ABSTRACT: Spices are often considered as one of the possible sources of meat products contamination with toxigenic moulds. Genera Aspergillus, Eurotium and Penicillium are most frequent xerophilic storage moulds that contaminate spices. Because spices are possible source of contamination of the final product and potential producers of mycotoxins, it is necessary to estimate the degree of moulds contamination and their ability to produce secondary metabolites – mycotoxins.

Mycological analysis was carried out on five samples of oregano and clove respectively. Presence of moulds was determined by parallel usage of Sabouraud maltose agar (SMA) and the medium that stimulates the growth of xerophilic species: malt – yeast extract agar with 50% of glucose (MY50G).

Isolated moulds were classified into five genera (Aspergillus, Alternaria, Cladosporium, Rhizopus and Penicillium) and 9 species.

Mycotoxins determination was carried out using ELISA test (commercial kits Tecna, Italy) for the presence of aflatoxin B1, ochratoxin, A and zearalenone.

The results showed the presence of aflatoxin B1, ochratoxin A, and zearalenone in almost all samples, except one sample of oregano and one clove sample.

We can conclude that it is necessary to introduce mandatory mycotoxins determination (aflatoxin B1, ochratoxin A), in raw material for meat industry, especially spices. These secondary metabolites are known as extremely toxic and are classified in group I of human carcinogens.

KEY WORDS: ELISA, moulds, mycotoxins, selective medium, spices
and processing all over the world. As is the case with many other agricultural products, spices and herbs may be exposed to a wide range of microbial contamination during pre- and post-harvest (Hashem and Alamri, 2010). Such contamination may occur during processing, storage, distribution, sale and/or use (Mc Kee, 1995).

Modern meat industry cannot be imagined without utilization of spices. However, spices, together with all other dried material of herbal origin, are never sterile. In most cases, they contain sporogenic bacteria and moulds. These microorganisms can cause spoilage of the product by their metabolic activity, consequently resulting in significant economic losses.

Presence of moulds in spices and later in sausages or other meat products can result in production of toxic metabolites – mycotoxins, independently of contamination degree (Kocić-Tanackov et al., 2007). In addition, changes in odor and other undesirable sensory changes can also occur in contaminated products. Mycotoxins are fungal secondary metabolites identified in many agricultural products screened for toxicogenic moulds (Cleveston and Ljunggren, 1985; CAST, 2003). Mycotoxins have been reported to be carcinogenic, teratogenic, tremorgenic, hemorrhagic, and dermatitic to a wide range of organisms, and known to cause hepatic carcinoma in man in humans and animals (Frisvad et al., 2005; Zinedine et al., 2006).

It has been reported that principal contaminants of spices are xerophilic moulds from the genera Eurotium, Aspergillus, and Penicillium (Dimić and Škriňar, 1995; Dimić et al., 2000; Romagnoli et al., 2007).

Production of toxins primarily depends on genetic factors; however, environmental conditions at the site of moulds growth (temperature, water activity, matrix composition, moisture content, pH of the medium, contamination and physical destruction of the substrate, antifungal properties and other factors) are considered highly significant.

The authors Škriňar and Boldocky (1994) determined the presence of 8 moulds species isolated from mixtures of spices intended for meat industry; Aspergillus species were dominant, while garlic had the highest degree of molds contamination, but only with two species (Eutotium herbariorum and Penicillium granulatum).

Dimić et al. (2000) established that about 46% of spices mixtures, 29% of black pepper and 25% of paprika was contaminated by moulds. According to this author, main sources of contamination were Eutotium herbariorum, Aspergillus versicolor, A. sydowii and A. flavus, Penicillium auratiogriseum and P. chrysogenum.

The same authors report that among 45 identified species responsible for fungal contamination, 55% was potentially toxigenic. Two samples of spices mixture contained high concentrations of ochratoxin A (32.00 µg/kg in mixture of spices for frankfurters production), as well as zearalenone in three samples of black pepper (192.00 µg/kg – 288.00 µg/kg).

