Black Aspergilli and fumonisin contamination in onions purchased in Hungary

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Abstract

Black mold rot caused by black Aspergilli is an important post-harvest disease of onion worldwide. Usually *Aspergillus niger* is cited as the causative agent based on morphological criteria. In this study, the mycobiota and fumonisin contamination of mouldy onion bulbs purchased in Hungary were examined. All except one of the examined mouldy samples were found to be contaminated with black Aspergilli, which could be isolated both from the outer dry and the inner fleshy scales of onion bulbs. Species assignment of the isolates was carried out using sequence analysis of part of the calmodulin gene. Sequence data revealed that all 35 black Aspergilli isolated from onions belong to the *Aspergillus awamori* species. The range of fumonisin isomers present in the onion samples was also examined using reversed-phase high-performance liquid chromatography/electrospray ionization – ion trap mass spectrometry. Two of the examined onion samples were found to be contaminated with fumonisins at a rate of about 0.3 mg kg^{-1}. This is the first report on fumonisin contamination of onion bulbs. The fumonisin isomers observed include fumonisins $B_{2\text{-}4}$, 3-epi-FB_4, iso-FB_1 (FB_6) and an iso-$FB_{2,3}$ form. The range of fumonisin isomers detected in the onion bulbs indicates that probably *A. awamori* is responsible both for mold rot and fumonisin contamination of onions in Hungary.

Running title: Black Aspergilli and fumonisins in onions

Keywords: onion, *Aspergillus* section *Nigri*, *Aspergillus awamori*, fumonisin, sequence-based identification, calmodulin, HPLC/ESI-ITMS

Introduction

Onion (*Allium cepa* L.) is considered as one of the most important crops in several countries. According to the United Nations Food and Agriculture Organization, onion varieties are cultivated throughout the world on an estimated 3.691.855 ha producing more than 72 million tons of onions in 2009 (FAOSTAT 2010). Leading onion producing countries are China, India, the United States, Pakistan and Turkey, respectively. In Hungary, onion is traditionally cultivated mainly in the eastern part of the country on 2.366 ha producing more than 60.000 tons of onion bulbs in 2009 (FAOSTAT 2010).

Black mold rot caused by black Aspergilli is often responsible for severe damage of onion bulbs during storage. Infected onion bulbs have a black discoloration at the neck, shallow lesions on the outer scales, streaks of black mycelium and conidia beneath the outer scales and a black discoloration in bruised areas. The disease commonly occurs on onions stored at high ambient temperatures (Hayden and Maude 1992; Burgess et al. 2008). Contaminated seeds and soil appear to constitute the main inoculum source (Kaul 1973; Gupta et al. 1984; Hayden et al. 1994; Köycü and Özer 1997). The species responsible for black mold rot is usually referred to as *Aspergillus niger*, a member of *Aspergillus* section *Nigri* (Samson et al. 2007). Indeed, RFLP analysis of the mitochondrial DNA of black Aspergilli isolated from onions in Hungary indicated that they belong to *A. niger* (Varga et al. 2003). However, black Aspergilli are one of the more difficult groups concerning classification and identification. Several new species have been described recently in section *Nigri*, which cannot be reliably distinguished based on morphological or physiological methods (Samson et al. 2007). Recent data indicate that sequence-based methods can be used successfully for species assignment in this group of organisms (Samson et al. 2007; Varga et al. 2007, 2010). Such an approach is important since some members of section *Nigri* are

mycotoxin producers and are able to contaminate several food products with ochratoxins and/or fumonisins (Samson et al. 2007; Frisvad et al. 2007; Varga and Kozakiewicz 2006; Varga et al. 2006; Noonim et al. 2009; Varga et al. 2010). To our knowledge, black Aspergilli infecting onion bulbs have not yet been reliably identified to species level using a sequence-based approach.

In this study we examined the mycobiota of onions purchased in markets in Hungary. Black Aspergilli were isolated from diseased bulbs, and calmodulin sequence-based identification was used to assign the isolates to species. Fumonisin content of the onion bulbs was also examined and correlated with the presence of potential fumonisin-producers in the bulbs.

Materials and methods

Isolation of Aspergilli from onion bulbs

Samples were taken both from the outer dry and the inner fleshy scales of the onions, and were plated onto Dichloran Rose Bengal Chloramphenicol (DRBC) agar (King et al. 1979). The plates were incubated for 5–7 days at 25 °C in the dark. Colonies of black Aspergilli isolates were isolated, purified and maintained on Malt Extract Agar (MEA) slants (Samson et al. 2004).

