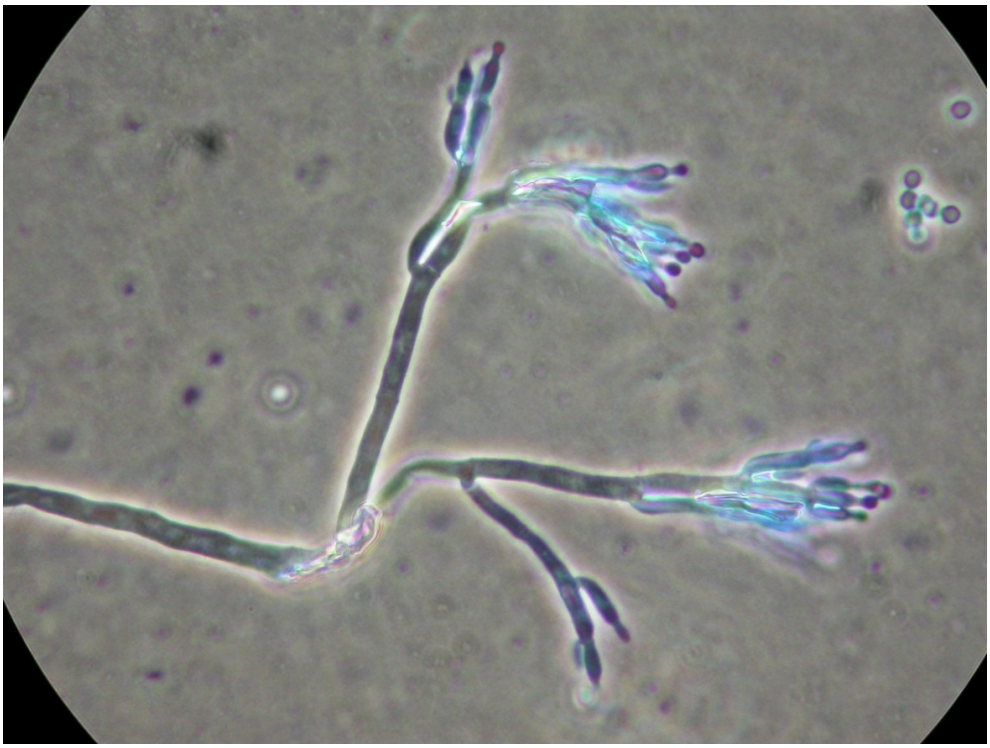
UPPSALA
UNIVERSITET

Survey of mycotoxin producing fungi in goji berries, oil seeds and walnuts on the Swedish market



Disa Eklöf

Degree project in biology, Master of science (1 year), 2013
Examensarbete i biologi 30 hp till magisterexamen, 2013
Biology Education Centre, Uppsala University, and the National Food Agency
Supervisors: Ann Gidlund and Elisabeth Fredlund

Abstract

In many foodstuffs the knowledge about contaminating moulds and their associated mycotoxins, is still fairly unknown. In this study the mycoflora and content of toxins in goji berries, oilseeds and walnuts on the Swedish market were examined. The foodstuffs were surveyed for their content of total fungi and xerophilic fungi and the concentrations of specific groups of potential toxin producers. Potential toxin producers were isolated and identified. The a_w was measured to evaluate storage stability and potential for toxin contamination. Levels of aflatoxins as well as ochratoxin A (OTA) in all commodities was measured by ELISA, and content of penitrem A in walnuts were measured by LC-MS/MS.

The a_w were below 0.6 in most samples (max 0.7) and the products were judged as storage stable. The average a_w was lowest in goji berries, and highest in pumpkin seeds, although one sample of walnuts had the highest measured a_w. No samples contained over 10^5 cfu/g total mould, but over 10^4 cfu/g mould was not uncommon. Goji berries had the highest yeast count, and lowest yeast count was seen in walnuts. Potential toxin producing species were isolated from all commodities, and all food stuffs contained *Penicillium spp.* although this genus dominated in sunflower seeds and walnuts. In sunflower seeds all samples contained *P. expansum*, and in walnuts both *P. crustosum* and *P. expansum* were common. Black and brown species of *Aspergillus* and *A. flavus/parasiticus* were seen in all commodities, but were most prevalent in walnuts. In goji berries the genus *Alternaria* was an important contributor to the mycoflora.

High prevalence of toxin producing species was detected in several commodities. This emphasis the importance of dry storage conditions to avoid toxin production. OTA was found in all samples of goji berries between 1.4-4.6 ppb. OTA was found in all sampled walnuts, and the concentration varied from 4.0 to 13.5 ppb. Aflatoxins were detected in 80% of the walnuts, with 4.3 ppb at the highest. No penitrem A was detected in the walnuts. The OTA content in goji berries and walnuts were worrying, and further studies should address these findings as well as the high content of *Alternaria* in goji berries.

(Picture on front page; *Penicillium chrysogenum* in sunflower seeds)

Index

1. Introduction

Foodstuffs are expected to be nutritional, appetizing and safe, but sometimes the process from field to store fails to provide a satisfactory product. Since foods are generally rich in nutrients they are also a desirable habitat for microorganisms (Pitt *et al.* 2009), and a large and common problem in the food industry is contamination and growth of fungi. In dried foods, which are often transported and stored for a long time, there is an enhanced risk of contamination and growth of microorganisms. Especially moulds are a problem due to their ability to grow in foods with a low content of water. The growth of fungi can lead to nutritional losses and deterioration of the food. Moulds which are able to produce toxic secondary metabolites can if the conditions are favorable, secrete potent and hazardous substances which will contaminate the commodity (Madigan *et al.* 2006). In this study, six different products with low content of available water were examined for mycoflora and mycotoxins. The examined foodstuffs were goji berries (wolfberries), pumpkin seeds, sesame seeds, sunflower seeds, melon seeds and walnuts.

1.1 Background

The goji berry or wolfberries (*Lycium barbarum*) belongs to the Solanaceae family, related to tomatoes and potatoes. The berries grow along the branches of the plant, which can grow up to 4.5 meters high, and is cultivated in Mongolia, Tibet and in Ningxia in China which is the largest producer (Gross 2006). Here it has been cultivated for over 600 years, and a yearly festival to celebrate the berry is held in August (Gross 2006, China Daily 2004). The berries are harvested by hand from June through October and are dried in the sun (Jing 2010). Goji berries have been used in traditional Chinese medicine for over 5000 years and has among many other applications, been used as a medicine for the vision (Gross 2006). It is eaten directly as dried fruit, or added to teas, soups or stews. In Europe the berries are commonly sold as a health beneficial commodity, since goji berries are rich in zeaxanthin which have anti oxidative properties (Cheng *et al.* 2005).

Melons and pumpkins belong to *Cucurbitaceae,* a family of tropic plants. Melons probably originate from east Africa and have been cultivated for thousands of years (Engstrand *et al.* 2002). During the Roman era it became famous in Europe, and today melons are cultivated all over the world with China and Turkey as the largest producers (Boriss *et al.* 2006). Pumpkins are especially cultivated in USA (Engstrand *et al.* 2002) and may originate from Central America, where 7500 years old seeds have been found (Kiple et al. 2000). Pumpkins cultivated for their seeds belong to *Cucurbita pepo var. styriaca* or *oleifera*. The seeds are in the first case only enveloped by a very thin shell. Pumpkin seeds are grouped as an oil seed containing up to 60 % oil. Most of the production goes to the food industry, but some is also used in the medical industry. When the fruit is mature the seeds are removed, rinsed with water and dried before the oil is pressed out (Bavec *et al.* 2007). Melon seeds of *Citrullus colocynthis* contain up to just over 50% oil (Milovanović *et al.* 2005), and oil from melon seeds are used in cooking, especially in African and Middle Eastern American countries (Akoh *et al.* 1992).

Sesame seeds are the seeds of the broad leaf plant *Sesamum indicum* (Sesaco Corporation 2008). It is the oldest known oil seed crop and the origin is not clear (Ram *et al.* 1990). Nayar et al (1970) suggested that the plant was domesticated in Africa. A systematic study however indicated south Asia as the origin (Venkataramana *et al.*1999). The plant can grow up to 1.8 m high and the seeds are produced in capsules (Sesaco 2008). At harvest the whole plant is gathered, and is stored upright. As the capsules dries they rupture and the seeds are shaken out. The seeds have an outer shell which is often removed (Naturland 2002). The seed contain 50 to 60% oil, which is very stable due to natural antioxidants. The plant is cultivated in many parts of the world but Asia accounts for the largest part of production (Ram *et al.* 1990). According to Rajendra *et al.* (2012) India, China, Sudan, Mexico, Turkey, Burma and Pakistan are the important sesame seed producing countries.

The seeds of sunflower (*Helianthus annuus),* may first have been cultivated by Indian tribes in North America, about 3000 B. C. The seeds were used as a food, medicine and as a religious symbol. The plant was brought by Spanish travelers to Europe sometime around 1500 AD. The sunflower thrives in temperate climate and is today cultivated in USA, Europe, Russia and Canada among others. For the food industry the seeds are largely cultivated for oil production, but the seeds are also used for food or bird feed (National Sunflower Association 2013). Sunflower seeds can contain up to 45% oil (Thomas Jefferson Agricultural Institute 2013).

Walnuts are the kernel of the fruit of the walnut tree *Juglans regina*. The tree grows wild both in eastern and western hemispheres and is the oldest known tree crop; dating back to 7000 B. C. Walnuts have traditionally been cultivated in the South of Europe (CFAITC 2011). Today large producers of walnuts are California (USA), China, Turkey, Iran, and Chile, and production is increasing in Asia. When the fruit matures, the flesh of the fruit breaks open, the kernel loosens and the harvest starts. At harvest the nuts are shaken from the tree into nets, after which they are washed dried and sorted and eventually distributed into the market (Risenta 2013).

1.2 Spoilage

As foods are spoiled its characteristics is altered so that the product is no longer acceptable, and according to (EC) 178/2002 food shall not be placed on the market if it is unfit for human consumption. This is a subjective assessment, and foods that may be safe for consumption is discarded, leading to economic losses for the food industry. Since foods are often rich in nutrients, they are also a good substrate for microorganisms (Pitt *et al.* 2009), and most spoilage is due to microbial activity (Adams *et al.* 2010). The microbial spoilage is often noticed by chemical products of the microbial metabolism. This is often flavors and off-odors, but can also be pigments, gas and polysaccharides (Adams *et al.* 2010). Growth of fungi on foods causes the foods to degenerate and reduces the quality of the food by decreasing the nutritional value. Chemical and physical properties of the product are factors which effect how susceptible the food will be to microbial activity (Adams *et al.* 2010). The speed of the process primarily depends on temperature and available water (Northolt *et al.* 1995).

1.3 Water activity (a_w)

The most important factor for microbial growth is availability of water. Water activity (a_w) is the measure of water available for use by microorganisms in their metabolic processes. Pure water has an a_w of 1.00 during which the water molecules are loosely ordered. The a_w decreases if the substrate is dried. Water molecules in this situation arrange themselves around molecules of solute in the food and become less available, and the microbial cells must compete with the solute for the water molecules (Adams *et al.* 2010). Fungi are better competitors for the free water then bacteria. Most bacteria are inhibited at about a_w 0.96, molds however compete well for water, and often have a good growth, at low a_w (Madigan *et al.* 2006). Growth of fungi can be prevented by drying and keeping the commodity at a_w below 0.65 (Northolt *et al.* 1995). Propagules can still survive below this a_w in inactive state, but spoilage of food below this value will be due to other factors such as chemical reactions or insect damage (Adams *et al.* 2010).

1.4 Fungi

Fungi are heterotrophic organisms (Adams *et al.* 2010) which degrade their substrate by secreting metabolic enzymes (Madigan *et al.* 2006). The process releases soluble nutrients, which the fungi absorb through the cell wall and membrane (Adams *et al.* 2010). The fungus in this way gains access to energy and carbon locked in molecules of the substrate, and during the primary metabolic process new cell material is built and metabolites are formed. Under certain conditions some of them accumulate. Secondary metabolites, which are not necessary for growth or energy supply of the fungi, are mostly formed in the end of the growth, and sometimes these are toxic substances (Northholt *et al.* 1995).

Filamentous fungi (moulds) grow by extension of the hyphal tip. The fungi grow through and over the substrate, forming a mycelium by branching and anastomosis. (Adams *et al.* 2010). Many of the most important moulds involved in food spoilage are dispersed by conidia; these are asexual spores often smaller than 4 µm, resistant to light damage and desiccation. They are produced in large quantities and are passively dispersed by the air through wind or disturbance. Both the genera *Penicillium* and *Aspergillus*, which are responsible for a large deal of food spoilage, are dispersed in this passive manner (Adams *et al.* 2010). Germination can take between 1 to 10 days depending on how favorable the conditions are (Northolt *et al.* 1995). In contrast to yeasts and bacteria, most moulds (including the genera *Penicillium* and *Aspergillus*) have an absolute requirement for oxygen (Sholte *et al.* 2004). Yeasts are single cell organisms, reproducing by budding (Pitt *et al.* 2009). The need for oxygen varies (Visser *et al.* 1990), and they can be obligate fermentative, facultative fermentative or non-fermentative (Barnett *et al.* 1983). Many types of yeast have good growth under strictly anaerobic conditions (Pitt *et al.* 2009), during which ethanol and organic acids are formed (Northolt *et al.* 1995). Commonly yeasts are found on surfaces of plants or fruits, where they exploit exudate with nutrients emitted from the plant (Adams *et al.* 2010).