Considering that spices are possible source of contamination of final product, the aim of this paper was to determine contamination degree of final product with xerophilic moulds by using various selective cultivating media,
resulting in recommendation of most suitable medium, and to determine ability of isolated moulds to produce mycotoxins using semiquantitative immunoassays.

2. MATERIALS AND METHODS

2.1. Sampling

Samples of spices used in meat industry (oregano and clove) were investigated. Presence and enumeration of moulds, their determination and investigation of ability to produce mycotoxins were carried out.

2.2. Mycological procedures

2.2.1. Mould isolation

Enumeration of moulds in samples of spices (cfu/g) was carried out using dilution technique by Koch (Harrigan, 1998). The enumeration of moulds was performed using 16-cm Petri dishes on both Sabouraud malt agar (SMA, Merck) with the addition of antibiotic (1ml of chloramphenicol, Sigma/100ml of medium) and the medium that stimulates the growth of xerophilic species – malt yeast extract agar with 50% of glucose (MY50G). SMA composition is as follows: peptone 10 g, D(+)-glucose 40 g, agar-agar 15 g; distilled water ad 1000 mL. pH after sterilization should be 5.6±0.2.

The composition of the other selective medium for detection of xerophilic moulds species, MY50G, is: malt extract, Difco 0186, 10 g; yeast extract, Difco 0127, 2.5 g; glucose, 50 g; distilled water, 500 ml; agar 10 g. pH value after sterilization should be 5.7. The inoculated agar media were incubated for 7 days in dark at 25±1 °C and inspected for genus identification using macro and microscopic morphological characteristics.

2.2.2. Mould identification

Moulds determination included recultivation of grown colonies on media used for determination (Czapek and malt agar). Macroscopic and microscopic morphological characters were used in the identification process. Colony color, texture and diameter, the production of diffusible pigments, and exudates were among macroscopic features, while conidia and conidiophore arrangements were the microscopic.

All the isolates were identified according to Samson and van Reenen-Hoekstra (1988), Samson and Pitt (2000), Samson et al. (2004) and Pitt and Hocking (1997).

2.2.3. Mycotoxicological investigations

Presence of aflatoxin B1, ochratoxin A, and zearalenone was determined simultaneously with enumeration and identification of moulds from oregano and clove samples. Mycotoxins were determined using semiquantitative test – ELISA. Commercial kits Tecna, Italy were used. Sample preparation was carried out according to internal protocol for each mycotoxin. The results were calculated based on calibration curve obtained during measurements. The results were expressed as μg/kg⁻¹.
3. RESULTS AND DISCUSSION

Table 1 shows the results of mycological analyses – enumeration of molds in samples of oregano and clove using different selective media, SMA, and MY50G. These media have different composition of added sugars and water activity (aw). SMA contains maltose and aw is in the range 0.98-0.99, while MY50G contains glucose and aw is 0.89.

The media that are traditionally used in isolation and enumeration of moulds (SMA, potato dextrose agar, Czapek agar, and other) have high water activity (about 0.99). Numerous xerophilic moulds, which are carriers of spic- es contamination, have optional aw below 0.90. Collaborative investigations with SMA and MY50G provide more accurate perspective on micropopulation and contamination of examined samples.

Tab. 1. – Total viable count (TVC) per 1g of oregano and clove

<table>
<thead>
<tr>
<th>Sample</th>
<th>Oregano</th>
<th>Clove</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SMA (TVC)</td>
<td>MY 50 G (TVC)</td>
</tr>
<tr>
<td>1</td>
<td>40</td>
<td>1.6 x 10^2</td>
</tr>
<tr>
<td>2</td>
<td>15</td>
<td>1.2 x 10^3</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>4.5 x 10^2</td>
</tr>
<tr>
<td>4</td>
<td>40</td>
<td>1.0 x 10^2</td>
</tr>
<tr>
<td>5</td>
<td>15</td>
<td>1.0 x 10^3</td>
</tr>
</tbody>
</table>

All investigated samples showed presence of moulds on both SMA and MY50G media (Table 1). However, significant differences were observed in enumeration of moulds grown on SMA and MY50G. Number of colonies was significantly higher on MY50G (from 1.0 x 10^2 to 1.2 x 10^3 cfu/g), compared to SMA (from 10 to 40 cfu/g).

