **Table 1**. Comparison of volatiles detected in both groups reveals that there was no specific compound or group of compounds related only to toxigenic strains. Such compounds should be present in all toxigenic strains and absent in nontoxigenic ones. When the amount of volatiles produced by strains was compared to quantity of OTA, a weak correlation was found ($r = 0.4369$, $P = 0.05$). There was a tendency for nontoxigenic strains, or those producing minute amounts of OTA, to produce lesser amounts of volatiles, whereas for strains producing >10 ppm of OTA, the amount of volatile compounds was generally 4—25 times higher. In a few cases, production of high levels of volatiles was not accompanied by abundant mycotoxin production and vice versa.

Among the volatiles, one compound related to the structure of OTA was detected—mellein (3,4-dihydro-8-hydroxy-3-methyl 1*H*-2-benzopyran-1-one). Its structure and mass spectrum are shown in **Figure 2**. It has been tentatively identified on the basis of NIST library match and literature spectral data (*27*). There have been no reports on the detection of mellein (ochracin) in the headspace of toxigenic *Aspergillus* strains, although it was

Table 1. Volatile Compounds Produced by 13 Strains of *A. ochraceus* of Various Abilities To Form Ochratoxin A (Compounds Were Isolated by SPME Technique Using PDMS Fiber)

		volatile compounds: toxigenic strains		volatile compounds: nontoxigenic strains	
compound	RI	frequency[a]	range[b]	frequency	range
3-methyl-1-butanal	647	1/10	10	0/3	
2,2,3,3-tetramethylbutane	676	3/10	42–84	0/3	
heptane[c]	700	3/10	11–43	0/3	
3-methyl-1-butanol[c]	735	10/10	15–38	3/3	13–24
1-octene	784	9/10	11–24	2/3	9–24
unidentified	821	9/10	14–169	3/3	7–41
p-xylene[c]	866	10/10	49–111	2/3	14–97
3,5,5-trimethyl-2-hexene	968	10/10	9–51	3/3	9–29
1-octen-3-ol[c]	973	10/10	294–9741	3/3	1072–2154
3-octanone[c]	977	5/10	88–176	3/3	6–410
3-octanol[c]	989	10/10	9–61	3/3	11–54
octanal[c]	1004	7/10	10–32	1/3	12
limonene[c]	1032	10/10	8–34	3/3	11–31
(Z)-2-octenal[c]	1057	6/10	14–21	1/3	10
2-octen-1-ol	1066	8/10	8–19	1/3	10
1-octanol[c]	1069	8/10	10–33	0/3	
octen-1-ol acetate	1106	2/10	10–21	0/3	
phenyl ethyl alcohol[c]	1120	6/10	14–24	2/3	8–12
1,3-nonadiene	1290	2/10	10	0/3	
undecanal[c]	1310	2/10	7–11	0/3	
unidentified	1370	4/10	8–36	0/3	
α-elemene	1372	1/10	11	0/3	
β-elemene	1421	5/10	10–34	0/3	
unidentified sesquiterpene	1439	1/10	11	0/3	
β-cubebene	1479	2/10	9–17	0/3	
unidentified sesquiterpene	1484	1/10	10	0/3	
γ-cadinene	1514	1/10	12	0/3	
unidentified sesquiterpene	1515	1/10	9	0/3	
δ-cadinene	1539	7/10	7–103	0/3	
mellein	1546	4/10	9–141	1/3	7
unidentified	1871	3/10	11–64	0/3	
ethyl palmitate	1965	1/10	10	0/3	
ethyl linoleate	2132	1/10	8	0/3	

[a] Frequency of occurrence in examined strains. [b] Volatiles content expressed in arbitrary units on the basis of comparison to tridecane peak area used for normalization. [c] Compounds identified by comparing their retention indices and mass spectra with those of authentic standards; remaining compounds were identified tentatively on the basis of comparison of mass spectra with those in NIST mass spectra library and comparison of retention indices with data available in the literature.