Genotypic studies

The fungal cultures used for the molecular studies were grown on malt peptone (MP) broth as described previously (Varga et al. 2010). DNA was extracted from the cells using the Masterpure™ yeast DNA purification kit (Epicentre Biotechnol., Madison, US) according to the instructions of the manufacturer. Amplifications of the partial calmodulin gene were set

up as described previously (Hong et al. 2006). Sequence analyses were performed with the Big Dye Terminator Cycle Sequencing Ready Reaction Kit for both strands. Sequences were analyzed on the ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Carlsbad, California, US).

DNA sequences were edited with the DNASTAR computer package. Calmodulin sequences of type strains of the *A. niger* species complex (Samson et al. 2007) were also included in the data set. Alignments of the sequences were performed using MEGA version 4 (Tamura et al. 2007). Alignment positions with gaps or missing data were excluded, and all characters were unordered and equal weight. The MP tree was obtained using the Close-Neighbour-Interchange algorithm with search level 3 in which the initial trees were obtained with the random addition of sequences (10 replicates). The tree is drawn to scale, with branch lengths calculated using the average pathway method. To assess the robustness of the topology, 1000 bootstrap replicates were run by maximum parsimony (Hillis and Bull 1993). Other measures including tree length, consistency index and retention index were also calculated. An *A. carbonarius* isolate was used as outgroup in these experiments.

Determination of fumonisins in onion samples

Onions including a blank, uninfected one (sample G in Fig. 1) were cut into thin slices, freeze-dried for 24 h and grinded (2 min) by using a commercial coffee grinder to obtain fine powders. The powdered samples (0.5 g each) were vortexed for 20 s in 4 ml of MeOH/H_2O 3/1 (v/v), sonicated for 3 min, extracted using an overhead shaker at 60 rpm for 90 min, and subsequently centrifuged at 13000×g for 10 min. The supernatants were filtered through 0.45 µm PTFE membranes into the HPLC autosampler vials. Recovery examinations with 0.5 g amounts of the blank onion sample spiked with FB_1 and FB_2 standards at two levels (0.1 and 1 mg kg^{-1} in MeCN/H_2O 3/1, v/v) were also performed. The spiked samples were left for two

hours in a fume hood to allow the solvent to evaporate, and were extracted as mentioned above.

The extracts of the onion samples were analysed by reversed-phase high-performance liquid chromatography/electrospray ionization – ion trap mass spectrometry (RP-HPLC/ESI–ITMS) using a Agilent 1090 Series II HPLC (Waldbronn, Germany) and a Varian (Palo Alto, CA, USA) 500 MS ion trap mass spectrometer as described in detail by Bartók et al. (2010a). The HPLC system was equipped with a binary DR5 solvent delivery system and an autosampler. A binary gradient of water (solvent A) and acetonitrile (solvent B, each supplemented with 0.1% (v/v) HCOOH) was applied for the separation of the compounds on a YMC-Pack J'sphere ODS H80 (YMC Europe GmbH, Dinslaken, Germany) HPLC column (250 mm x 2.1 mm, 4 μm) at a flow rate of 0.2 ml/min. The gradient elution started with 24% B and increased linearly to 40% B at 79 min, and then to 100% B at 94 min, which value was held for 10 min and finally, the solvent composition reached again the starting value for 2 min. The temperature of the HPLC column was adjusted at 40 °C by using a Jones (Jones Chromatography Ltd., Hengoed, Mid Glamorgan, UK) Model 7990 Space column heater placed in the optimum position between the injector and the MS nebulizer in order to decrease the length of the connecting capillaries. The injection volume was 4 μl.

The mass spectrometer were equipped with an ESI source and operated in positive ion mode, at normal scan speed. The quantitative evaluation was performed by external standard calibration based on the extracted ion chromatograms (EIC) at *m/z* 722 ($[M+H]^+$ of FB_1) and *m/z* 706 ($[M+H]^+$ of FB_2) of the full scan MS measurements of eight solutions in the range of 10 pg – 50 ng (on-column) of FB_1 and FB_2 calibration standards dissolved in $MeCN/H_2O$ 1/1 (v/v). The identity of fumonisin isomers was confirmed as published formerly (Bartók et al. 2006, 2010b) by examining the product ion spectra of the protonated molecules ($[M+H]^+$. The

HPLC and the ESI–ITMS systems were controlled by Agilent ChemStation version A09.03 and Varian Workstation 6.6 (SP1) software, respectively.