1.5 Fungi as food spoilers

At normal conditions fungi can't compete with the much faster growing bacteria, but in foodstuff with low pH levels the fungi are given free rein since they tolerate more acidic conditions then bacteria (Pitt *et al.* 2009). Dry foods are poor habitats for bacteria (Adams *et al.* 2010) and most species of yeasts does not either tolerate low a_w, since the budding type of reproduction limits spread on solid substrates. In these conditions the filamentous fungi are favored (Pitt *et al.* 2009). Yeasts have also compared to filamentous fungi more fastidious nutritional requirements, and prefer sugar rich substrates (Pitt *et al.* 2009). Still, yeasts are responsible for enormous losses of foods every year, and especially if the manufacturing practice is neglected they can be a problem (Pitt *et al.* 2009).

The natural amount of fungi varies between commodities and environments (Pitt *et al.* 2009). Predominance of a species can be a result of abundant contamination from an ecological niche where a specific species of fungi is highly represented. The inoculum can be spores, conidia or mycelium fragments, and the food stuff can be contaminated from the environment at all stages of the production (Northholt *et al.* 1995). Generally total fungal counts are poor indicators for spoilage potential, since many of the fungi isolated will not be responsible for spoilage or even grow in the food (Adams *et al.* 2010), but for the food industry it is important to be aware of the natural levels, since heightened levels of mould or yeast could indicate unsanitary production conditions as well as poor conditions during cultivation or storage (Sholte *el al.* 1995). Risenta uses a total count of 10^4 cfu/g as their maximum allowable level for total mould. Above this count the food will also have a taste of mould. Usually below 10^3 cfu/g are found in the products from Risenta (personal communication, Monica Demorior, Risenta). The propagules are not in them self a problem, but if growth are allowed both quality and storage stability of the product can be effected, wherefore content of fungi is preferred low (Sholte *el al.* 1995). Storage stability is achieved if the crop is

harvested with little damage and dried to an appropriate a_w, and as long as storage temperature and moisture are held moderate. The food should also be kept safe from insects and rodents. Damage from these (both in storage and in field) can lead to secondary invasion by moulds and subsequent spoilage, since propagules can be carried by the pests (Adams *et al.* 2010). Most molds have an optimal growth around 30°C, and in storage temperature is held low to restrict germination and microbial growth (Adams *et al.* 2010). Keeping the temperature uniform is also important; the a_w can otherwise increase locally since the moisture can migrate to colder areas, which can led to growth (Northolt *et al.* 1995). Also, if foods with low a_w is stored in high humidity, water is transferred to the food increasing the a_w. In both cases condensation on surfaces creates local regions with higher a_w, in which still viable propagules are able to propagate. As a result of respiration water is produced, and the a_w is then locally increased even more, making it possible for more demanding microorganisms to grow (Adams *et al.* 2010).

1.6 Storage fungi

Fungi associated with food and feed are divided into two groups; field- and storage fungi (Northholt *et al.* 1995). The term field fungi refer to fungi, of which many are plant pathogens, inoculating and growing on the nut or seed in the field before harvest. These seldom cause further deterioration after harvest since the storage fungi becomes dominant (Pitt *et al.* 2009). Storage fungi germinate in the food during processing or storage (Pitt *et al.* 2009). In non-living foods, conditions and properties of the product correlates with species domination, and the best adapted fungi will propagate (Northholt *et al.* 1995). In dried foods xerophilic fungi are commonly isolated. These are moulds and yeasts which are able to grow at low a_w. Many of the xerophilic fungi have an optimal growth at quite high a_w, but due to their slow growth, they are not able to compete in mixed cultures at higher a_w. Common moderate xerophilic fungi are the filamentous fungi *Eurotium spp., Aspergillus spp.* and *Wallemia spp.* (Pitt *et al.* 2009), but also *Penicillium spp*. belongs to this group (Northolt *et al.* 1995).

Species of *Aspergillus* (and its sexual reproductive state *Eurotium*) tolerate higher temperatures and lower a_w then *Penicillium spp. Penicillium* has an advantage in their species diversity, but Aspergilli are usually less fastidious and grow faster producing less fragile conidia. *Aspergillus* thrives in warm and humid climate and dominates the spoilage in tropic and subtopic regions (Pitt et al. 1997), while *Penicillium* species dominate in temperate zones like Sweden, having a lower minimal temperature for growth as well as production of toxins then Aspergilli (Northolt *et al.* 1995). Some fungi commonly isolated from stored foods with low a_w are presented in table 1.

Table 1. Minimal a_w:s for germination and growth of some moulds commonly isolated from stored foods and their minimal a_w for growth (table 1; Northolt *et al.* 1995).

Species	a_w
Wallemia sebi	0.69-0.75
A. candidus	0.75-0.78
A. ochraceus	0.76-0.83
A. niger	0.77[1]
A. sydowii	0.78
A. tamari	0.78
A. flavus	0.78-0.80
P. crysogenum	0.78-0.81
P. citrinum	0.80-0.82
P. verrucosum	0.81-0.83
P. expansum	0.82-0.85
P.roqueforti	0.83
Alternaria Alternata	0.85-0.88

[1] Avari *et al.* 1983

1.7 Fungal flora in selected commodities

Nuts and oilseeds are commodities rich in oils, generally dried in the field before harvest. The crops have similar microbial problems, and are vulnerable to spoilage by fungi (Adams *et al.* 2010). The reason is the low content of carbohydrates able to bind water, with the consequence that only a small rise in moisture will give a considerable rise in a_w (Pitt *et al.* 2009). Many moulds colonizing these commodities have strong lipolytic activity which increases with a_w. Oils are metabolized into fatty acids, and the fatty acids can undergo oxidation which can result in rancidity. *Aspergillus* and *Penicillium* are important lipolytic genera at lower a_w, and *A. niger* is a common problem. If the nut or seed is damaged the process is even faster, since the damage may give chance for more contact with enzymes and substrates (Adams *et al.* 2010). Crops protected by a shell in the field rarely suffer from invasion pre-harvest (Pitt *et al.* 2009). But if the shell is damaged inoculation of fungi will also be possible (Bayman *et al.* 2002, Fuller *et al.* 1977).

Spoilage specific to walnuts, is especially caused by growth of *Eurotium* spp., but also *A. flavus* which is equally adapted to both field- and storage conditions, is a common problem (Pitt *et al.* 2009). Bayman *et al.* (2002) found that *A. flavus, A. niger, Penicillium ssp.* and *Rhizopus,* are common in store bought nuts, and a few times also *A. ochraceus, A. fumigatus* and *A. tamaarii* were found (Bayman *et al.* 2002). In a study of Indian walnuts, especially three species were identified as the major contributors to the mycobiota; *A. flavus, A. niger,* and *P. citrinum. Rhizopus spp.* was also commonly found (Singh *et al.* 2008). Khayria *et al.* (1993) found Penicillium to be a common genus in walnuts. Most common species were however *A. flavus* and *A. niger*.

There are few studies done on the mycoflora of small oil seeds. In sunflower seeds Shahnaz *et al.* (1991) and Sharfun-Nahar *et al.* (2005) found high incidence of *A.flavus* and *A. niger*, as well as *Penicillium* spp. (Sharfun-Nahar *et al.* 2005). A*. ochraceus* along with several other species was also seen as a contributor to the mycoflora of sunflower seeds (Sharfun-Nahar *et al.* 2005). *Alternaria* and *Fusarium* was also found (Shahnaz *et al.* 1991, Sharfun-Nahar *et al.* 2005). Jimenez *et al.* (1991) found highest counts of *A. niger* and *Penicillium spp*.

The most important species in sesame seeds are according to Chakrabarti (1987) *A. flavus, A. niger, A. tamarii, Cladosporium herbarum, Macrophomnia phaseoli, Penicillium funiculosum* and *Penicillium citrinum*. Venkatesagowda *et al.* (2012) isolated *A. niger* from 27.3 % of samples, *Mucor racemosus* from 48.6 % and *Rhizopus stolonifer* from 21.3 % of sampled sesame seeds. In a study from 1988 of sesame seeds from Sierra Leone five common *Aspergillus ssp.* were identified, these were *A. flavus, A. tamari, A. ochraceus, A. japonicas* and *A. niger* (Jonsyn 1988).

Widenböner (2001) examined the mycoflora of pumpkin seeds, and found *Penicillum* to be the dominating genus, followed by *Eurotium* and *Cladosporium.* In melon seed Bankole *et al.* (2004 and 2006) found *Aspergillus* and *Penicillium* to be the dominating genera, with *A. flavus* as the most common species. *A. niger* was also commonly found.

Since fruits have a high pH the spoilage is mostly due to fungi and not bacteria. It can be yeasts, but moulds are usually the largest contributor (Adams *et al.* 2010). In dried fruits and berries *P. expansum* and species of *Aspergillus* is often seen (National Food Agency 2007). The knowledge about mycoflora in dried goji berries is very limited. One Chinese study, published in Chinese, examined the seed borne mycoflora in goji berries from the Ningxia region. From the external parts of the berry 8 genera and 11 species were found, and from the internal parts of the berry 6 genera and 6 species were found. No difference in mycoflora was found in berries stored 2 or 3 years (Zhang 2008).

1.8 Mycotoxins

As moulds grow in a commodity, it does not create the putrefactive degradation associated with bacteria, and therefore the foods is sometimes eaten even though infected, which can result in ingestion of toxins (Adams *et al.* 2010). The fungi themselves are not toxic (Madigan *et al.* 2006), but their secondary metabolites can sometimes be hazardous substances. These are mycotoxins such as aflatoxins, ochratoxin A ,penitrem A, sterigmatocystein, roquefortin C, PR toxin and cyclopiazonic acid (Northholt *et al.* 1995). Yeasts are not known to produce mycotoxins (Pitt *et al.* 2009). There are hundreds of known mycotoxins produced by a large number of mould species. For production of toxins the demands on the substrate, as well as on the environmental factors, is different than for growth. Toxin production often requires a higher a_w than growth, as well as more available oxygen. Less favourable conditions can also result in less potent or stable toxins, or limited production (Pitt *et al.* 2009, Northholt *et al.* 1995). The chemistry of the substrate can also affect production of toxins. For example production of aflatoxins is stimulated by the presence of fatty acids, specific amino acids and zinc. Other microorganisms can also inhibit growth and formation of toxins (Northolt *et al.* 1995).

The total count of fungi is a poor indication for toxin contamination. Especially when foods are stored for a long time fungi will gradually die, while the stable toxin remains. The fungi may also grow on structures like shells that are removed during processing while the toxin is gathered in the core product (Pitt *et al.* 2009). High content of certain toxin producing fungi can however be alerting, giving an indication that a toxin analysis should be performed for evaluation of potential health risk. Some species are more important, *Aspergillus flavus/parasiticus, Penicillium verrucosum* and black or brown species of Aspergilli, are all species that are not desired in high concentrations (Pitt *et al.* 2009). The mycoflora and associated mycotoxins vary between commodities. In nuts aflatoxins, ochratoxin A (OTA) and penitrem A is a potential risk, and in oil seeds aflatoxins could also be a threat. Dried fruits and berries risk containing patulin and OTA (National Food Agency 2007).

1.8.1 Aflatoxins and aflatoxigenic species

The interest for mycotoxins started with the death of several thousand poultry in East Anglia, and was initially called turkey X disease. The origin of the outbreak was revealed to be groundnut meal in the pelleted feed for the birds, which was contaminated with aflatoxin producing *A. flavus.* The toxin was discovered to be a problem in many commodities, especially maize and groundnuts. In 1974 about 1000 people were poisoned by aflatoxin B_1 in mouldy maize in India. Nearly 100 of them died. A similar event occurred in Kenya in 2004, where 317 poisonings and 125 deaths occurred, also originating from maize (Adams *et al.*2010).

There are several types of aflatoxin and all are genotoxic and carcinogenic substances (European Union 2013). Aflatoxin B_1 and B_2 is produced by *A. flavus* as well as *A. parasiticus* and *A. nomius* which also produces G_1 and G_2. Aflatoxins (especially B_1) are mainly known as a liver carcinogen, but can also be acutely toxic. Acute effects are liver damage, and in worst case death. It has been shown that aflatoxin and hepatitis B in combination, in some parts of the world increases the risk for liver cancer, wherefore vaccination against the virus could reduce the magnitude of liver cancer (JECFA 1999). Kupier-Goodman (1995) states that because aflatoxins are genotoxic carcinogens agencies choose not to establish a tolerable daily intake (TDI). This is done out of a safety approach, and levels of genotoxic carcinogens are instead recommended to be held as low as it is technically possible. As guidance to industries Kuiper-Goodman establishes the TDI to 0.11-0.19 ng/kg body weight of Aflatoxin B_1, based on epidemiological data from countries where consumers in addition to Aflatoxin are exposed to the Hepatitis B virus (Kuiper-Goodman 1995).

Since species of *Aspergillus* are especially widespread in the tropics and subtropics aflatoxins are common contaminants in products produced and stored in these areas (Adams *et al.*2010). Optimal conditions for toxin production by *A. flavus* and *A. parasiticus* is at 33 °C and a_w 0.99 (Magan *et al.* 2004). But they can be produced from a_w 0.86 (Pitt et al. 2009). Production of aflatoxins is often a result of poor storage at high temperature and humidity, but the toxin can also be produced in growing crops before harvest. The toxin producing species of *Aspergillus* are able to establish an endophytic relationship with the plant, and can if the plant is stressed produce low but still significant amounts of toxin. This can for example occur during drought (Adams *et al.*2010). The risk of toxin production is also enhanced if harvest is performed in humid weather, and if drying of the commodity is poorly executed. Also if a crop is dried in the sun there is an enhanced risk for toxin production, partly because the drying will take long time (Pitt *et al.* 2009).