The results of clove analysis showed somewhat different aspect (Table 1). The growth was determined in all samples cultivated on SMA, while no growth was observed in four samples cultivated on MY50G. Total count of moulds was between 2.0 x 10^2 (sample 4) and 2.4 x 10^3 (sample 1), while only one sample (number 1) showed moulds contamination using MY50G (7 x 10^3). Therefore, in this series of investigations, SMA was more appropriate for qualitative and quantitative determination of moulds.

These results pointed to the fact that media with limited amount of free water suppressed the growth of moulds that are not extremely xerophilic in nature. It is also known that xerophilic moulds can be divided into fast-growing and slow-growing forms (P i t t and H o c k i n g, 1985). Slow-growing moulds, even under optimal conditions can be overgrown by fast-growing xerophiles.

After monocultivation of different colonies formed on SAM and MY50G media and examination of their morphological and other properties, seven different species were identified. The majority belonged to genus Aspergillus,
in oregano samples (57.14%) and clove samples (28.57%), respectively. Other identified species in oregano samples were Alternaria alternata (14.28%), Rhizopus stolonifer (14.28%), and Penicillium sp. (14.28%).

Even percentage share (28.57%) was recorded in moulds from genus Aspergillus and Penicillium in clove samples. Other identified moulds were from genera Alternaria (A. alternata), 14.28%, Rhizopus (R. stolonifer), 14.28%, Penicillium (P. aurantiogriseum, Penicillium sp.), 28.57% and Cladosporium (Cladosporium sp.) with the share of 14.28%.

Tables 2 and 3 show the percentage of moulds species in oregano and clove samples.

**Tab. 2. – Presence of moulds species in oregano samples**

<table>
<thead>
<tr>
<th>Genus</th>
<th>Species</th>
<th>Share %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspergillus</td>
<td>A. flavus</td>
<td>57.14</td>
</tr>
<tr>
<td></td>
<td>A. niger</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A. rubrum</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A. candidus</td>
<td></td>
</tr>
<tr>
<td>Alternaria</td>
<td>A. alternata</td>
<td>14.28</td>
</tr>
<tr>
<td>Rhizopus</td>
<td>R. stolonifer</td>
<td>14.28</td>
</tr>
<tr>
<td>Penicillium</td>
<td>P. sp.</td>
<td>14.28</td>
</tr>
</tbody>
</table>

**Tab. 3. – Presence of moulds species in clove samples**

<table>
<thead>
<tr>
<th>Genus</th>
<th>Species</th>
<th>Share, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspergillus</td>
<td>A. flavus</td>
<td>28.57</td>
</tr>
<tr>
<td></td>
<td>A. rubrum</td>
<td></td>
</tr>
<tr>
<td>Cladosporium</td>
<td>Cladosporium sp.</td>
<td>14.28</td>
</tr>
<tr>
<td>Penicillium</td>
<td>P. aurantiogriseum</td>
<td>28.57</td>
</tr>
<tr>
<td></td>
<td>Penicillium sp.</td>
<td></td>
</tr>
<tr>
<td>Alternaria</td>
<td>A. alternata</td>
<td>14.28</td>
</tr>
<tr>
<td>Rhizopus</td>
<td>R. stolonifer</td>
<td>14.28</td>
</tr>
</tbody>
</table>

The emergence of Aspergilli, Penicillia, and Rhizopus species on the three different media greatly indicated the presence of these moulds as the dominant mycoflora of different spices. This observation was greatly in agreement with other authors who studied mycoflora of spices and medicinal herbs (El-Kady et al., 1995; Dijmic et al., 2008). Early Takatori et al. (1977) and Ayres et al. (1980) found the Aspergillus and Penicillium spp. the main mycopopulation of cardamom, cinnamon, fennel, coriander, cumin, black cumin and white pepper all of which are common in the food industry. The contamination with fungal species resulted from neutral extraneous contamination by dust following storage in humid conditions (Domsch et al., 1981). Moulds fall into two ecological categories, e.g., field and storage moulds. Field moulds were observed to invade development or mature seeds while it is on the plant, the major field moulds genera being Alternaria, Fusarium and
Cladosporium. On the other hand, storage moulds are those encountered on plants at moisture conditions routinely found in stored products. These moulds principally belong to species Aspergillus and Penicillium (Abou Donia, 2008). The spices can undergo fungal contamination mainly during spice processing, storage and transport (Đimić et al., 2008).