Results and discussion

Species assignment of black Aspergilli isolated from onions

Altogether six infected onion samples were examined in this study (Fig. 1). Black Aspergilli were successfully isolated from all of them, except for sample D, which was found to be contaminated by *A. fumigatus*, *A. versicolor* and Penicillia. Black Aspergilli could be isolated from both the outer and inner scales of onion bulbs in samples A-C and E-F. Other Aspergilli identified on the onion samples include *A. ochraceus* (samples A and C), *A. fumigatus* (sample B) and *A. flavus* (samples E and F). Sequence analysis of part of the calmodulin gene of the black *Aspergillus* isolates was used to assign the isolates to species (Samson et al. 2007). The evolutionary history was inferred using the Maximum Parsimony method. The calmodulin data set consisted of 250 characters (excluding those with gaps or missing data), including 26 parsimony informative sites; MP analysis resulted in 653 most parsimonious trees (length = 81, consistency index = 0.74419, retention index = 0.9052), one of which is presented in Figure 2. All 35 isolates recovered from the onion samples were found to belong to the *Aspergillus awamori* species. This species has recently been found to represent a phylogenetic species closely related to *A. niger* based on a multilocus sequence approach and AFLP analysis (Perrone et al. 2010, Perrone et al. submitted). This species can readily be distinguished from *A. niger* based on molecular data including sequences of several protein coding genes (β-tubulin, calmodulin, translation elongation factor α; Perrone et al. 2010), AFLP analysis and RAPD analysis (unpublished observations). Similarly to *A. niger*, a large

proportion of *A. awamori* isolates are also able to produce ochratoxins and fumonisins (Varga et al. 2010; Frisvad JC, personal communication).

Fumonisin contamination of onion samples

The extracts of freeze-dried and powdered onion samples including an uninfected blank one were analysed for the presence of fumonisins using RP-HPLC/ESI–ITMS. Previously, this hyphenated technique was found to be very useful for the identification of new fumonisin isomers (Josephs 1996; Bartók et al. 2006, 2008, 2010a, 2010b). The potential of this procedure was also demonstrated successfully during the detection of several fumonisin isomers in raisin samples (Varga et al. 2010). One of the main advantage of the ion trap mass spectrometers is the exceptional sensitivity in full scan MS or MS^n mode (necessary for the identification of minute amount of unknown compounds), however, there is significant disadvantage of this technique: the less linear dynamic range (generally 2-3 order of magnitude) of the ion trap mass analyzer on a particular compound than that of mass spectrometers equipped with e.g. an QqQ analyzer most frequently applied for quantitative examinations. Because of the less dynamic range the preparation of multilevel calibrations (1 pg – 50 ng on-column) of FB_1 and FB_2 standards were necessary. Similarly to our previous investigation (Varga et al. 2010), the calibration curves for FB_1 and FB_2 toxins were linear in the 10 pg to 10 ng range with r^2=0.9989 and 0.9971, respectively (data not shown). The observed recovery values calculated based on the analysis of spiked (0.1 and 1 mg kg^{-1}) freeze-dried and powdered blank onion bulb samples were 93.7% - 96.7% for FB_1 and 90.4% - 99.4% for FB_2, respectively (data not shown).

Fumonisin isomers were detected in two of the samples in total quantities of 0.32 and 0.33 mg kg^{-1}, respectively (Table 1). The fumonisin isomers observed include fumonisins $B_{2\text{-}4}$, 3-epi-FB_4, iso-FB_1 (FB_6 according to Mansson et al. 2010) and iso-$FB_{2,3}$ (1) (Varga et al.