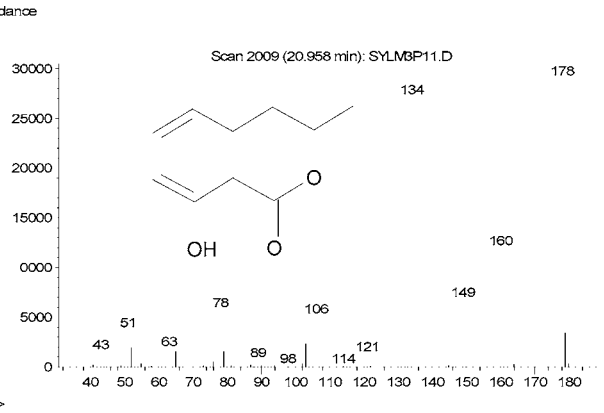

Figure 2. Structure and electron impact (EI) mass spectrum of mellein produced by *A. ochraceus* strain KA-10.

detected in extracts of *A. ochraceus* (*28*) and *A. melleus* (*29*). Harris and Mantle (*27*) observed the presence of mellein as a transitory compound in a shaken liquid culture of *A. ochraceus* on potato dextrose broth; however, no mellein was detectable in a 2 week culture of shaken solid state (shredded wheat) fermentations. Related isocoumarin derivatives have been reported from other fungi: 8-hydroxy-3-methylisocoumarin by *Marasmius ramealis*, 8-hydroxy-6-methoxy-3-methylisocoumarin by *Ceratocystis fimbriata*, ramulosin and rydroxyramulosin by *Pestlotia ramulosa*, *cis*-4-hydroxymellein by *Lasiodiplodia theobromae*, and *trans*-4-hydroxymellein by *Apiospora camptospora* (*29*).

The most abundant volatiles were degradation products of fatty acids—eight-carbon alcohols and ketones (1-octen-3-ol, 3-octanol, 2-octen-1-ol, and 3-octanone). In particular, 1-octen-3-ol and 3-octanone were the compounds present in highest amounts in almost all samples. These compounds contribute to the aroma of *Basidiomycetes* and were also isolated from numerous molds (*13*). In cultures of *A. oryzae*, *A. ochraceus*, and *A. niger* Kamiński and co-workers (*1*, *2*) identified 1-octen-3-ol, 2-octen-1-ol, 3-octanol, 1-octanol, and 3-octanone, which comprised 67−97% of the volatile compounds fraction. It was noted that during the growth of *Penicillium* and *Aspergillus* on a coarse wheat medium a decrease in triacylglycerols was accompanied by free fatty acids increase and subsequent increase of volatile compounds derived from them—mainly 1-octen-3-ol (*30*). The presence of 1-octen-3-ol and 3-methyl-1-butanol in stored grains is often regarded as an indicator of the presence of molds (*11*). Both of these metabolites were detected in toxigenic and nontoxigenic *A. ochraceus* strains. Therefore, they cannot be used as unique markers for the differentiation between toxigenic and nontoxigenic strains. Similarly, the most characteristic metabolites for toxigenic *A. ochraceus* strains that were present in all analyzed strains (*p*-xylene, 3,5,5-trimethylhexene, and limonene) were present also in nontoxigenic isolates. Xylene was reported earlier in *A. niger*, *A. flavus*, *A. versicolor*, and *A. candidus*, whereas limonene was reported in strains *A. flavus*, *A. versicolor*, and *A. candidus* (*7*, *31*). In the majority of samples 1-octene, 2-octen-1-ol, and 1-octanol have been detected. There was a low incidence of sesquiterpene production: the most frequently observed were δ-cadinene and β-elemene.

Volatile Compounds Produced by Strains of the *A. ochraceus* Group and Other *Aspergillus* Species. To test whether the profile of *A. ochraceus* was unique for this species only, 11 strains of *Aspergillus* were chosen for comparison—3 from the *A. ochraceus* group (*A. melleus*, *A. ostianus*, and *A. alliaceus*) and 8 other *Aspergillus* species (**Table 2**). Strains belonging to the *A. ochraceus* group were known to be OTA producers. The remaining strains were nontoxigenic. Volatile compounds produced by all of these strains were similar to those of *A. ochraceus*, with 1-octen-3-ol and 3-octanone being dominant. Limonene, 2,2,3,3-tetramethylbutane, and *p*-xylene were detectable in most strains. 3-Methyl-1-butanol was detected in all strains of the *A. ochraceus* group, and it was also detectable in other strains. The profile of volatiles was not differentiated between *Aspergillus* strains. Although there are instances when volatile secondary metabolites can be helpful in the chemotaxonomy of *Penicillium* (mainly due to sesquiterpenoid fraction) (*32*), in the case of the investigated *Aspergillus* strains the differences were not pronounced.