Jonsyn (1988) found aflatoxin B_1 producing *A. flavus* in sesame seeds from Sierra Leone (Jonsyn 1988). Bankole *et al.* (2004) found that 32% of melon seeds from forested areas in Nigeria contained 14.8 µg/kg aflatoxin B_1, and 21% of melon seeds from the savanna contained 11.3 µg/kg aflatoxin B_1. Bankole *et al.* (2006) found similar amounts in the areas and above 5 µg/kg of the toxin was detected in 32.2% of the samples. Jimenez (1991) found 68% of sampled sunflower seeds to be contaminated with *A. flavus*. 54 isolates of *A. flavus* were found, of which 4 produced aflatoxins. The samples did however not contain any toxin (Jimenez *et al.* 1991). In a study from 2008 where walnuts from India were examined, *A. flavus* were commonly isolated. 39% of the isolates exhibited production of aflatoxin. 83% of stored walnuts contained aflatoxin B_1 with a concentration of 140-1220 µg/kg (Singh *et al.* 2008). Gürses (2006) tested 24 samples of walnut from retails and markets in Turkey, for aflatoxin B_1 and found 6 samples positive. The mean were 22.1 with a range of 3-28 µg/kg. Khayria *et al.* (1993) found however no aflatoxins in analyzed walnuts from Saudi Arabia.

1.8.2 OTA and responsible fungi

OTA was first isolated in South Africa from *Aspergillus ochraceus* (Adams *et al.*2010) in 1965, during an extensive scan of fungal metabolites, designed to detect new mycotoxins (Kumar *et al.* 2008). The toxin is now known to be produced by several other fungi (Adams *et al.*2010), including a few black and brown species of *Aspergillus* comprising *A. ochraceus, A. sulphureus, A. niger* as well as *A. carbonarius*, which is the most important toxin producer (Samson *et al.* 2004). It is also produced by two species of *Penicillium; P. verrucosum* and *P. nordicum* (NMKL 2005). OTA is a quite stable and potent nephrotoxin, which may cause kidney cancer (Adams *et al.*2010). The toxin is also recognized to be teratogenic, immunotoxic, and possibly also neurotoxic (NMKL 2005). According to Joint FAO/WHO Expert Committee on Food Additives (JECFA), a tolerable weekly intake of ochratoxin A is 100 ng/kg of body weight (JECFA 2001).

OTA is a widespread toxin occurring in many commodities (Adams *et al.*2010). Most OTA contamination in temperate countries is due to Penicillia. Among them, *P. verrucosum,* can produce the toxin from a_w 0.83 (Sanchis *et al.* 2004). When OTA is present in foods from tropic or subtopic regions the contamination is usually due to species of *Aspergillus* (Adams *et al.*2010), and *A. ochraseus* needs a minimum a_w of 0.83 for OTA production (Sanchis *et al.* 2004) *A. carbonarius* needs a_w 0.95 for toxin production, producing the toxin optimally at cooler temperatures (Pitt *et al.* 2009).

Research in toxin content of goji berries is very limited. One recent study examined OTA content of 36 samples of Chinese wine, made from Chinese goji berries. The samples were shown to contain low levels of OTA. 41.7 % of the samples contained OTA, below the limit of quantification, one sample were shown to contain 0.24 ng/ml (Kuang *et al.* 2012). For wines the legislated limit value of ochratoxin A is 2 ng/ml (EC 1881/2006). In 2010 a survey of mycotoxins in Jordan foods detected OTA in one of three sampled sunflower seed, but not in any of the two samples of sesame seeds (Salem *et al.* 2010). However Jonsyn (1988) found OTA producing *A. ochraceus* in sesame seeds from

Sierra Leone (Jonsyn 1988). In a study of OTA content in 20 samples of Moroccan walnuts, the toxin content were found to vary between 0.04 (±0.01) µg/kg to 0.23 (±0.01) µg/kg (Zinedine *et al.* 2006).

1.8.3 Other mycotoxins and toxin producers

Tremogens is a group of toxins which includes penitrem A and roquefortin C. These toxins can affect the central nervous system, and the symptoms can be prolonged tremors, vomiting or in extreme cases death (National Food Agency 2007). Roquefortine C is a metabolite of many common *Penicillum* species (Scott 2004). A few examples are *P. roqueforti, P. crysogenum*, and *P. expansum* which also produce patulin (Samson *et al.* 2004). Penitrem A is only produced by *P. crustosum*. In 1981 a case of acute poisoning in a dog from Penitrem A, originating from *P. crustosum* in walnuts, were examined (Richard *et al.* 1981), however there is still little knowledge about these toxins, and few studies have been performed. Most strains of *P. crustosum* are able to produce Penitrem A, however a_w over 0.92 is necessary for the production of toxins (National Food Agency 2007). In a survey at the National Food Agency, performed from 2004-2005, *P. crustosum* was found to be a common species in walnuts.

The genus *Fusarium* includes species which produce a large number of toxins, mainly in the field. Grains and maize are commonly contaminated with these fungi. Important toxins which are also regulated by law are deoxynivalenol and zearalenon. These toxins are mainly produced by *Fusarium graminearum* and *F.culmorum* (Adams *et al.*2010). Also fumonisins, produced by *F. proliferatum* and *F. verticilloides*, as well as T2 and HT2 produced by *F. langsethiae* have maximum allowable levels and recommended values (EC 1881/2006).

1.9 Control of quality

Quality control of food and food production is regulated at several levels. A series of EU-legislations (Commission Regulations) regulates hygiene, safety and content of foreign substances including mycotoxin in some selected products. The food producing company has according to (EC) 852/2004 the primary responsibility for the safety of the product, and is obligated to have an HACCP plan. Critical control points (CCP) in the production are established, and safe production can therefore be guaranteed (EC 852/2004). Since the company has the primary responsibility, control of the quality of their products is done by demanding product data as well as quality specifications from their suppliers. In many cases the supplier has a quality certificate (BRC or ISO22000 are examples) which verifies good quality of the production. But if these are lacking, the company may visit the supplier and evaluate the safety. As shipments arrive to the company the commodities are visibly examined (which is the first CCP in the production), and if quality is questioned the product is sent for analysis (personal communication, Monica Demorior, Risenta). The European Commission has an alert system, which is used to inform a network of governmental agencies, if food hazardous for health has entered the market. It is called Rapid Alert System for Food and Feed (RASFF) (National Food Agency 2013) If levels of toxin over a maximum allowable value is detected this is reported and documented in the RAFF- statistics and the product is not allowed on the market.

In (EC) 1881/2006 maximum allowable levels for contaminants including some mycotoxins are laid down, for commodities where high levels of these toxins have been seen. There is also specified the lowest maximum levels which are achievable, if Good Hygiene Practice, Good Manufacture Practice and Good Agricultural Practice practices are followed (EC 1881/2006). Since many toxins including aflatoxins are very heterogeneously distributed in commodities (especially in nuts and other products of larger particle sizes), control and sampling is of great importance to ensure a trustworthy result. In (EC) 401/2006 methods for sampling are defined for food stuff in which maximum levels are set. For oil seeds, walnuts (the amount applies the edible parts only) and dried fruit intended for direct consumption the limit for B_1, is 2.0 µg/kg, and for the sum of the aflatoxins 4.0 µg/kg. The toxin levels in nuts and dried fruit can be reduced by sorting or other mechanical

treatment, and to limit the impact on trade, higher levels of aflatoxin is allowed in commodities not intended for direct consumption. For those 5 respectively 10 µg/kg are the maximum levels for B_1 and the combined aflatoxins (EC 1881/2006). OTA has maximum limits for dried grapes; 10.0 µg/kg, grains and grain products intended for direct consumption; 3.0 µg/kg, and unprocessed cereals; 5 µg/kg (EC 1881/2006). There are no maximum limits regarding OTA established for the foodstuffs included in this study, and no maximum allowable levels are set for penitrem A and roquefortine C.

1.10 Purpose of the survey

The knowledge about incidence of toxin producing fungal contaminants as well as toxin levels, are in many commodities limited. As a result the legislated limit values on mycotoxins in food stuffs are limited to a few mycotoxins in the most important types of food (EC 1881/2006). It is therefore of importance to continuously examine and analyze foods with respect to fungi and their secondary metabolites. This study was an exam work, but the purpose was also to provide a basis for further studying and analysis by the National Food Agency.

The objectives were;
A: To examine the total amount of fungi present in 10 samples of goji berries, pumpkin seeds, sesame seeds, sunflower seeds and walnuts, as well as one sample of melon seeds.
B: To identify presence of some of the relevant toxin producing fungi.
C: To identify the a_w of the commodities to evaluate storage stability.
C: To find the amount of aflatoxins, ochratoxin A, and for walnuts also penitrem A.
D: To examine correlations between results from a_w measurement with mycological and chemical analysis

1.11 Hypothesis

Previous studies at the National Food Agency indicated that goji berries were highly contaminated with mould. Therefore it was of interest to examine the mycoflora and evaluate risk of toxins. It has been discovered that melon seeds are often contaminated with aflatoxins, and an increased import control per year on 50% of melon seeds from Nigeria (EU 91/2013) and Sierra Leone (EU 1235/2012), has been introduced, while no control on sesame seeds, sunflower seeds or pumpkin seeds is done (1-2 RASFF). Therefore it was of interest to examine these similar products, to evaluate potential problems and risks. Many studies have encountered significant contamination of fungi as well as toxins in walnuts, and content of aflatoxin is regulated (EC 1881/2006). Other non-regulated toxins could however also be a risk.

2. Material and methods

Analysis of the samples was performed in three parts; 1) quantification of fungi, 2) identification of toxigenic mould species and 3) chemical determination of mycotoxins. The aim of the first part was to determine the amount of total fungi (cfu/g) as well as amount of specific species present in the samples. The second part included identification of some potential toxin producing *Penicillium* and *Aspergillus* species by morphological criteria. The chemical analysis was performed in order to determine the concentration of ochratoxin A and aflatoxins in the samples.

2.1 Samples and sampling

A total of 53 samples were sampled and analyzed (table 2). 10 samples of goji berries (wolfberries), walnuts and sesame seeds, and 11 samples of shelled sunflower seeds and shelled pumpkin seeds were collected. One sample of melon seeds was also analyzed. The sesame seeds included shelled and unshelled, and white and black seeds. The samples where dried but not further processed, except for two samples of roasted sunflower and pumpkin seeds, which were not included in the microbiological analysis. No products with preservatives were included. Goji berries of the brand Risenta were not sampled, since these contain sulfur dioxide, an antioxidant that also inhibits growth of bacteria and fungi (Shee *et al*. 2010). Some samples were certified as organic which ensures specific environment friendly standards as well as livestock welfare with restrictions regarding fodder and medications. Chemical fertilizers, chemical pesticides, irradiation or GMO is not allowed. Standards on the production of foods are regulated by the council regulation (EC) 834/2007 and the Commission regulation (EC) 889/2008 of the European Union (National Food Agency 2013).

The samples were all purchased in Uppsala except for one sample of goji berries that was sent to the National Food Administration, due to complaints from a consumer. The samples were purchased in January and February 2013, in larger supermarket chains, smaller local stores, health food stores, and stores with predominantly ethnic products. The samples were mostly bought as pre-packed bags, but a few samples were bought in bulk. To obtain representative samples, a minimum of 400 grams from each batch was purchased. However, there were two exceptions, the sample from the consumer only contained 67 grams, and for one sample of goji berries only 335 grams was possible to collect from one batch. In many cases several sub-samples (small packages of the same batch) were bought to obtain appropriate sample amount. For each sample a delivery note was written with sample information (table 2). At arrival to the laboratory, the sample was transferred to sealable plastic bags, and the total weight was established. The samples were stored in dark below 5 °C until further handled.

Table 2. Sampling information of the sampled foodstuff including name of manufacturer, type of store, sample origin and agricultural practice (organic/conventional). The MI numbers is given to all products when included in a survey at the National food agency, and is used so that later identification will be possible. 53 samples in total of goji berries (G), pumpkin seeds (P), sesame seeds (Se), sunflower seeds (Su), melon seeds (M) and walnuts (W) were sampled from larger supermarket chains (1), stores with predominantly ethnic products (2), health food stores (3) and smaller local stores (4). All samples were sampled in Uppsala, except for sample 65 which was sent to the National Food Agency after a consumer complaint.

MI-nr.	Sample type	Manufacturer	Origin	Type of store	Packaging	Organic/ conventional
38	W	Ica gott liv	unknown	1	pre-packed	conv.
39	G	Ica gott liv	unknown	1	pre-packed	conv.
40	Su	Risenta	unknown	1	pre-packed	conv.
41	Su	Saltå Kvarn	China	1	pre-packed	organic [a]
42	P	Risenta	unknown	1	pre-packed	conv.
43	Se [b]	Risenta	unknown	1	pre-packed	conv.
44	Se [c]	Risenta	unknown	1	pre-packed	conv.
49	G	AGLOB	China	2	pre-packed	conv.
50	Se [d]	Marque Depose	Thailand	2	pre-packed	conv.
58	W	X-tra	unknown	1	pre-packed	conv.
59	P	Saltå Kvarn	China	1	pre-packed	organic [a]
60	G	Superfruit	China	3	pre-packed	conv.
63	W	Alesto	California	1	pre-packed	conv.
64	G	Supernature	China	3	pre-packed	conv.
65	G	Superfruit	China	Unknown	pre-packed	conv [f]
78	G	Delta Food	unknown	2	pre-packed	conv.
79	W	unknown	Spain	2	bulk	conv.
80	Su	Granat	unknown	2	pre-packed	conv.
81	P	Delta Food	unknown	2	pre-packed	conv.
82	W	Prima	USA	4	pre-packed	conv.
83	Se [c]	Marque Depose	Thailand	2	pre-packed	conv.
84	W	Delta Food	unknown	2	pre-packed	conv.
85	Se [c]	Delta Food	unknown	2	pre-packed	conv.
86	W	unknown	unknown	2	bulk	conv.
87	P	Biofood	China	3	pre-packed	organic [a]
98	G	Eneboga Nötter AB	China	3	pre-packed	conv.
99	M	Risenta	unknown	1	pre-packed	conv.
100	Se [c]	coop änglamark	unknown	1	pre-packed	organic [a]
101	Su	Urtekram	unknown	1	pre-packed	organic [a]
102	P	Urtekram	unknown	1	pre-packed	organic [a]
103	Su	Biofood	China	1	pre-packed	organic [a]
104	G	Urtekram	unknown	1	pre-packed	organic
113	Se [c]	Saltå Kvarn	Bolivia	1	pre-packed	organic [a]
114	Se [b]	Urtekram	unknown	1	pre-packed	organic [a]
115	P	coop änglamark	unknown	1	pre-packed	organic [a]
116	G	unknown	China	1	pre-packed	conv.
126	Se [c]	ica i love eco	Italy	1	pre-packed	organic

127	P	Kung Markatta	unknown	1	pre-packed	organic [a]
128	Su	Kung Markatta	unknown	1	pre-packed	organic [a]
142	W	ATCO	unknown	1	pre-packed	conv.
143	Su	Risenta	unknown	1	pre-packed	conv.
144	P [e]	Go green	China	1	pre-packed	conv.
145	Su [e]	Saltå Kvarn	China	1	pre-packed	organic [a]
146	Su	Risenta	unknown	1	pre-packed	organic
147	P	Risenta	unknown	1	pre-packed	organic
148	Su	Ica gott liv	unknown	1	pre-packed	conv.
149	P	Ica gott liv	unknown	1	pre-packed	conv.
150	W	Parrots	unknown	1	pre-packed	conv.
151	Se [d]	Longevity	China	2	pre-packed	conv.
152	G	Nice Crown	China	2	pre-packed	conv.
153	W	Tant Grön	Moldavia	1	pre-packed	organic
154	P	Tant Grön	China	1	pre-packed	organic [a]
155	Su	Risenta	unknown	1	bulk	conv.