In clove samples (Table 4), besides aflatoxicogenic moulds from genus Aspergillus, Penicillium aurantiogriseum has been determined as dominant species. This microorganism belongs to ochratoxin A producers (Škrinjar and Horvat-Skenderović, 1992).

Original medium, MY50G has shown better efficiency in determination of Aspergillus species, based on results of micropopulation quantitation in oregano samples. The frequency of Aspergillus species in overall population was 100% on MY50G, while frequency was only 51% on SMA (Figure 1). The highest frequency of Penicillium genera was determined on SMA medium (14.5%). On MY50G medium, this value was 3.3% (Figure 2).

![Fig. 1. – Frequency of Aspergillus spp. on SMA (1) and MY50G (2)](image1)

![Fig. 2. – Frequency of Penicillium spp. on SMA (1) and MY50G (2)](image2)
The variation in frequency of mycopopulation of oregano and clove cultivated on SMA and MG50Y media is most probably related to the strain type within one species. Environmental factors also have significant effect and can induce the growth of mycopopulation on lower aw values (optimal temperature and type of nutritive components in the medium). Xerophiles, especially selective ones tend to be very sensitive on environmental conditions.

As k u n et al. (2007) used Rose-Bengal Chloramphenicol Agar (Oxoid, CM 549) and Dichloran-Glycerol (DG18) Agar (Oxoid, CM 729) for determination of xerophilic moulds. Other media can also be used such as Dichloran-Glycerol (DG18) Agar Base (P i t t and H o c k i n g, 1985), MY70FG and MY50FG (B e u c h a t and H o c k i n g, 1990), MY50S and MY40S (B e u c h a t, 1998).

Presence of mycotoxins was determined in examined samples (5 oregano and 5 clove samples) in 80% of cases (Table 4).

<table>
<thead>
<tr>
<th>Oregano</th>
<th>Clove</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Aflatoxin B₁</strong></td>
<td><strong>Ochratoxin A</strong></td>
</tr>
<tr>
<td>(µg/kg)</td>
<td>(µg/kg)</td>
</tr>
<tr>
<td>7.5</td>
<td>22.4</td>
</tr>
<tr>
<td>17</td>
<td>10</td>
</tr>
<tr>
<td>10</td>
<td>12</td>
</tr>
<tr>
<td>Not detected</td>
<td>4</td>
</tr>
<tr>
<td>5.3</td>
<td>5.7</td>
</tr>
</tbody>
</table>

The origin of mycotoxins in spices samples can be the result of their previous synthesis during storage of these products. However, there is the possibility of their production by moulds that contaminated the spices during storage. This ability is characteristic for moulds belonging to *Penicillium* genus since they can reproduce at lower temperatures (around +5°C), and in some cases even produce toxins in these environmental conditions (Š k r i n j a r et al., 1998).

The risk of contamination by mycotoxins is an important food safety concern for grains and other field crops. Mycotoxins affecting groundnuts/peanuts, cereals (maize, rice, sorghum, wheat, barley and oats), spices (black pepper, ginger and nutmeg) and chili are considered to be of greater significance for human beings (B h a t and V a s a n t h i, 2003; CAST, 2003; B r y d e n, 2007).

Over the last two decades various international evaluation on maximum residue limits and regulations for mycotoxins were published. A study by the United Nations’ Food and Agriculture Organization (FAO) on worldwide regulations for mycotoxins revealed that at least 77 countries now have specific regulations for mycotoxins (FAO, 2004). In the Republic of Serbia, maximum residue limits for mycotoxins in spices are set to 30 µg/kg for total aflatoxins and 10 µg/kg for ochratoxin A.
CONCLUSION

Based on obtained results, it can be concluded that it is necessary to use selective media adjusted to specific requirements of xerophiles in order to achieve proper isolation and accurate contamination degree of spices by xerophilic moulds. Utilization of selective media enables acquiring representative insight in spices mycopopulation.