Dynamics of Volatile Compounds Formation by *A. ochraceus*. To investigate the relationships between formation of selected metabolites by *A. ochraceus*, two strains were selected: KA-10 (producer of 150 mg/kg of OTA) and KA-192 (which produced ∼200 times less OTA—0.78 mg/kg). **Figure 3** shows changes in the amount of produced volatile compounds, mellein, OTA, and ergosterol in the course of 14 days of incubation on sterilized wheat kernels. A comparison of the amount of mycelium, based on the ergosterol level, showed that KA-10 formed twice as much fungal biomass as KA-192. An increase in ergosterol content in KA-10 was particularly

Table 2. Volatile Compounds Produced by Strains Representing the *A. ochraceus* Group and Other *Aspergillus* Species (Compounds Were Isolated by SPME Technique Using PDMS Fiber)

		volatile compounds (normalized units)										
compound	RI	KA-7 *A. melleus*	KA-12 *A. ostianus*	KA-132 *A. alliaceus*	KA-30 *A. flavus*	KA-148 *A. glaucus*	KA-162 *A. terreus*	KA 172 *A. amstelodami*	KA-224 *A. fisherii*	KA-229 *A. repens*	KA-240 *A. versicolor*	KA-261 *A. niger*
3-methylbutanal	647	33.7B		13.6C								
2,2,3,3-tetramethylbutane	676		119.2B	54.7A	11.1B	2.3B	9.6A	6.1B	4.8C	11.4A	6.0B	12.3B
heptane[a]	700	11.4B	33.5B	12.3A	2.9A	9.8B	2.7B			1.9A		2.5A
3-methyl-1-butanol[a]	735	3.8A	16.9A	13.2A	3.6B				6.1A			15.0B
1-octene	784	2.6C										
unidentified	821	25.9C	12.4B						49.1C		6.4A	
p-xylene[a]	866	71.9A	57.4A	86.3B	4.7B	2.6A	18.4B	13.9B	88.7A	15.8A	43.7B	
3,5,5-trimethyl-2-hexene	968	23.5A							32.2B			
1-octen-3-ol[a]	973	18.14A	858.9A	53.2A	69.1B	26.7B	139.6A	66.3A	1132.2A	52.2B	346.3B	11.4A
3-octanone[a]	977	79.9B	130.3A	16.3B	42.7B	26.9B	86.7A	14.8B	315.7A	26.4B	123.7A	13.3A
3-octanol[a]	989	24.1A	21.1B		13.6B		7.6C		11.7B	7.6C	10.9A	
limonene[a]	1032	25.5B	10.3A	19.3A	1.4C	6.8C	1.5B	9.4C	11.8C	63.9B	11.1C	17.4A
1-octanol[a]	1069	4.4A										
linalol[a]	1096									3.0C		
octen-1-ol acetate	1106						5.3B					
1,2,3-trimethoxybenzene	1369					42.5B				64.1C		
melilatin	1394		13.9B	9.6B								
β-elemene	1421	8.9C										
β-cubebene	1479											1.3C
unidentified sesquiterpene	1483	18.2B										
γ-cadinene	1514	21.1B										
mellein	1546		12.2B									
unidentified diterpene	1783			4.4A								
ethyl palmitate	1965			4.1A								11.5A
ethyl linoleate	2132			7.1A								18.3C

[a] Compounds identified by comparing their retention indices and mass spectra with those of authentic standards; remaining compounds were identified tentatively on the basis of comparison of mass spectra with those in the NIST mass spectra library and comparison of retention indices with data available in the literature. Samples were run in triplicates; A, RSD $\leq$ 25%; B, 25% < RSD < 50%; C, RSD $\geq$ 50%.