[a] KRAV- certified product, a Swedish labeling of organic foods with high demands on the production.
[b] Unshelled white seeds
[c] Shelled white seeds
[d] Black seeds
[e] Roasted sample
[f] The sample was sent to the National Food Agency from a consumer, and the sampling data was therefore unknown.

2.2 Water activity (a_w)

Before the samples were stored the water activity (a_w) was measured in an AquaLab series 3 device. In this the precise dewpoint temperature of the sample is determined by an infrared beam focused on a tiny mirror. The dewpoint temperature is then translated into a_w (aqualab 2013). The average a_w, as well as standard deviation for the a_w was calculated for each sample type.

2.3 Quantification of fungi

Each sample was analyzed for the total amount of moulds and yeasts and for the concentration of potentially toxigenic species. The quantification of fungi in the samples was performed according to NMKL (2005). At first 40.0 grams of each sample was weighed in a stomacher filter bag. After 30 minutes of soaking in peptone water without salt, the sample was crushed in a stomacher device for 2 minutes. Each sample was diluted and the 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} dilutions were spread on DG18 plates (Samson *et al.* 2004). To lower the limit of quantification, the 10^{-1} dilution was also analyzed by pipetting 1 ml of sample suspension directly in a petri dish after which approximately 30 ml of liquid DG18 was poured (NMKL 2005). Samson *et al.* (2004) recommend DG18 as a general substrate for foods of a_w >0.90, and as a selective substrate for xerophilic fungi in foods of a_w <0.90. The selective principles is the high concentration of carbohydrates (Samson *et al.* 2004), and the dichloran which inhibits the growth of fast growing fungi (Hocking *et al.* 1980) enabling quantification of colony forming units (cfu). DG18 is also a diagnostic media for *Penicillium verrucosum*, which is recognized by a characteristic read reverse, a result from the reaction between fungal metabolites and trace elements added in the substrate (Samson *et al.* 2004).

The plates were incubated for 7 days at 25 °C and then examined. Number of colonies of both filamentous fungi and yeasts were counted, and cfu/g was calculated. The number of cfu was also calculated for the genera *Aspergillus* (including potential *A. flavus/parasiticus*, black and brown Aspergilli and potential *A. ochraseus*), *Penicillium* (the total count and potential *P. verrucosum*), *Fusarium* and *Alternaria*. For the genus *Penicillium* only the cfu/g of the genus as and *P. verrucosum*

was calculated since species of the genus are hard to distinguish from each other on a plate with a mixture of isolates. Two individuals of the same species can also vary to some extent in their morphology, and the age of the colony can also result in varying appearance. The cfu/g of *P. verrucosum* could be calculated based on its characteristic read reverse on DG18 (Samson *et al.* 2004).

The cfu/g was calculated according to NMKL, rap. nr. 1: $\#cfu/ml = \frac{C_1+C_2+\ldots+C_n}{W_1+W_2+\ldots+W_n}$

C_1=number of colonies on plate 1
W_1=volume from the original sample on plate 1
The detection limit was 10 cfu/g.

2.4 Identification of toxigenic species

The mould identification was performed in order to detect molds responsible for production of mycotoxin, notably aflatoxins and OTA, in the food samples. Because of the limited project time it was not possible to identify all isolates or even all suspected toxin producing isolates. Potential isolates of toxin producing species within the genus *Penicillium* and *Aspergillus* where isolated from the DG18 plates to malt extract agar (MEA) (appendix), a general substrate suitable for most species (Samson *et al.* 2004). Since *Penicillium* species are hard to distinguish from each other, isolations on MEA of the most dissimilar colonies were made.

Potential species of *Fusarium* ssp. were isolated on MEA and Potato-Dextrose Agar (PDA)(appendix) for further identification since this genus also includes toxin producing species. PDA is recommended by Samson *et al.* (2004) and commonly used for *Fusarium* diagnostics, where the color of the reverse is an important morphological character. The isolation to new plates was done to obtain pure cultures. Streak-inoculation is the best method according to Samson *et al.* (2004) since contaminants are difficult to detect in cultures created by point inoculation. The plates were incubated for 7 days at 25 °C. After incubation the plates were examined for contamination, and if necessary re-isolated. The MEA plates were analyzed directly and kept below 7 °C.

2.4.1 Identification of Penicillium

To identify species of *Penicillium*, the isolates were first inoculated in tubes with inoculation agar (MEA) to a semi solid suspension; this procedure helps to prevent the formation of colonies from stray spores (Pitt. 1979). With an inoculation loop the inoculum was then placed on six plates, “the *Penicillium*-plate package” including one plate respectively of MEA, Czapek Agar (CZ), Creatine Agar (CREA), and Yeast Extract Sucrose Agar (YES), and two plates of Czapek Yeast Autolysate Agar (CYA) (appendix). The inoculation was performed with three sticks on each plate. The plates were incubated for 7 days at 25 °C, and 37 °C (one plate of CYA).The morphological identification was performed according to Samson *et al.* (2004). The diameter of the colony on MEA, YES and CYA 25°C was measured and color of colony and reverse noted, as well as color of exudate droplets if present. CREA was studied for growth and acid/base production. The CYA plates incubated at 37 °C were also checked for growth. The MEA colonies were assessed to be either velvety or aggregated and microscopically characters were studied on preparations. Fresh colonies were used to obtain representative data.

Conidiophores were considered to be monoverticillate (lacking metulae), biverticillate (with metulae, one stage branched), terverticillate (with metulae, two-stage branched) or quarterverticillate (with metulae, three-stage branched). The phialides were measured, as well as conidia and stipe diameter. The conidia were studied for shape and assessed to be smooth, finely rough or rough, as well as the stipe (figure 1). Colonies of *P. crustosum*, *P. expansum* and *P.*

roqueforti, from the culture collection at the National Food Agency, were cultivated in parallel on the substrates in the "*Penicillium*-plate package", to be used as reference strains, for comparing with unknown isolates. Potential toxin producers, including isolates of *P. crustosum* and *P. expansum* suspected on the MEA plates by macroscopic evaluation, were further analyzed. Confirmation of suspected isolates of *P. verrucosum* was performed by inoculation on MEA and YES, where if positive gave a typical read reverse on YES.

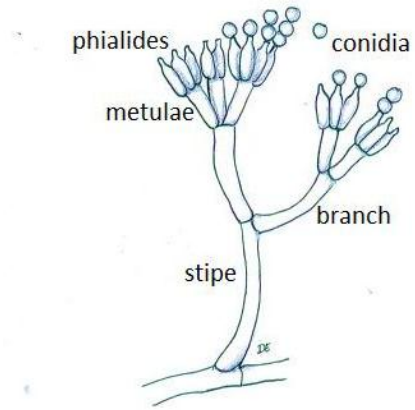


Figure 1. An illustration of the morphological structures of the conidiophore of *Penicillium*. The figure shows a terverticillate conidiophore, metulae is present on a two-stage branched stipe. The stipe and conidia are smooth (Samson *et al.* 2004, drawing by Disa Eklöf).

2.4.2 Identification of Aspergillus

Suspected colonies of *A. flavus/parasiticus* were re-inoculated on AFPA (appendix) plates at 30 °C for 48 hours according to NMKL (2004) for confirmation. AFPA is a diagnostic media for *A. flavus* and *A. parasiticus*. The aflatoxin producing species can be recognized by its sharp orange coloration of the reverse, which indicates production of a Ferri chelate of aspergillic acid. Aflatoxin is not produced on this medium, but the aspergillic acid indicates ability to produce the toxin (Samson *et al.* 2004).

Black or brown species of *Aspergillus* are very characteristic as a group, and the identification was not made to species level. The isolates were therefore described as belonging to the "black group" in the initial quantification.To identify other species of *Aspergillus,* colonies and preparations from the MEA plates were studied in the microscope and dissecting microscope. Morphological characters were assessed according to Samson *et al.* (2004). Characters studied were color of colony and reverse. Presence and coloration of exudate droplets were also noted. The formation of conidia on MEA-colonies was studied in the dissecting microscope, and color of the conidial head was established, as well as shape (radiate or columnar). Presence of sclerotia was also assessed. In the microscope the type of conidial head was determined; uniseriate (one layer of phialides), biseriate (two layers of phialids) or mixed. The conidial diameter was measured and form and ornamentation noted (smooth, finely rough or rough). The vesicle was established to be either round or club shaped, and the stipe studied for ornamentation.

2.4.3 Identification of Fusarium

Suspected colonies of *Fusarium* were isolated on MEA as well as PDA plates on which a pink or red coloration of the latter substrate indicates the genus (Samson *et al.* 2004). Microscopy studies of the MEA grown isolate was performed to confirm that the isolate belonged to the *Fusarium* genus, according to Samson *et al.* (2004). No further analyses of the *Fusarium* isolates or their potential toxins were performed.

2.5 Chemical analysis

2.5.1 ELISA analysis of aflatoxins and OTA

The content of aflatoxins and ochratoxin A in the samples were determined by competitive direct enzyme linked imunosorbent assays (CD-ELISA) by using the quantitative kits Veratox HS for aflatoxin (8031) and Veratox for ochratoxin (8610) fromNeogen, Auchincruive, Scotland.

The test contained micro wells which were coated with antibodies internally specific for the mycotoxin tested for. The detection principle is that in the coated wells, the toxin and conjugate (the toxin tested for labeled with an enzyme) compete for the binding sites on the antibodies. More toxin in the sample means that less of the conjugate is allowed to bind to the antibodies. The substrate which is added reacts with the bound enzyme-conjugate, and blue color is produced. A stronger blue color means that more conjugate has been able to bound, and lower amounts toxin is present. In contrast weak color means that a higher amount of toxin is present (Neogen 2012).

Before analysis, the samples were prepared according to the instructions supplied with the tests. Between 20 and 30 grams of sample was ground in an electric grinder to a powder, with particle size of fine instant coffee (at least 75% of the grinded material have to be able to pass through a 20 mesh sieve). 5 grams was weighed in for the aflatoxin test and 10 grams was weighed in for the ochratoxin A test. The grinded samples were placed in falcon tubes and kept dark at below 5°C until further handled. The grinded sample was mixed with a methanol solution, 25 ml of a 70% solution for the aflatoxin test, and 40 ml of a 50% solution for the ochratoxin A test. The methanol was added to the falcon tube which was then shaken vigorously for 3 min (for the Aflatoxin test) or 5 min (for the ochratoxin A test). When this procedure was not performed directly before the tests, the sample-methanol mixture was kept in refrigerator, below 5°C.

The ELISA-tests were performed according to the instructions included in the test material, with a small change to optimize testing time. Instead of first adding conjugate to the empty wells, samples and controls were first added. After this the conjugate was added and the suspension mixed. The suspension was transferred to the antibody wells and the micro wells were incubated, washed, and finally a substrate was added. After incubation with the substrate a stop solution was added as a final step. A Multiscan EX micro well reader from Thermo Electron Corporation was used for the reading of the reactants at 620nm, and a standard curve of optical densities was created from the controls provided. The optical densities from the sample were then plotted against this curve to calculate exact concentration of toxin in Veratox Software for windows version 3.3 (Neogen, Auchincruive, Scotland). The detection limit for aflatoxin in the sample was 0.5 ppb, the quantification level 1 ppb, and the controls provided contained 0, 1, 2, 4 and 8 ppb toxin. The detection limit of ochratoxin was 1 ppb, the quantification level was 2 ppb and the controls provided contained 0, 10, 20 and 50 ppb toxin. The aflatoxin test was validated for coconut, figs, peanuts, pumpkin seeds and sunflower meal among many other matrixes, and the OTA test was validated for various grains, and fruit products (apricots, dates, figs) but no matrixes similar to oilseeds or nuts (Neogen, Auchincruive, Scotland).

2.5.2 LC MS/MS analysis of Penitrem A

The walnut samples were analyzed for the presence of penitrem A by staff from the Chemical Unit 2 at the National Food Agency by using triple quadruple mass spectrometry (Triple quad LC/MS/MS) according to internal method SLV K2-m396.1, based on Berthiller (2007). In the liquid chromatography (LC) column the different toxins are separated, so that they will reach the detector at different times. The mass spectrometric (MS) detection is based on the fact that the toxin molecule is broken up into ions and the ions in turn are broken up into daughter ions, which are

detected based on their mass. The two-stage disassembling of the substance makes this type of detection very specific (personal communication Anna-Maria Thim, National Food Agency).