2010). These isomers were found to be produced by *A. awamori* in a previous study (Varga et al. 2010). Previously another fumonisin producing fungus, *Fusarium proliferatum* has also been found to be able to infect onion bulbs (Dissanayake et al. 2009a, 2009b; Du Toit et al. 2003; Galvan et al. 2008; Stankovic et al. 2007). However, this species was not identified in any of the onion samples examined in this study (data not shown). Furthermore, the main fumonisin isomer produced by *F. proliferatum* is fumonisin B_1, which was not detected in the contaminated onions. Instead, FB_2 and FB_4 were found in largest quantities, which are the main fumonisin isomers produced by *A. niger* and *A. awamori* (Frisvad et al. 2007; Mogensen et al. 2010; Varga et al. 2010). These data indicate that presumably *A. awamori* caused fumonisin contamination of the examined onion bulbs. Previous studies indicate that about two-third of *A. niger/A. awamori* isolates are able to produce fumonisins (Noonim et al. 2009; Logrieco et al. 2009; Mogensen et al. 2010; Varga et al. 2010). Interestingly, the amount of 3-epi-FB_4 exceeded the amount of FB_4 in sample A, although in our previous investigations we found that the amount of FB_4 exceeded that of 3-epi-FB_4 in all raisin samples (Varga et al. 2010). Previous studies indicated that both naturally and artifially contaminated samples containing either FB_3 and/or FB_4 usually also contain the corresponding 3-epi isomers (Gelderblom et al. 2007; Varga et al. 2010). According to our experience, 3-epi-FB_3 and 3-epi-FB_4 can be separated from FB_3 and FB_4, respectively, if an efficient HPLC column and an isocratic or a relatively flat gradient separation is applied.

Due to the presence of alliin and other antimicrobial compounds in onions, relatively few fungi are able to colonize the bulbs (Filtenborg et al. 1996). Consequently, mycotoxins are relatively rarely detected in onion. Ochratoxins have not yet been detected (Filtenborg et al. 1996), and even artificial inoculation with patulin-producing *Penicillium expansum, P. urticae* or *Byssochlamys nivea* strains did not result in patulin contamination in onions (Frank 1977). Malformin, a secondary metabolite of *A. niger* was reported to be present in the outer

scales of onion, not in central portions of the bulb (Curtis et al. 1974). Although fumonisin B_1 has been detected in garlic bulbs infected by *F. proliferatum* (Seefelder et al. 2002), fumonisins have not yet been detected in onion bulbs to our knowledge. Although the amount of fumonisins detected was relatively low (ca. 0.3 mg kg^{-1}), further studies are needed to clarify the significance of these observations.

Previously, *A. niger* and *A. awamori* have been found to produce fumonisins at low water activities, in contrast with Fusaria (Frisvad et al. 2007; Varga et al. 2010). Besides agricultural products with high sugar content (e.g. dried vine fruits, figs), the relatively dry outer layers of onion bulbs seem to be also able to promote fumonisin production in these fungi. Further studies are needed to clarify whether *A. niger* contaminates onions during storage or in the field as recently it has been proposed that *A. niger* has an endophytic life style in onions (Tuffley and Lorbeer 2002; Lorbeer et al. 2002; Palencia et al. 2010). Besides, further work is also in progress to examine the possible role of black Aspergilli in ochratoxin contamination of onions.

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Figure legends

Figure 1. The onion samples examined (samples A-F: infected samples, sample G: control sample).

Figure 2. Phylogenetic tree of the black *Aspergillus* isolates came from onions, and the type strains of species assigned to the *A. niger* species complex (Samson et al. 2007) based on calmodulin sequence data. Numbers above branches indicate bootstrap values. Only values above 70% are indicated.