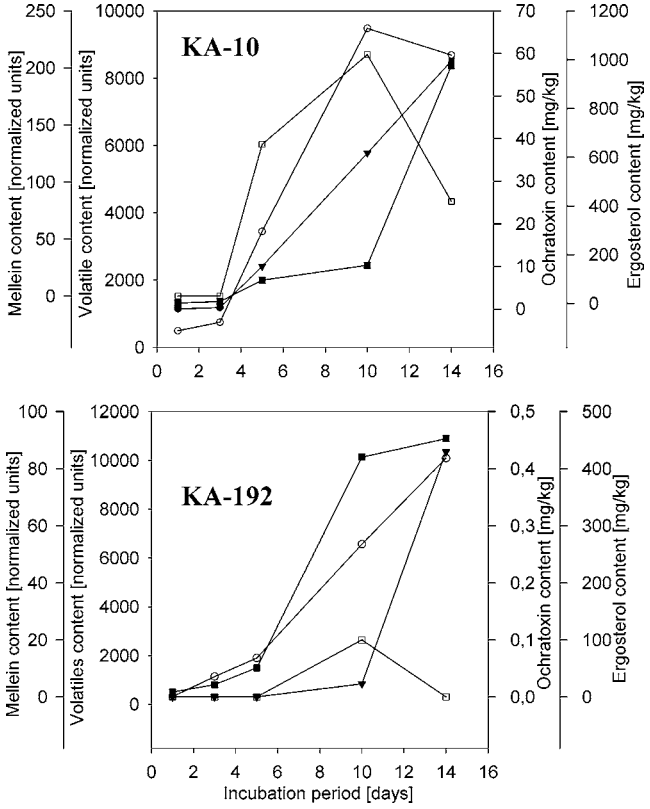

Figure 3. Dynamics of the formation of volatile compounds, including mellein, ergosterol, and ochratoxin A, by *A. ochraceus* strains KA-10 and KA-192 during 14 days of growth on sterilized wheat kernels medium: (▼) ochratoxin A; (■) ergosterol; (○) total volatile compounds; (□) mellein. Results are the mean of three replicates.

intensive between 3 and 5 days (12-fold) and between 10 and 14 days (6-fold). For strain KA-192, the highest (8-fold) increase in ergosterol level was noted between days 5 and 10. Ochratoxin was detectable in KA-10 as early as 1 day after inoculation and after 14 days reached 58.3 mg/kg. After 14 days, strain KA-192 produced only 0.43 mg/kg of ochratoxin and it first appeared only after day 10. In KA-192, an intensive increase in ochratoxin content was noted after the maximum in mycelium growth, whereas in strain KA-10 intensive production of OTA was accompanied even by minimal mycelium growth. The correlation coefficient for ergosterol contents and OTA formation during incubation was 0.7058 ($P = 0.05$). It was observed earlier that the formation of ochratoxin is not related to the increase of biomass (*32*).

Despite the vast differences in toxigenicity, the abilities of both strains to synthesize volatile compounds were similar—KA-10 produced 8700 units after 14 days, whereas KA-192 produced 10100 units. Mellein was detected after 5 days of KA-10 strain growth; the maximal amount was produced at day 10 and then decreased 2-fold at day 14. Mellein was detected in cultures of KA-192 only after 10 days at a level 10 times lower than in KA-10 strain. During the first 10 days of growth total volatile compounds and OTA were highly correlated 0.9985 ($P = 0.01$) for KA-10. However, it is clear that strains capable of forming huge amounts of OTA do not have to produce vast amounts of volatile compounds. A much higher concentration of mellein was detected in the toxigenic strain, and its decrease suggests that it is consumed during the formation of ochratoxin. However, data from mellein incorporation experiments do not support its role as an intermediate in ochratoxin biosynthesis (*27*).

Influence of Incubation Temperature and Water Content in a Medium on Production of Volatile Metabolites and Ochratoxin A by *A. ochraceus*. To investigate the influence of incubation temperature on the ability of *A. ochraceus* KA-10 to produce volatile metabolites, the incubation was carried out at 20, 24, and 27 °C. The amount of volatile compounds

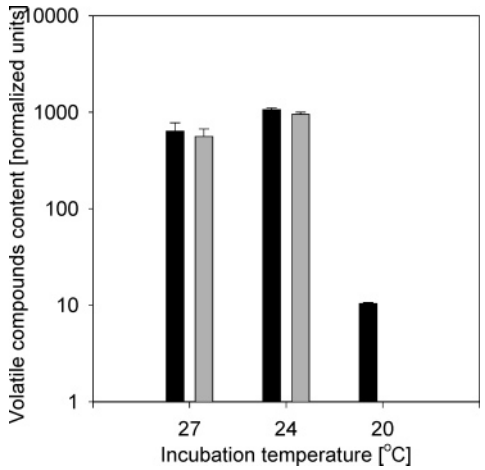

Figure 4. Influence of incubation temperature on the formation of volatile compounds by *A. ochraceus* strain KA-10. Volatile compounds were analyzed after 14 days of incubation on sterilized wheat kernels medium containing 40% water: (black bars) total volatile compounds; (gray bars) sum of 1-octen-3-ol and 3-octanone. Results are the mean of three replicates.