Prior analysis the samples were grinded and 5.0 g were weighed in and placed in 50 ml pyrex bottles. The bottles were stored dark below 5°C. Preparations for the test were done by adding 20 ml of extraction solution (79% CH_3CN, 20% mQH_2O, 1% HAc) to the grinded sample and shaking for 30 minutes on a shaking table with 250 shakes/min. The sample was then filtrated and 100 µl filtrated sample was then transferred to vials and analyzed. Sample 38 was selected to be used as a blank, since this had a low content of total cfu/g mould, and was therefore judged to be least likely to contain toxins. This sample was analyzed once; prior the other samples of walnuts, to ensure that it did not contain penitrem A. After this was confirmed 3 Pyrex flasks with sample 38 was prepared. To 2 of the flask known amounts of toxin was added to be used as standards.

2.6 Statistical analysis

The average a_w, as well as standard deviation for the a_w was calculated for each sample type using Microsoft excel 2010.The standard deviation is a method used for estimating the distribution of a quantitative variable (University of Gothenburg 2002). It is the average deviation from the average variable, which means that a low value expresses a uniform data set (SCB 2011). The total count of moulds and yeasts were analyzed using Minitab 15, 2006, and boxplot of log 10 of the total content of mould and yeast was done.

3. Results

3.1 Water activity (a_w)

The a_w was measured in all samples before storage (Table A1-A5 Appendix). The average a_w and standard deviations were calculated for each group of sample type (table 3). Only one sample of melon seeds was sampled (sample 99), which had an a_w of 0.523. The a_w of the goji berries were lower and less variable than for the other commodities. In each of the other sample types (pumpkin seeds, sesame seeds, sunflower seeds and walnuts) one of the samples had an a_w above the critical value of 0.6.

Table 3. Average a_w, standard deviation, maximum and minimum value for each sample type.

Sample type	Average (standard deviation)	Minimum	Maximum
Goji berries	0.32(0.03)	0.29	0.38
Pumpkin seeds	0.50(0.09)	0.40	0.62
Sesame seeds	0.47 (0.13)	0.28	0.68
Sunflower seeds	0.48(0.08)	0.35	0.63
Walnuts	0.45(0.11)	0.27	0.71

3.2 Total content of fungi

The samples were analyzed with respect to total concentration of filamentous fungi and yeasts, by spreading sample dilutions on DG18, and counting the number of colony forming units after 7 days of incubation at 25 °C. Cfu/g of both moulds and yeasts were calculated (table A6-A10 Appendix). The total amount of moulds and yeast differed more between samples within each food commodity than between commodities, however some differences between food types were identified by analyzing the data with Minitab, shown as boxplot figures (figure 2-3). Sesame seeds varied most in mould content while walnuts and sunflower seeds had the highest mould content. Goji berries had highest content of yeast.

In Goji berries the content of filamentous fungi varied from 10^2 to 10^4 cfu/g, and the content of yeast varied from 0 to 10^4 cfu/g. All samples contained yeast. In the pumpkin seeds the total mould count varied between $<10^1$ to $9.09x10^4$ cfu/g, and the yeast count varied between $<10^1$ to $7.86x10^4$ cfu/g. The content of mould and yeast in the samples of sesame seeds varied between 0-10^4 and 0-10^3 cfu/g. The shelled samples (44, 83 and 113) did not contain any fungi above the detection limit. The two samples with most cfu/g fungi were both Krav-certified. Sunflower seeds had a content of moulds, varying from 10^2 to 10^4, only one samples exhibited growth of yeast above the detection limit at 10^3 cfu/g. The total amount of mould (cfu/g) in the walnut samples were between 10^2 and 10^4 cfu/g. Three samples contained $<10^2$ cfu/g yeast.

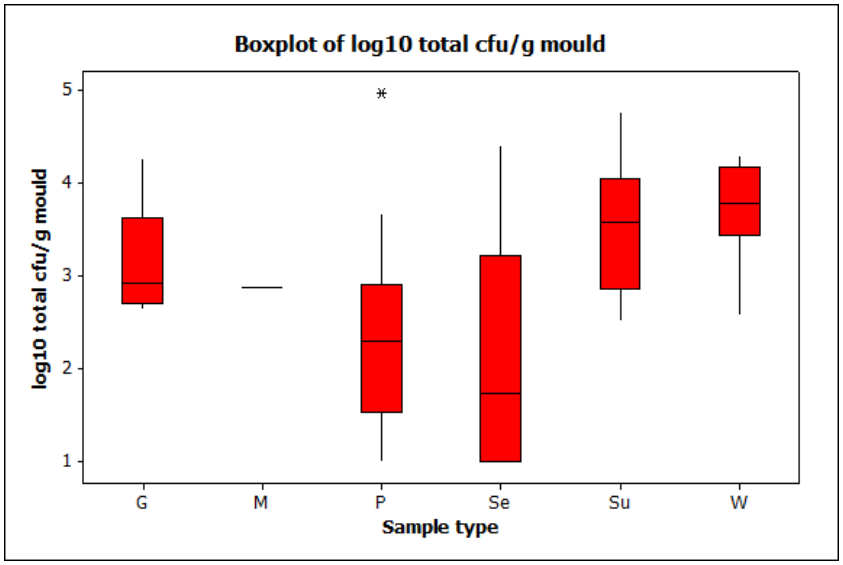


Figure 12. Boxplot illustration of the total amount (log cfu/g) of mould in the samples of goji berries (G), melon seeds (M), pumpkin seeds (P), sesame seeds (Se), sunflower seeds (Su), and walnuts (W).

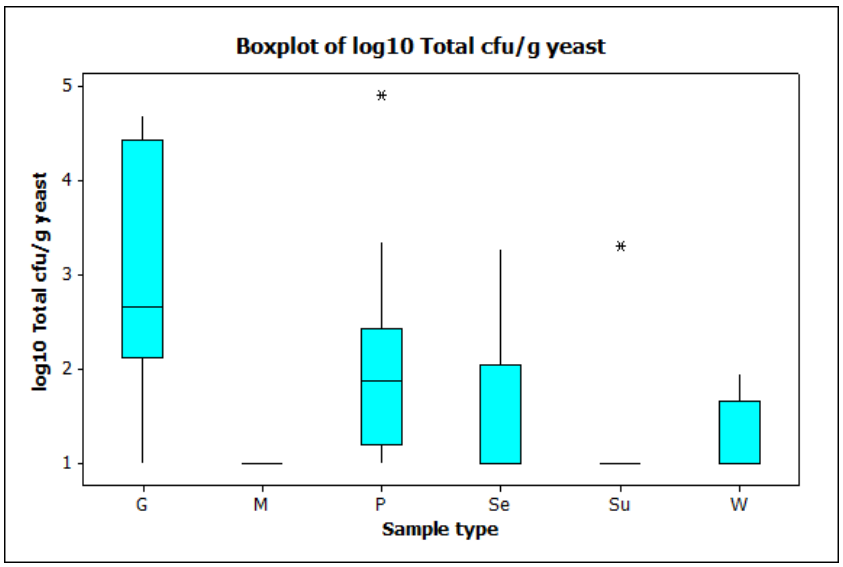


Figure 23. Boxplot illustration of the total amount (log cfu/g) of yeast in the samples of goji berries (G), melon seeds (M), pumpkin seeds (P), sesame seeds (Se), sunflower seeds (Su), and walnuts (W).

3.2.1 Correlation between a_w and fungal content

Average total mould and yeast was calculated for the sample types (table 4), and to evaluate the correlation between a_w and fungal content, a_w for each sample was plotted against total content of mould. No correlation between the amount of mould and a_w was seen (data not shown).

Table 4. The average (ave) content (cfu/g) of moulds and yeasts.

Sample type	Ave mould	Ave yeast
Goji berries	$3.24x10^3$	$1.04x10^4$
Pumpkin seeds	$9.68x10^3$	$8.13x10^3$
Sesame seeds	$4.38x10^3$	$2.88x10^2$
Sunflower seeds	$1.00x10^4$	$2.00x10^2$
Walnuts	$8.16x10^3$	$1.80x10^1$

3.3 Identification of toxigenic species and chemical analysis of toxin content

Each sample was analyzed with respect to concentration of potentially toxigenic species (species of *Penicillium*, *Aspergillus* and *Fusarium*). Cfu/g of relevant species and genera was calculated, detection limit was set to 10 cfu/g, and isolation of relevant species on MEA (for *Fusarium* also PDA) was performed with subsequent morphological analysis in order to identify toxin producing species. Black and brown species of *Aspergillus* were grouped and named "the black Aspergilli group". The content of aflatoxins and ochratoxin A in the samples were determined by CD-ELISA, in which free toxin in the sample were allowed to compete with a conjugate for binding sites on toxin-specific antibodies on the walls of the micro wells. A Multiscan micro well reader was used for the reading of absorbances, and exact concentration of toxin was calculated. The detection limit for aflatoxin in the sample was 0.5 ppb and quantification limit was 1 ppb. The detection limit of ochratoxin was 1 ppb, the limit of quantification was 2 ppb (Neogen 2013).

3.3.1 Goji berries

Common genera in goji berries were *Cladosporium, Alternaria, Aureobasidium, Eppicoccum* and *Eurotium*. *Penicillium spp.* was present, *A. flavus/parasiticus* occurred as well as black or brown species of *Aspergillus*. Some findings of potential *A. ochraceus* were also made (table 5). Cfu/g of potential toxin producers are presented in table x.

Table 5. Amount of potential toxin producing moulds (cfu/g) in the sampled goji berries. Presence of fungi below the detection limit is marked <10.

Sampled goji berries	*Penicillium spp.*	Black or brown *A. spp.*	*A. flavus /parasiticus*	Potential *A. ochraceus*	*Alternaria*
39	<10	14	<10	<10	153
49	32	<10	<10	<10	180
60	<10	<10	50	23	541
64	63	<10	22.5	<10	126
65	<10	<10	<10	<10	149
78	27	27	90	<10	901
98	27	54	68	18	360
104	45	<10	<10	<10	333
116	<10	<10	<10	<10	396
152	<10	<10	<10	<10	<10

From assessment of macroscopical and microscopial characteristics and comparing the appearance of unknown isolates with reference strains, potential presence of the toxin producing species *P. expansum* and *P. chrysogenum* were detected in the samples listed in table 6.

Table 6. Samples of goji berries with potential presence of the toxin producing species *P. expansum and P. chrysogenum*. The isolates were assessed by visual comparing with isolates from the culture collection at the National Food Agency.

Sample	*P. expansum*	*P. chrysogenum*
39		
49		
60		
64	+	
65		
78	+	
98		
104	+	
116		+
152		

In goji berries aflatoxins were detected in one sample (Table 7). Ochratoxin A was detected in all samples. 40% of the samples had levels above the limit of quantification, and at highest 4.6 ppb was detected (table 7).

Table 7. Levels of toxins in sampeled goji berries (ppb).

Sample	Aflatoxins	Ochratoxin A
39	<0.5	2.3
49	<0.5	1.8
60	<0.5	1.5
64	<0.5	1.9
65	<0.5	1.7
78	<0.5	1.4
98	<0.5	1.4
104	<0.5	2.3
116	<0.5	3.6
152	0.5-1	4.6

3.3.2 Pumpkin- and melon seeds

Eurotium species were found in 90% of samples of pumpkin seeds, as well as in the melon seeds, and were in many cases dominating. Findings of potential toxin producers are presented in table 8 and 9. *Penicillium spp.* was found in 70% of sampled pumpkin seeds (table 8). In melon seeds the dominating genera were *Eurotium* and 45 cfu/g *Aspergillus* were also seen. No potential toxin producers were isolated, and the pumpkin- and melon seeds did not contain toxin above the detection limit.

Table 8. Amount of potential toxin producing moulds (cfu/g) in the sampled pumpkin seeds. Presence of fungi below the detection limit is marked <10.

Sample	*Penicillium spp.*	Black or brown A. spp.	A. flavus /parasiticus
42	63	<10	<10
59	<10	<10	<10
81	27	36	<10
87	27	<10	<10
102	<10	<10	<10
115	14	<10	<10
127	630	<10	90
147	<10	<10	<10
149	<10	<10	<10
154	<10	<10	<10

Potential toxin producing species of *Penicillium* were suspected in two samples (table 9). 90 cfu/g of *Alternaria spp* was also seen in sample 127.

Table 9. Samples of pumpkin seeds with potential presence of the toxin producing species *P. expansum and P. verrucosum*. The isolates were assessed by visual comparing with isolates from the culture collection at the National Food Agency.

Sample	*P. expansum*	*P. verrucosum*
42		
59		
81	+	
87		
102		
115		
127	+	+[a]
147		
149		
154		

a The sample contained potential *P. verrucosum*, but since the colonies on the DG18 plates were too over-grown by zygomycetes, it was not possible to isolate the suspected strain.

3.3.3 Sesame seeds

The mycoflora in the sesame seeds were variable, but also the sorts of the sesame seeds were mixed. Sample 43 and 114 were unshelled white seeds. Sample 50 and 151 were black seeds. In half of the samples no potential toxin producers were found and two samples did not exhibit any growth at all. In sample 50 (black seeds) the dominating genus were *Aspergillus* and in sample 100 (shelled white seeds) the dominating genus were *Cladosporium.* In sample 114 which were unshelled seeds, Krav-certified, the dominant fungi were *Wallemia. A. flavus* or *A. parasiticus* was isolated from 50 % of the samples (table 10). Black or brown *Aspergillus* was found in 2 samples, and 1 sample contained suspected *A. ochraceus* colonies (table 10). Two samples contained *Alternaria* spp., in one sample 10^3 cfu/g was found. *Fusarium* spp. was also identified (table 10). Three of the 10 samples showed a higher amount of potential toxin producing fungi; Sample 50 which were black seeds purchased in a store with predominantly ethnical products, and sample 100 (shelled) and 114 (unshelled) of white seeds with Krav-certification and bought in a lager supermarket chain (table 10).