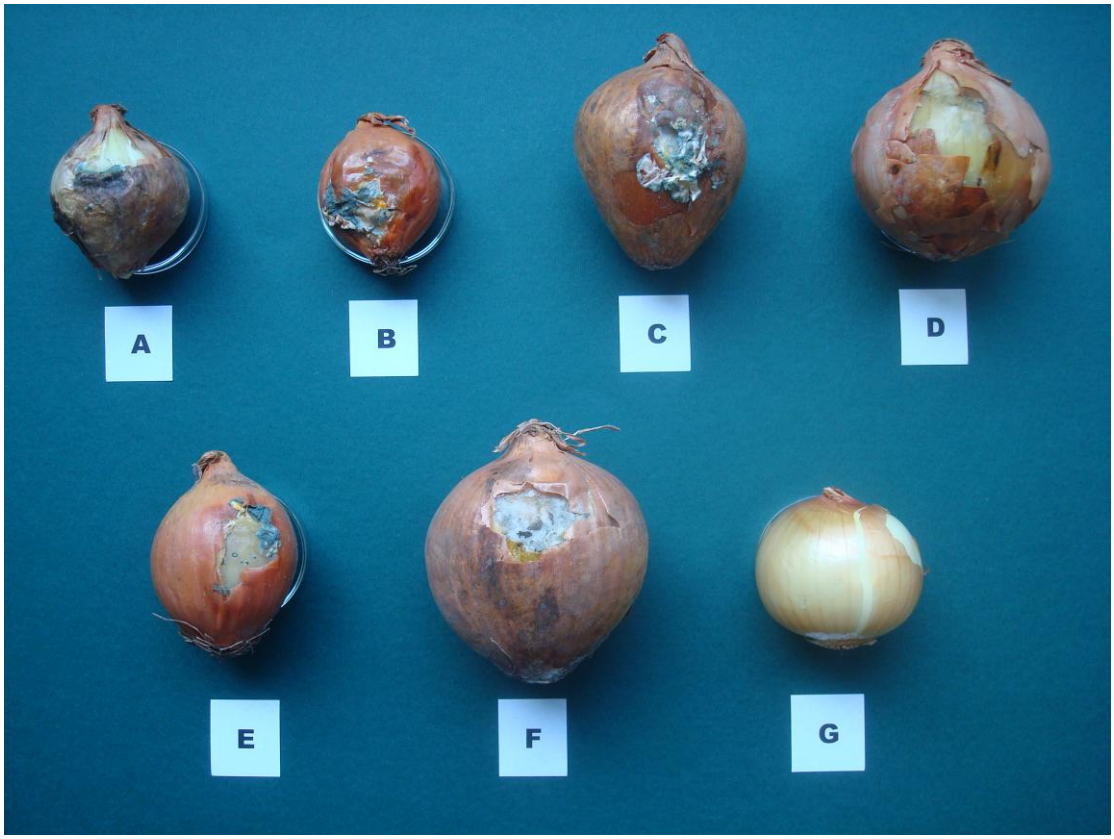
Figure 3. RP-HPLC/ESI-ITMS extracted ion chromatogram (EIC, *m/z* 690+706+722) of onion samples A and C.

Table 1. Fumonisin content of the onion samples.

Sample No.	**Amount of fumonisin isomers detected (ng g^{-1})[a]**						
	FB_2	**FB_3**	**FB_4**	**3-epi-FB_4**	**iso-FB_1 (FB_6)**	**iso-$FB_{2,3}$(1)**	**Total fumonisin content**
A	**203.75**	**9.50**	**48.46**	**58.58**	**6.21**	**16.14**	**324.64**
B	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**
C	**16.73**	**25.06**	**288.22**	**ND**	**ND**	**ND**	**330.01**
D	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**
E	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**

F	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**
G	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**