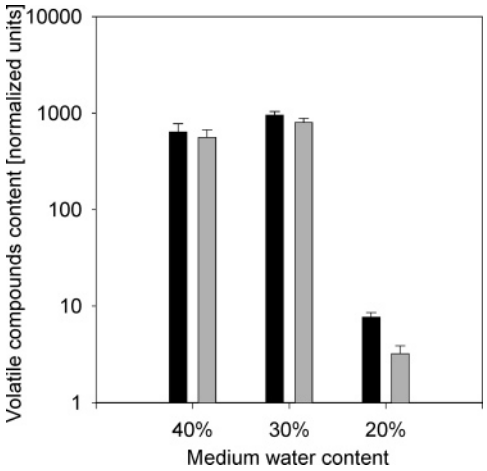

Figure 5. Influence of medium water content on the formation of volatile compounds by *A. ochraceus* strain KA-10. Volatile compounds were analyzed after 14 days of incubation at 27 °C: (black bars) total volatile compounds; (gray bars) sum of 1-octen-3-ol and 3-octanone. Results are the mean of three replicates.

produced (total and 1-octen-3-ol and 3-octanone) for different incubation temperatures is shown on **Figure 4**. The profiles of volatiles produced at 24 and 27 °C were very similar. The prevailing compounds were 1-octen-3-ol and 3-octanone. Their peaks at high concentrations were partially overlapping when using an HP-5 column. At 20 °C the contents of volatile compounds were very low, and neither 1-octen-3-ol nor 3-octanone was detected. In all samples regardless of temperature, a mixture of 3-methyl-1-butanol, 2-methyl-1-butanol, and benzaldehyde was detected.

For the evaluation of water content on the amount of metabolites produced, the incubation was carried out at 27 °C for 14 days with water content in a medium of 20, 30, and 40%. **Figure 5** shows that the most compounds were generated when the medium water content was 30%. At 20% there was a decrease in 1-octen-3-ol concentration and an absence of 3-octanol and 3-octanone.

Production of OTA for strain KA-10 was dependent upon water content with levels of 3.8, 82.5, and 256.3 mg/kg produced on the medium with water contents of 20, 30, and 40%, respectively. The data confirm the crucial role of water content (or rather water activity) in the medium on the formation of volatile compounds and OTA. It has been well recognized that apart from competing fungi presence, the most important factors in *A. ochraceus* growth and OTA formation are water activity and temperature (*34*, *35*).

There were also high differences in the dependence of OTA on the incubation temperature: an increase in temperature from 20 to 24 °C resulted in a 2-fold increase in OTA concentration (103.7 and 212.5 mg/kg, respectively). The conditions providing the highest OTA production did not result in the highest levels of volatile compounds. The influence of temperature on the biosynthesis of OTA by *A. ochraceus* was described by Häggblom (*33*): for similar mycelium content at 10 °C, no OTA was detected, whereas the same strains at 25 °C formed significant amounts of OTA. Damoglou and co-workers showed that no OTA was formed at 10 °C on sterilized barley kernels with 20% water content (0.85 a_w) (*36*). Wheeler et al. (*37*) defined the best temperatures for *A. ochraceus* growth as 25 and 30 °C at pH 4. These temperatures were also regarded as optimal by Ramos et al. (*38*).

The results of presented model experiments lead to a conclusion that there are no volatile compounds uniquely related to the formation of OTA, as was previously shown in the case of *A. flavus*, *P. roqueforti*, or *Fusarium* (*14*, *15*, *23*). Due to its similarity of structure mellein might play such a role; however, this compound is probably not incorporated into OTA, and it was also detected in nontoxigenic strains. In a natural environment where *Aspergillus* strains coexist with other fungi and bacteria, the pattern of volatile metabolites can be far more complicated. In such cases, fingerprinting based on chromatographic data can be beneficial. Appropriate data processing based on multivariate analysis applied to GC-MS results may help to discriminate between samples, as was demonstrated by Olsson et al. (*39*) for sound and OTA-contaminated barley.

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