Table 10. Amount of potential toxin producing moulds (cfu/g) in the sampled sesame seeds. Presence of fungi below the detection limit is marked <10.

Sample	*Penicillium spp.*	Black or brown *A. spp.*	*A. flavus /parasiticus*	*Potential A. ochraseus*	*Alternaria*
43	<10	<10	<10	<10	<10
44	<10	<10	<10	<10	<10
50	14	41	77	<10	<10
83	<10	<10	<10	<10	<10
85	<10	<10	<10	<10	<10
100	1170	<10	18	32	135
113	<10	<10	<10	<10	<10
114	1890	123	99	<10	1171
126	<10	<10	<10	<10	<10
151	<10	<10	<10	<10	<10

Only one colony of *Penicillium* spp. was isolated from sample 151 (black seeds), and was identified as *P. crustosum*. In sample 43, unshelled white seeds, an isolate were identified as *A. sydowii*, this species is not a toxin producer (Samson *et al.* 2004). Only sample 114 contained aflatoxins above the detection limit (but below the quantification limit). No samples contained OTA above the detection limit.

3.3.4 Sunflower seeds

The genus *Penicillium* dominated in most of the samples, but also *Eurotium* spp. was a common contributor to the mycoflora. Potential toxin producers were isolated (table 11). Black or brown species of *Aspergillus* were found in 70% of the samples. Potential findings of A. ochraceus were present in low amounts in 70 % of the samples, and *A. flavus/parasiticus* were also common (40% of the samples). *P. verrucosum* colonies also seemed to be a common contributor to the mycoflora of sunflower seeds and were found and identified in 60% of the samples (table 11).

Table 11. Amount of potential toxin producing moulds (cfu/g) in the sampled sunflower seeds. Presence of fungi below the detection limit is marked <10.

Sample	*Penicillium spp.*	Black or brown *A. spp.*	*A. flavus /parasiticus*	*Potential A. ochraseus*	*P. verrucosum*	*Alternaria*
40	32	<10	<10	14	<10	<10
41	1210	<10	<10	14	32	<10
80	55900	90	<10	<10	<10	<10
101	789	<10	<10	<10	45	50
103	108	<10	<10	<10	<10	<10
128	729	<10	<10	<10	<10	<10
143	6950	68	31.5	23	<10	<10
146	3910	18	<10	<10	36	<10
148	267	41	<10	<10	14	<10
155	3870	36	36	<10	<10	<10

In all samples potential toxin producing *Penicillium* species were found and, to some extent identified (table 12). P. expansum were potentially present in 100% of the samples, and in sample 146 *P. expansum* was identified as the dominating species. In sample 155 the potential *P. expansum* was recognized to be a common contributor to the mycoflora. Fusarium was isolated from sample 40. Some other species were also identified, which are all potential toxin producers (table 13).
No tested sunflower seeds contained toxins above the detection limits.

Table 12. Isolates of P. crustosum, P. expansum and P chrysogenum in sunflower seeds.

Sample	*P. crustosum*	*P. expansum*	*P. chrysogenum*
40		+[b]	
41		+[b]	
80		+[b]	+[b]
101		+[b]	
103		+[b]	+[a]
128	+[a]	+[b]	
143		+[b]	+[b]
146		+[a]	+[b]
148		+[b]	+[b]
155		+[b]	

a: Isolates which were identified by inoculation on "the *Penicillium* plate package" and morphological characterization
b: Isolates which were assessed by visual comparing with isolates from the culture collection at the National Food Agency

Table 13. Other species of *Penicillium* identified in the sampled sunflower seeds by inoculation on "the *Penicillium* plate package" and morphological characterization.

Sample	Identified species
40	
41	*P. albocoremium*
80	*P. brevicompactum*
101	*P. citrinum*
103	
128	*P. rugulosum, P. polonicum*
143	
146	
148	
155	

3.3.5 Walnuts

Species of *Penicillium* dominated in all of the walnut samples, and in many cases the plates of the lowest dilution were totally covered with *Penicillium.* Black or brown species of Aspergilli were found in 90% of the samples and *A. flavus/parasiticus* were identified in 80% of the samples (table 14).

Table 14. Amount (cfu/g) of potential toxin producing moulds in the sampled walnuts. Presence of fungi below the detection limit is marked <10.

Sample	*Penicillium spp.*	Black or brown *A. spp.*	*A. flavus /parasiticus*	*Potential A. ochraceus*	*P. verrucosum*
38	230	68	<10	<10	<10
58	3600	18	<10	<10	<10
63	293	221	14	<10	<10
79	12300	342	586	766	455
82	3150	292	<10	<10	<10
84	5140	<10	<10	<10	<10
86	18100	405	135	<10	<10
142	14000	27	36	<10	<10
150	6260	<10	<10	<10	<10
153	9460	50	23	<10	<10

P. crustosum was identified in 80% of the samples and in sample 150 *P. crustosum* were also confirmed, and judged as a common contributor to the mycoflora. *P. expansum* were potentially found in 50% of the samples. In sample 150 *P. expansum* was confirmed, and assessed as dominating. Potential isolates of *P. chrysogenum* were found in 40% of the samples (table 15).

Table 15. Isolates of *P. crustosum, P. expansum* and *P. chrysogenum* in the 10 samples of walnuts.

Sample	*P. crustosum*	*P. expansum*	*P. chrysogenum*
38	+[a]		
58	+[a]	+[b]	+[b]
63	+[a]		+[b]
79	+[a]		+[b]
82	+[a]		+[b]
84	+[a]	+[b]	
86		+[b]	
142	+[a]		
150	+[a]	+[a]	
153		+[b]	

a: Isolates which were identified by inoculation on "the *Penicillium* plate package" and morphological characterization
b: Isolates which were assessed by visual comparing with isolates from the culture collection at the National Food Agency

In 80% of the walnut samples aflatoxins above the limit of detection (0.5ppb) was seen. 50% of the samples contained aflatoxins above the limit of quantification. One sample had a value over the maximum allowable value of 4 ppb in Europe (EC 1881/2006). All walnut samples contained OTA above the maximum allowable limit of 3 ppb for grains and grain products intended for direct consumption, and 3 samples were above 10 ppb (table 16).

Content of penitrem A was analyzed by LC-MS/MS by staff at the chemical unit 2 at the National Food Agency. No samples were positive for this toxin. The detection limit was 1.7 µg/kg, and the method was not previously tested on nuts.

Table 16. Levels of toxins (ppb), in samples of walnuts. The levels were set to; not detected, <2, 2-4, and >4 ppb for aflatoxins, and; not detected, < 3, 3-5, 5-10 and >10 ppb for ochratoxin A. The level divisions were based on maximum allowable levels (EG 2006).

Sample	Aflatoxins	Ochratoxin A
38	<0.5	7.3
58	0.5-1	11.5
63	0.5-1	6.5
79	1.1	7.7
82	<0.5	7.9
84	0.5-1	9.7
86	1.6	13.5
142	1.5	4.9
150	1.9	4.0
153	4.3	11.8

4. Discussion

About 50 samples of goji berries oil seeds and walnuts were analyzed for colony forming units of fungi. The total amount of fungi as well as potential toxin producing genera and species were identified. Because of the limited time it was not possible to identify all possible toxin producers and focus was laid at some selected. The a_w was also measured to evaluate storage stability, and content of some fungal toxins were examined with chemical analysis.

4.1 Water activity (a_w)

The a_w for all commodities was low, and only exceeding 0.6 in 4 samples. However these levels were not especially high and very few species of fungi would be able to grow and produce toxins at these levels. The highest a_w detected (0.71) was found in one sample of walnuts, which is known to be vulnerable to growth of moulds (Pitt *et al.* 2009).

4.2 Total fungal counts in commodities

The average count did not differ much between samples, however sunflower seeds had the highest level. The average content of mould and yeasts in the samples were 10^3-10^4, and 10^1-10^4, and the total count of mould reached 10^4 cfu/g in at least one sample of each commodity. In sunflower seeds and walnuts 10^4 cfu/g was seen in three samples, and in pumpkin seeds almost 10^5 cfu/g moulds was found in one of the samples. This could be a result of a slow drying process. According to Risenta, a Swedish retail company, the maximum allowable level is 10^4 cfu/g total mould content in their products, and levels between 10^2 and 10^3 is often seen (Personal communication Monica Demorior, Risenta). Since the a_w of the samples were low and the commodities therefore storage stable, the content of fungi is of no concern, as long as the taste is still good. A level above 10^4 cfu/g mould is however not preferable, since a product with this amount can have a mouldy taste (Personal communication Monica Demorior, Risenta).

Sunflower seeds had the highest average count of mould, but greatest variability in fungal content was seen in sesame seeds. This could be because the structural properties of this commodity were the most variable. Some of the seeds were shelled, some unshelled and some were also of a black variety. Three samples of sesame seeds which did not contain any cfu/g fungi were unshelled. This was expected, since moulds often grow on outer structures of the sesame seeds.

Goji berries had the highest content of yeasts of all the commodities. Since yeasts commonly live of the juices secreted from plants, and goji berries also are a moister crop (before it is dried) then other tested products, the yeast may propagate in the fruit before the drying is completed. The berries are dried in the sun (Stiegter 2010) and the process may also vary in time depending on climatic factors which can give the yeast an opportunity to grow.

4.3 Fungal flora and toxins in goji berries

Very little is known about the mycoflora of goji berries. Common genera were found to be *Cladosporium, Alternaria, Aureobasidium, Eppicoccum* and *Eurotium*. *Alternaria* is a genus with toxin producing species (Scott 2004), and the high content of these fungi could therefore be of interest. The genus belongs to the field flora (Pitt *et al.* 2009), and several species are plant pathogens. They have also been reported to contaminate foods after harvest (Scott 2004). *A. flavus/parasiticus* was found in 40% of the samples but in low amounts ($<10^2$ cfu/g). These fungi can grow above a_w 0.82 and produce aflatoxins at a_w >0.86. 0.5-1 ppb Aflatoxin was seen in one sample of the goji berries. The low a_w of the commodity indicates that growth and toxin production probably occurred before the berries were dried. *A. flavus* can also occur endopytic on crops as a field fungus (Adams *et al.*2010), which means that it is possible that growth and toxin production occurred before harvest. The level of toxin was below both the maximum allowable level for aflatoxin B_1 (2ppb) and the maximum allowable level for the combined toxins (4ppb) (EC 1881/2006), which means that the

aflatoxins in these goji berries is of no health concern. Some species of the black or brown Aspergilli (especially *A. carbonarius*) produces OTA (Samson *et al.* 2004). 30% of the samples contained low levels of black or brown Aspergilli. 20% of the goji berries also contained low levels of potential *A. ochraceus*, a storage fungus which is also often able to produce the toxin (Samson *et al.* 2004). OTA was detected in all sampled goji berries, and 40% contained levels above the limit of quantification (2 ppb). The highest level detected were 4.6 ppb and the ELISA method was validated for similar products (apricots, dates, figs)(Neogen 2012). Since the black and brown Aspergilli are storage fungi (Pitt *et al.* 2009), the growth and toxin production must have occurred after harvest but before the berries were completely dried. This indicates an inefficient drying process or improper handling before drying. There is no general maximum allowable level for OTA in products such as goji berries, but the maximum allowable level for grains intended for direct consumption is 3.0 ppb and for dried grapes 10.0 ppb (EC 1881/2006).

Some other interesting species were also seen in goji berries; *P. expansum* was found in 30% of the samples. This species can produce patulin, which is regulated for apple products. A maximum allowable level of 25 µg/kg is set in these products intended for direct consumption (EC 1881/2006). The toxins roquefortine C and citrinin can also be produced by this species (Samson *et al.* 2004), which may both be neurotoxic (Scott 2004). *P. crysogenum* was also identified which can potentially produce roquefortine C (Samson *et al.* 2004).

4.4 Fungal flora and toxins in pumpkin and melon seeds

The type and level of potential toxin producing fungi were variable in this commodity. Black or brown *Aspergillus*, *A. flavus, P. expansum, P. verrucosum* and *Alternaria* spp. were found, however no toxins were detected from these samples. Pumpkin seeds appeared to be a product with little problems, storage stable and probably dominated by non-toxigenic fungi. A previous study of the mycobiota of pumpkin seeds found *Penicillium*, *Eurotium* and *Cladosporium* to be dominating genera (Weidenbörner 2001), therefore species of these genera were expected in Pumpkin seeds. In 90% of pumpkin seeds and in the melon seed sample, *Eurotium* species were found and in many cases dominating. *Eurotium* are not toxin producers, and are therefore not of health concern. Weidenbörner (2001) found 15 potential mycotoxigenig moulds in pumpkin seeds including *A. flavus, A. ochraceus, Alternaria spp.* Venkatesagowda (*et al.* 2012) found that approximately 27% of examined sesame seeds contained *A. niger.*

Only one sample of melon seed was included in this study due to the limited availability of unprocessed melon seeds on the marked. *Eurotium* dominated the sample, and no potential toxin producers were isolated. No toxins were detected. Bancole (*et al.* 2004) found *Aspergillus* to be the dominating genus and *A. flavus* to be the most common species in melon seeds, and also detected levels above 5 ppb aflatoxin B_1 in 32.2% of the samples. Bancole (*et al.* 2006) also found *A. flavus* to be the most frequent species, with *Aspergillus* and *Penicillium* as the dominating genera. The study found that 32% of melon seeds from forested area contained a mean level of 14.8 ppb aflatoxin B_1, and 21% of seeds from the savannah contained mean level of 11.3 ppb aflatoxin B_1. Among the oil seeds melon seeds are the product most often subject to RASFF notification (1-2 RASFF) official control (EU 91/2013, EU 1235/2012).