[a] FB_1 calibration curve and recovery values were used for quantifying iso-FB_1, while FB_2 calibration curve and recovery values were used for quantifying FB_2, FB_3, FB_4, 3-epi-FB_4 and iso-$FB_{2,3}$(1); ND, not detected.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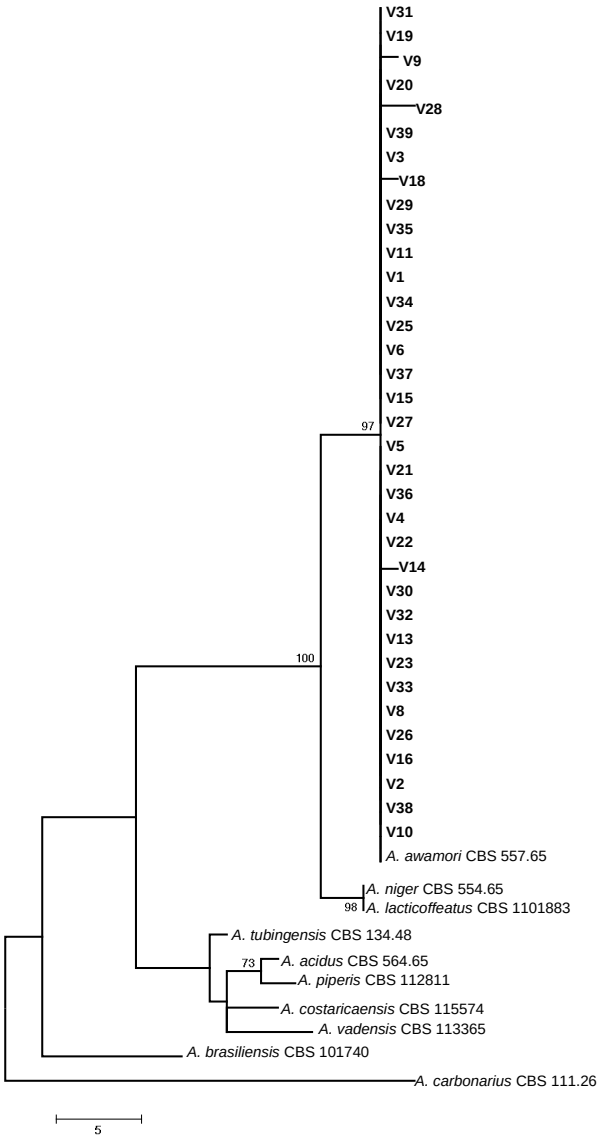
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V26
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V2
V38
V10
A. awamori CBS 557.65
A. niger CBS 554.65
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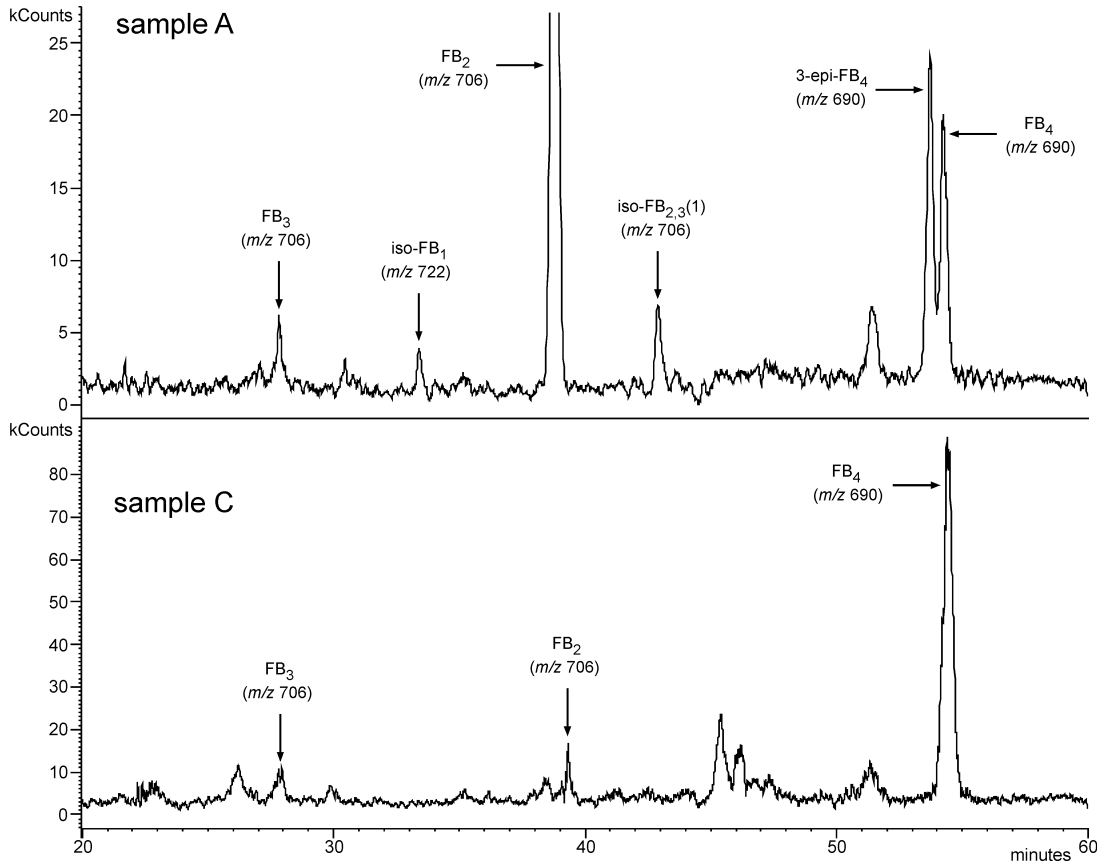
A. lacticoffeatus CBS 1101883
A. tubingensis CBS 134.48
73
A. acidus CBS 564.65
A. piperis CBS 112811
A. costaricaensis CBS 115574
A. vadensis CBS 113365
A. brasiliensis CBS 101740
A. carbonarius CBS 111.26
5


kCounts
sample A
FB2
(m/z 706)
3-epi-FB4
(m/z 690)
FB4
(m/z 690)
FB3
(m/z 706)
iso-FB1
(m/z 722)
iso-FB2,3(1)
(m/z 706)
kCounts
sample C
FB4
(m/z 690)
FB2
(m/z 706)
FB3
(m/z 706)
minutes