Spices are potential source of mycotoxins, hence the necessity of regular mycotoxicological analysis of these products with the aim of consumers’ protection, prevention of food spoilage and consequent significant economic losses. For this reason, such analyses should be mandatory in evaluation of food safety parameters.

The ELISA test was used as an initial screening procedure in order to determine the presence of ochratoxin A and other mycotoxins.

REFERENCES


B e u c h a t, L. R. and H o c k i n g, A. D. (1990): Some considerations when analysing foods for the presence of xerophilic fungi. J. Food Protect., 53: 984–989.


C l e v s r t o n, G., L j u n g g r e n, H. (1985): Aflatoxin formation and the dual phenomenon. Mycopathologia 92:129–139.


D i m i č, G., Š k r i n j ar, M., D o š e n - B o g i ē v i č, V. (2000): Plesni, potencijalni proizvođač sterigmatocistina u začinima. Tehnologija mesa 41(4–6), 131–137.


КСЕРОФИЛНЕ ПЛЕСНИ ИЗОЛОВАНЕ ИЗ ЗАЧИНА КОЈИ СЕ КОРИСТЕ У ИНДУСТРИЈИ МЕСА КАО ПОТЕНЦИЈАЛНИ ПРОДУЦЕНТИ МИКОТОКСИНА

Марија М. Шкрњар1, Славица М. Весковић Морачанин2, Весна В. Јанковић2, Јелена Б. Вукојевић3

1 Технолошки факултет, Универзитет у Новом Саду, Булевар цара Лазара 1, 21000 Нови Сад, Србија
2 Институт за хигијену и технологију меса, Каћанског 13, 11000 Београд, Србија
3 Институт за ботанику, Универзитет у Београду, Таковска 43, 11000 Београд, Србија

Резиме

Како један од могућих извора контаминације производа од меса токсигеним плеснима често се наводе зачини. врсте које се појављују као контаминенти зачини су ксерофилне, складишне плесни, најчешће из родова Aspergillus, Eurotium и Penicillium. Узимајући у обзир чињеницу да зачини представљају могућ извор контаминације финалног производа, као и да су потенцијални продуценти микотоксин-на, неопходно је уочити степен контаминације плеснима и њихово потенцијално физиолошко својство да продукују секундарне метаболите – микотоксине.

Миколошким анализама обухваћено је по пет узорака оригана и каранфилића. Присуство плесни испитано је паралелним коришћењем Sabouraud малтозног агара (SMA) и подлоге која фаворизује раст ксерофилних врста – сладни квашчев екстракт агар са 50% глукозе (MY50G).

Изоловане плесни сврстане су у пет родова (Aspergillus, Alternaria, Cladosporium, Rhizopus и Penicillium) и 9 врста.

Микотоксиколошка испитивања обухватали су утврђивање присуства афлатоксина B1, охратоксина A и зеараленона. Испитивања су вршена употребом ELISA теста и комерцијалних китова произвођача Тецна, Италија.

Резултати испитивања су показали присуство афлатоксина B1, охратоксина A и зеараленона у готово свим узорцима, осим у једном узорку оригана и у једном узорку каранфилића.

Како закључак наведених испитивања намеће се потреба за обавезним микотоксиколошким испитивањима сировина намењених изради производа од меса, првенствено зачини. Нарочито обавезним сматрају се испитивања присуства афлатоксина B1 и охратоксина A. Наведени секундарни метаболити познати су као веома токсични микотоксини који су уврштени у групу 1 хуманих карциногена.

КЉУЧНЕ РЕЧИ: ELISA, зачини, микотоксини, плесни, селективне подлоге

ACKNOWLEDGEMENT

The results from this paper are part of the Project III, No 46009 Improvement and development of hygienic and technological procedures in production of animal originating foodstuffs with the aim of producing high-quality and safe products competitive on global market, funded by the Ministry of Science of Serbia.