4.5 Fungal flora and toxins in sesame seeds

The variable mycoflora of sesame seeds in this study made it difficult to evaluate the mycoflora. The genera that were often seen were *Aspergillus, Cladosporium, Wallemia* and *Penicillium*. According to Chakrabarti (1987) *A. flavus, A. niger, A. tamarii, Cladosporium herbarum,* and some *Penicillium spp.* are common fungi in sesame seeds, which were consistent with the findings in this study. In a study from 1988 from Sierra Leone *A. flavus, A. tamari, A. ochraceus, A. japonicas* and *A. niger* were identified (Jonsyn 1988). In this study *A. flavus/parasiticus* was found in 30% of the

samples, black or brown species of *Aspergillus* were found in 20% of the samples and *A. ochraceus* was found in 32% of the samples. *P. crustosum* was also identified in one sample. Though many potential toxin producing species were present, only one sample contained aflatoxin (< 1 ppb), and no samples contained OTA. Since the product was storage stable the content of potentially toxin producing fungi should not be a problem.

4.6 Fungal flora and toxins in sunflower seeds

Species of *Penicillium* dominated in the samples of sunflower seeds, black or brown species of *Aspergillus* was found in 50% of the samples and *A. flavus* were found in 20%. 30% contained potential *A. ochraceus* isolates. *Alternaria* was also found in one sample. *Fusarium* spp. was isolated as well as several potentially toxin producing species of *Penicillium*. All seeds contained *P. expansum*, 50% contained *P. chrysogenum* and 40% contained P*. verrucosum. P. crustosum*. *P. albocoremium, P. brevicompactum, P. citrinum, P. rugulosum* and *P. polonicum* were also identified which are all toxin producing species. The mycoflora of sunflower seeds appeared to be diverse, with many toxin producing species as common contributors. The analysis of toxins did however not detect any toxins, and since the commodity was storage stable (the highest a_w detected was 0.63), there did not seem to be any risk involved with consuming these products. However, if the commodity would be exposed to moisture there would be a risk of toxin production. Abdullah (*et al.* 2010) found common genera in sunflower seeds to be *Aspergillus, Alternaria, Fusarium,* and *Penicillum. A. niger* and *A. flavus* were the most common species, but also *Penicillium expansum* were common. Jimenez (*et al.* 1991) found highest counts of *A. niger* and *Penicillium spp*. and Shahnaz *et al.* (1991) found high incidence of *A.flavus* and *A. niger* which Sharfun-Nahar *et al.* (2005) also found, along with high incidence of *Penicillium* spp. The study also revealed *A. ochraceus* as a contributor to the mycoflora of sunflower seeds, and both studies found species of *Alternaria* and *Fusarium.*

4.7 Fungal flora and toxins in walnuts

In walnuts *Penicillium* dominated, but black or brown species of Aspergilli was also common (80%). *A. flavus* found in 50% of the samples. Similar results have been shown by others. Khayria (*et al.* 1993) also found *Penicillium* to be a common genus. However, the most common species were *A. flavus* and *A. niger*. Bayman *et al.* (2002) also found *A. flavus, A. niger,* and *Penicillium ssp.* To be the most dominating fungi. Singh *et al.* (2008) identified A. flav*us, A. niger,* and *P. citrinum* as the major contributors to the mycobiota.

According to Pitt *et al.* (2009) *A. flavus* can often be a problem since the species is equally adapted to both field- and storage conditions. Findings of aflatoxins vary between studies; Gürses (2006) tested 24 samples of walnuts for aflatoxin B_1 and found 6 samples positive. The mean level was 22.1 with a range between 3-28 ppb. Khayria (*et al.* 1993) did not find any aflatoxins in the walnut samples while Singh (*et al.* 2008) found that 83% of the stored walnuts included in the study contained aflatoxin B_1 with a concentration range between 140-1220 µg/kg. In this study, only one sample of organically grown walnuts exceeded the maximum limit (4.3 ppb aflatoxins).

No OTA ELISA tests available on the market were validated for nuts. In this study, OTA analysis was performed with the Veratox ELISA test, which was validated for grains and fruits. With this test, OTA was detected in all samples between of 4.0 and 13.5 ppb. There is no maximum allowable level for OTA in walnuts, but grains and grain products intended for direct consumption has a maximum allowable level of 3 ppb while dried grapes has 10 ppb as the limit (EC 1881/2006). Both *P. verrucosum* and black and dark species of Aspergilli, all potential producers of OTA, were detected in the samples. Due to the dominance of Aspergilli, these species are likely the most important source of the toxin. *A. ochraceus* was potentially found in one sample. *P. verrucosum* was found in one sample at fairly high amount ($4.6x\ 10^2$ cfu/g). This could indicate that drying of the sample was not

performed fast enough to a safe a_w, and risk of OTA content is noteworthy. The toxin analysis must be verified with a validated method before any conclusions can be drawn.

P. expansum was found in 50% of the walnut samples and *P. crustosum* in 80% of which the latter sometimes dominated completely. *P. crustosum* is able to produce penitrem A at an a_w above 0.92 (National Food Agency 2007) and can cause tremors in animals (Scott 2004). Measured a_w was however far lower, and when analysis with LC MS/MS was performed, no toxin was detected. This indicated that the a_w of walnuts were held below this level from drying and through processing and storage. However the LC MS/MS method had not previously been tested on nuts.

In this study; varying amounts of total fungi was seen in the sampled products, and toxigenic species was seen in all commodities. A_w was low and the products therefore storage stable. However the broad range of different toxigenic species stresses the importance of storage conditions. If the dry product is moisturized and the a_w increases, the fungi can grow and toxins may be produced.

High amounts of *Alternaria* were seen in goji berries. *Penicillium* species were present in all commodities, but especially high amounts were seen in sunflower seeds and walnuts, where also findings of especially *P. expansum* were noteworthy. Black and brown species of *Aspergillus* were seen in all commodities, and walnuts had high amounts. Also *A. flavus* was found in all products with the highest amount in walnuts.

Toxin analysis revealed that goji berries and walnuts were contaminated with OTA, and walnuts with aflatoxins. Walnuts are from previously known to be a problematic commodity with respect to mould and toxin contamination.

4.8 Further analysis

- For goji berries the high content of *Alternaria* intrigues to examine levels of *Alternaria* toxins further, especially since there are no regulations for these. The content of OTA as well as aflatoxins in goji berries should also be further examined.

- The broad spectrum of potentially toxin producing species in oil seeds (especially sunflower seeds) could be further examined. It may also be an idea to analyze larger amount of seeds to ensure that the crop is generally storage stable and free from toxin contamination.

- The content of OTA in walnuts must be further examined. The ELISA method was not validated for nuts, and the result has to be confirmed with an accredited method.
  If the content of OTA is confirmed more samples should be analyzed and an evaluation of dietary intake should be performed, and perhaps a maximum allowable level regarding OTA content of walnuts should be considered. Since *P. crustosum* were often found in walnuts and sometimes dominated, and the LC MS/MS had not previously been tested on nuts, content of penitrem A could be more thoroughly examined.

Many thanks:

To my supervisors Ann Gidlund and Elisabeth Fredlund, who shared their knowledge, inspired and encouraged me during my time at the National Food Agency. Thanks also to Jimmy Sahlin who supplied me with substrates, and to all the other friendly staff on the microbial unit. Thanks also to the personal at the chemical unit 2 and to Monica Demorior at Risenta. And thanks, Mattias Eklöf for making this possible.

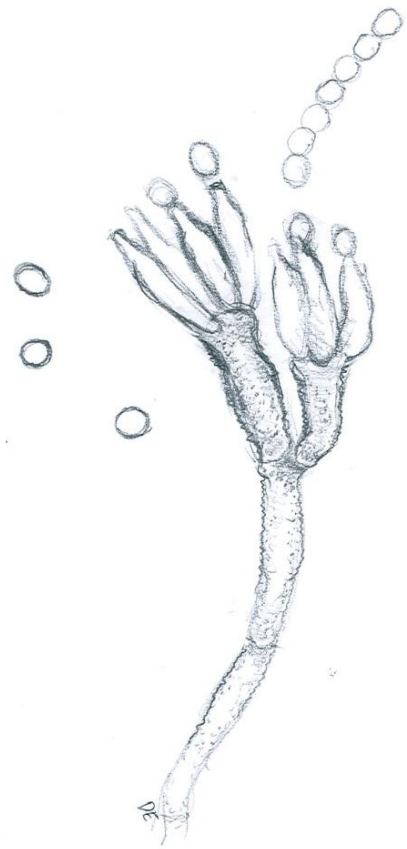


Figure 4 *Penicillium crustosum* isolated from sampled walnuts (D-nr 150). Drawing by Disa Eklöf

"The prediction of which toxins will be produced in which foods may be based on knowledge of the specific mycoflora of foods, laboratory experiment and data from chemical analyses for mycotoxins in foodstuffs under different conditions." (Northholt *et al.* 1995)

References

Adams M. R., Moss M. O. 2010. Food Microbiology. 3rd Ed. The Royal Society of Cemistry. Camebridge

Abdullah S. K., Al-Mosawi K. A. 2010. Fungi Associated with seeds of sunflower (*Helianthus Annuus*) Cultivars grown in Iraq. In: Phytopathologia **57:** 11-20

Akoh C.C., Nwosu C.V. 1992. Fatty Acid Composition of Melon Seed Oil Lipids and Phospholipids, Journal of the American Oil Chemists' Society 69: 314-317.

Aqualab. 2013. Aqualab Dew Point Water Activity Meter 4TE. WWW-document 2013: http://www.aqualab.com/products/water-activity-meters/aqualab-dew-point-water-activity-meter-4te/ visited 2013-06-04

Bankole S. A., Ogunsanwo B. M., Mabekoje O. O. 2004. Natural occurrence of moulds and aflatoxin B_1 in melon seeds from markets in Nigeria. Food and Chemical Toxicology **42:** 1309-1314

Bankole S. A., Ogunsanwo B. M., Osho A., Adewuyi G. O. 2006. Fungal contamination and aflatoxin B_1 of "egusi" melons seeds in Nigeria. Food Control **17:** 814-818

Barnett, J. A., Pyne R. W., Yarrow D. 1983. Agide to identifying and classifying yeasts. Cambrige university Press. Camebridge.

Bavec F., Grobelnik Mlakar S., Rozman Č., Bavec M. 2007. Oil Pumpkins: Niche for Organic Producers. In: Issues in new crops and new uses. Janick J., Whipkey A. (ed). ASHS Press, Alexandria, VA.

Bayman P., Baker J. L., Mahoney N. E. 2002. *Aspergillus* on tree nuts: incidence and assosiations. Mycopathologia **155:** 161-169.

Boriss H., Brunke H., Kreith M. 2006. Commodity Profile: Melons. Agricultural Issues Center, University of California.

CFAITC California Foundation for Agriculture in the Classroom. 2011. Walnuts, Information compiled by the California Walnut Board. CFAITC. Sacramento

Chakrabarti D. K. 1987. A Revew of Deterioration of Oil-seeds by Fungi with special Reference to India. International Biodeterioration **23:** 137-157

Cheng C. Y., Chung W. Y., Szeto Y. T., Benzie I. F. F. 2005. Fasting plasma zeaxanthin response to *Fructus Barbarum* L. (wolfberry; Kei tze) in food based human supplementation trial. British Journal of Nutrition. **93:** 123-130

China Daily. 2007. Wolfberry festival to be held in Ningxia. WWW-document 2004-07-19: http://www.chinadaily.com.cn/chinagate/2004-07/19/content_349679.htm visited 2013-05-24

EC 178/2002. Laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety. Official Journal of the European Communities

EC 852/2004 On the hygiene of foodstuff. Official Journal of the European Communities

EC 401/2006 Laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuff. Official Journal of the European Communities

EC 1881/2006. Setting maximum levels for certain contaminants in foodstuff. Official Journal of the European Communities

EC 834/2007. On organic production and labeling of organic products and repealing Regullation (EEC) No 2092/91. Official Journal of the European Communities

EC 889/2008. Laying down detailed rules for the implementation of Council Regulation (EC) 834/2007 0n organic production and labeling of organic products with regard to organic production, labeling and control. Official Journal of the European Communities

Engstrand L., Widén M. 2002. Frukter från främmande länder. Liber Distribution AB. Stockholm

EU 1235/2012. Amending Annex I to Regulation (EC) No 669/2009 implementing Regulation (EC) No 882/2004 of the European Parliament and of the Council as regards the increased level of official controls on imports of certain feed and food of non-animal origin. Official Journal of the European Communities

EU 91/2013. Laying down specific conditions applicable to the import of groundnuts from Ghana and India, okra and curry leaves from India and watermelon seeds from Nigeria and amending Regulations (EC) No 669/2009 and (EC) No 1152/2009. Official Journal of the European Communities

European Union, official website. 2013. Summaries of EU lagislation. WWW-document 2013-04-10: http://europa.eu/legislation_summaries/food_safety/contamination_environmental_factors/l21290_en.htm visited 2013-0410

Fuller G., Spooncer W. W., King A. D., Scade J., Mackey B. 1977. Survey of aflatoxins in California tree nuts. Journal of the American Oil Chemists' Society. 54: 231-234

Gross P. M., Zhang X., Zhang R. 2006. Wolfberry Nature´s Bounty of Nutrition and health. A Nutritional Reference for China´s Aincient Herbal Tresure. ISBN 1-4196-208-7 BookSurge, LLC

Gürses M. 2006. Mycoflora and Aflatoxin Content of Hazelnuts, Walnuts, Peanuts, Almonds and Roasted Chickpeas (LEBLEBI) Sold in Turkey. International Journal of Food Properties **9:** 395-399

JECFA. 1999. Risk analysis of mycotoxins by the Joint Food and Agriculture Organization of the United Nations/World Health Organization Expert Committee on Food Additives (JECFA). WWW-document 2013 04 26: http://www.fao.org/docrep/X2100T/X2100T04.HTM visited 2013-04-26

JECFA. 2001. Safety evaluation of certain mycotoxins in food. WHO Additives Series 2001; **74:** 505-506

Jimenez M., Mateo R., Querol A., Huerta T., Hernandez E. 1991. Mycotoxins and mycotoxigenic moulds in nuts and sunflower seeds for human consumptin. Mycopathologica. **115:** 121-127.

Jing J. 2010 Forecasting fungus disease conditions for wolfberries.In: Ed. Stigter K. 2010. Applied Agrometeorology. 217-219. Springer. Heidelberg, Dordrecht, London, New york

Jonsyn F. E. 1988. Seedborne fungi of sesame (sesamum indicum L) in Sierra Leone and their potential aflatoxin/mycotoxin production. Mycopathologia **104:** 123-127

Kerje T., Grum M. 2000. The origin of melon, *Cucumis melo*: a review of the literature. Acta Horticulturae. **510:** 37-44

Khayria M. A., Zohri A. A. 1993. Fungal flora and mycotoxins of six kinds of nut seed for human consumption in Saudi Arabia. Mycopathologia **124:** 55-64.

Kiple K. F., Ornelas K. C. 2000. The Cambridge World History of Food. Cambridge University Press. New York

KRAV. 2013. KRAV-märkningen. www-document 2013-05-08: http://www.krav.se/krav-markningen visited 2013-05-08

Kuang Y., Qiu F., Kong W., Yang M. 2012. Natural occurrence of ochratoxin A in wolfberry fruit wine marketed in China. Food Additives and Contaminants: Part B: Surveillance. **5:** 70-74

Kumar V., Basu M. S., Rajendran T. P. 2008. Mycotoxin research and mycoflora in some commercially important agricultural commodities. In: Crio Protection. **27:** 891-905

Kuiper-Goodman. 1995. Mycotoxins: risk assessment and legislation. Toxicology Letters 82/83: 853-859

Madigan M. T., Martinko J. M. 2006. Brock biology of microorganisms, 11th Ed. Pearson Education inc.

Milovanović M.,Pićurić-Jovanović K. 2005. Characteristics and composition of melon seed oil. Journal of Agricultural Sciences. **50:** 41-47

National Food Agency. 2007. Livsmedelsprovtagning i offentlig kontroll och mikrobiologisk bedömning av livsmedelsprov. The National Food Agency.

National Food Agency. 2013. Rapid Alert System for Food and Feed. WWW- Document: 2013 01 28 http://www.slv.se/sv/grupp1/Livsmedelskontroll/Resultat-av-kontrollen/RASFF/ visited 2013 05 20

National Food Agency. 2013. Ekologisk mat. WWW-document 2013-05-08: http://www.slv.se/sv/grupp1/Markning-av-mat/Ekologisk-mat/#reg visited 2013-05-08

National Sunflower Assosiation. 2013. All about Sunflower. WWW-document 2013: http://www.sunflowernsa.com/all-about/ visited 2013-06-03

Naturland. 2002. Organic farming in the Tropics and Subtropics: Sesame. WWW- Document 2002: www.naturland.de/fileadmin/MDB/documents/Publication/English/sesame.pdf visited 2013-06-03

Nayar M. N., Mehra K. L. 1970. Sesame: its uses, botany, cytogenetics and origins. Economic Botany **24:** 20-31

Neogen Corporation. 2012. Mycotoxin handbook. 4th. Ed. The dairy School. Auchincruive

NMKL, Nordisk Metodik-Kommitté för Livsmedel. 1983. Rap. no. 1. Statistical evaluation of results from quantitative microbiological examinations. 2th. Ed.

NMKL, Nordic Committee on Food Analysis. 2004. *Aspergillus flavus* and *A. parasiticus*. Determination in food and feed. Rap. no. 177: 1-4.

NMKL, Nordic Committee on Food Analysis. 2005. Molds and yeasts. Determination in food and feed. Rap. no 98, 4th. Ed.: 1-7.

NMKL, Nordic Committee on Food Analysis. 2005. *Penicillum verrucosum* – Ochratoxin Aproducing. Detection in food and feed. Rap. no. 142, 2nd. Ed.: 1-6.

Northholt M. D., Frisvad J. C., Samson R. A. 1995, Occurrence of food-borne fungi and factors for growth. In: Samson R. A., Hoekstra E. S., Frisvad J. C., Filtenborg O. 4th. Ed 1995. Introduction to food-borne fungi, p. 243-250. Centraalbureau voor Schimmelcultures. Baarn.

Pitt J. I., Hocking A. D. 1997. Fungi and Food Spoilage. 2nd. Ed. University Press. Cambridge

Pitt J. I., Hocking A. D. 2009. Fungi and Food Spoilage. 3rd. Ed. Springer. New York.

Rajendra B. K., Badar K. V., Pawar S. M., Chavan A. M. 2012. Storage mycoflora of oilseeds: a review. International Multidiciplinary Reseach Journal. **2:** 39-42

Ram, R., D. Catlin, J. Romero, and C. Cowley. 1990. Sesame: New approaches for crop improvement. p. 225-228. In: J. Janick and J.E. Simon (Ed), Advances in new crops. Timber Press, Portland.

Richard J. L., Bacchetti P., Arp L. H. 1981. Moldy walnut toxicosis in a dog, caused by the mycotoxin, penitrem A. Mycopathologia. **76:** 55-8

Risenta. 2013. Valnöt. WWW- document: http://www.risenta.se/expeditioner/valnot/ visited 2013-05-25

Salem N. M., Ahmad R. 2010. Mycotoxins in food from Jordan: preliminary survey. In Food Control. **21:** 1099-1103

Samson R. A., Hoekstra E. S., Lund F., Filtenborg O., Frisvad J. C. 2004, Methods for the detection, isolation and characterisation of food-borne fungi. In: Introduction to food-borne fungi. 7th. Ed: 2004, p. 283-297. Centraalbureau voor Schimmelcultures. Baarn.

Samson R. A., Hoekstra E. S., Frisvad J. C., Filtenborg O. 2004. Introduction to food- and airborne fungi. 7th. Ed. Centraalbureeau voor Schimmelcultures. Utrecht

Sanchis V., Magan N. 2004. Environmental conditions affecting mycotoxins. In: Magan N., Olsen M. (ed). Mycotoxins in food. 174-189.Woodhead publishing Ltd. Camebridge.

Sesaco Sesame Coordinators. 2008. Sesame Grower Guide. Sesaco Corporation. San Antonio

SCB. 2011. Standardavvikelse och kvartiler. WWW-document. 2011-08-15: http://www.scb.se/Pages/List 310894.aspx visited 2013-05-11

Scott P. M. 2004. Other mycotoxins. In: Magan N., Olsen M. (ed). Mycotoxins in food. 406-440.Woodhead publishing Ltd. Camebridge.

Shahnaz D., Ghaffar A. 1991 Detection of the seed-borne mycoflora of sunflower. Pakistan journal of botany. 23: 173-178.

Shaochun Chen, Yiju Song, Jinling Cao, Gang Wang, Hua Wei, Xushi Xu, Ling Lu. 2009. Localization and function of calmodulin in live-cells of Aspergillus nidulans. Fungal Genetics and Biology 47: 268-278

Sharfun-Nahar, Mustaq M., Hasami M. H. 2005. Seed-borne mycoflora of sunflower (*Helianthus Annuus L.*). Pakistan journal of botany 37: 451-457.

Scholte, R.P.M., Samson R. A., Dijksterhuis J. 2004. Spoilage fungi in the industrial processing of food. In: Samson, R.A., Hoekstra, E.S., Frisvad, J.C. and Frisvad J. C., Introduction to food- and airborne fungi. 7th .Ed. 2004, p 339-356. Centraalbureeau voor Schimmelcultures. Utrecht

Shee A. K., Raja R. B., Sethi D., Kunhambu A., Arunachalam K. D. 2010. Studies on the antibacterial activity potential of commonly used food preservatives. International Journal of Engineering Science and Technology. **2:** 264-269

Singh P. K., Shukla A. N. 2008. Survey of mycoflora counts, aflatoxin production and induced biochemical changes in walnut kernels. Jornal of Stored Products Research. **44:** 169-172

Thomas Jefferson Agricultural Institute. 2013. Sunflower- A Native Oilseed with Growing Markets WWW-document: http://www.jeffersoninstitute.org/pubs/sunflower.shtml#top visited 2013-05-26

University of Gothenburg. 2002. Standardavvikelse WWW-document. 2002-10-08: http://infovoice.se/fou/bok/10000049.shtml visited 2013-05-11

Venkatesagowda B., Ponugupaty E., Barbosa A. M., Dekker F. H. 2012. Diversity of plant oil seed-associated fungi isolated from seven oil-bering seeds and their potential for the production of lipolytic enzymes. World Journal of Microbiology and Biotechnology. **28:** 71-80

Venkataramana B. K., Babrekar P. P., Lakhanpaul S. 1999. Study of genetic diversity in Indian and exotic sesame (*Sesmum idicum L.*) germplasm using random amplified polymoephic DNA (RAPD) markers. Euphytica **110:** 21- 34

Visser W., Alexander S., Batenburg-van der Vegte W., Van Dijken J. P. 1990. Oxygen Requierments of yeasts. Applied and Environmental Microbiology. 56: 3785-3792

Weidenbörner M. 2001. Pumpkin seeds – the mycobiota and potential mycotoxins. European Food Research and Technology. **212:** 279-281

Zhang P. 2008. Testing of Seed Borne Fungi of Wolfberry in Ningxia. Nothern Horticulture **12:** 176-177

Zinedine A., Soriano J. M., Juan C., Mojemmi B., Molto J. C., Bouklouze A., Cherrah Y., Idrissi L., El Aouad R., Manes J. 2006. Incidence of ochratoxin A in rice and dried fruits from Rabat and Salé area, Morocco. Food Additives and Contaminants. **24:** 285-291

Appendix

Substrates

AFPA (Aspergillus flavus/parasiticus agar)

AFPA Base (Oxiod CM 731) (Oxoid LTD, Basingstoke , Hampshire, England)	45.5g
Millipore water	1000ml
Antibiotic solution according NMKL no 152	10 ml

CREA (Creatin sakaros agar)

According Samson et al. 2004
Trace metal solution according NMKL no 98

CZ (Czapek agar)

CZ agar (oxoid CM 97) (Oxoid LTD, Basingstoke , Hampshire, England)	45.5 g
Millipore water	1000ml
Trace metal solution according NMKL no 98	1 ml

CYA (Czapek Yeast extract agar)

According Samson et al. 2004
Trace metal solution according NMKL no 98

DG18 (Dichloran 18% glycerol agar)

According NMKL 98
Trace metal solution according NMKL no 98
Antibiotic solution according NMKL no 152

MEA (Malt extract agar)

According Samson et al. 2004

Water activity

A_w in the samples are presented in the tables A1-A5. Standard deviation and average was calculated using Microsoft excel 2010.

Table A1. A_w in goji berries

Sample	a_w
39	0.337
49	0.354
60	0.331
64	0.287
65	0.296
78	0.314
98	0.320
104	0.335
116	0.288
152	0.375
ave:	0.3237
stdev:	0.0286824

Table A2. A_w in pumpkin seeds

Sample	a_w
42	0.428
59	0.615
81	0.579
87	0.541
102	0.366
115	0.414
127	0.466
147	0.568
149	0.395
154	0.592
ave:	0.4964
stdev:	0.0876484

Table A3. A_w in sesame seeds

Sample	a_w
43	0.530
44	0.485
50	0.438
83	0.349
85	0.678
100	0.568
113	0.282
114	0.570
126	0.541
151	0.247
ave:	0.4688
stdev:	0.1313231

Table A4. A_w in sunflower seeds

Sample	a_w
40	0.528
41	0.346
80	0.433
101	0.459
103	0.555
128	0.514
143	0.433
146	0.476
148	0.628
155	0.413
ave:	0.4785
stdev:	0.0763456

Table A5. A_w in walnuts

Sample	a_w
38	0.462
58	0.461
63	0.422
79	0.470
82	0.462
84	0.714
86	0.424
142	0.268
150	0.421
153	0.414
ave:	0.4518
stdev:	0.1091348

Total amount of fungi (cfu/g) in sampled commodities

Total amount of fungi in the samples are presented in tables A6-A10.

Table A6. Amount of fungi in goji berries (cfu/g)

Sample	Cfu/g mold	Cfu/g yeast
39	572	194
49	18100	131
60	4100	26800
64	432	126
65	468	180
78	509	25900
98	4680	47700
104	856	0
116	1840	1040
152	811	1490

Table A7. Amount of fungi in pumpkin- and melon seeds (cfu/g)

Sample	Cfu/g mold	Cfu/g yeast
42	185	85.5
59	90900	2210
81	432	122
87	49,5	18
99 (melon seeds)	740	0
102	243	131
115	212	35
127	4680	78600
147	99	0
149	4.5	63
154	0	0

Table A8. Amount of fungi in sesame seeds (cfu/g)

Sample	Cfu/g mold	Cfu/g yeast
43	94.5	0
44	0	0
50	743	0
83	0	9
85	108	54
100	17700	574
113	0	0
114	25100	1890
126	4.5	63
151	31.5	0

Table A9. Amount of fungi in sunflower seeds (cfu/g)

Sample	Cfu/g mold	Cfu/g yeast
40	8240	1980
41	1710	0
80	56800	0
101	1100	9
103	320	0
128	797	0
143	10800	0
146	12100	0
148	545	0
155	7880	0

Table A10. Amount of fungi in walnuts (cfu/g)

Sample	Cfu/g mold	Cfu/g yeast
38	374	0
58	4600	0
63	540	0
79	14300	45
82	4730	0
84	5500	90.1
86	19300	0
142	16100	0
150	6290	45
153	9900	0