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Investigation of patulin and citrinin in grape must and wine from grapes naturally contaminated by strains of *Penicillium expansum*

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ABSTRACT

Twenty three strains of *Penicillium expansum*, as a predominant species, were isolated from 23 (92%) out of 25 grape samples of 17 different grape varieties. The results of the identification of *P. expansum* strains were confirmed by a PCR method. Most of the isolates of *P. expansum* (21/23, 91%), when tested for toxigenicity, were bi-toxigenic: they produced citrinin (CIT) and particularly high amounts of patulin (PAT). A validated UPLC-MS/MS method for the determination of PAT and CIT was applied. The limits of quantification (LOQ) for PAT and CIT in grape must and toxigenicity testing samples were 100 and 2 ng/g, respectively. The results of PAT and CIT quantification in 23 grape must samples demonstrated the occurrence of PAT in 10 (43%) grape must samples (mean: 171 ng/g; median: 50 ng/g; and range: 143–644 ng/g) and the occurrence of CIT in two (9%) grape must samples (mean: 1 ng/g; median: 1 ng/g; and range: 2.5–3.5 ng/g). This is the first report on the natural occurrence of CIT in grape must. A validated HPLC-UV-VIS method for the determination of PAT in wine samples was applied, and concentrations in all 23 wine samples were below the LOQ (< 10 ng/g).

1. Introduction

The climate change is a widely acknowledged fact (Botana and Sainz, 2015; Marroquín-Cardona et al., 2012; Paterson et al., 2018). Overall, it is necessary to consider how climate change will impact viniculture as this will in turn affect mycotoxin contamination (Paterson et al., 2018). Recently, there has been intensive monitoring of the effects of climate change and global warming on the possible occurrence and distribution of toxigenic microfungi - producers of important mycotoxins e.g. ochratoxin A (Battilani et al., 2003; Bellí et al., 2007; Delage et al., 2003), fumonisin B_2 (Abrunhosa et al., 2011; Logrieco et al., 2009; Mogensen et al., 2010), patulin (Bragulat et al., 2008; Majerus et al., 2008; Sanzani et al., 2016) and *Alternaria* mycotoxins (Asam et al., 2009), in grapes, grape juice, grape must and wine in subtropical and temperate climates.

The potential risk of exposure to patulin (PAT) and citrinin (CIT) in the grape-wine chain has been revealed recently after a report on the presence of *Penicillium expansum* in grapes and its possible ability to produce PAT and CIT (Bragulat et al., 2008; Ostry et al., 2017a).

Penicillium expansum Link is one of the most common microfungi on Earth, occurring in all kinds of food, in almost all climates. *P. expansum* is a postharvest rot that affects a number of different hosts, including apples, citrus fruits, pears, plums, peaches, apricots, cherries, blackcurrants, melons, strawberries and grapes (Larsen et al., 1998; Snowdon, 1990).

PAT (PubChem CID: 4696) (Fig. 1) is a water soluble lactone produced via the polyketide metabolic pathway by many species of microfungi (e.g. those within *Penicillium, Aspergillus* and *Byssochlamys*) (Frisvad et al., 2007).

PAT is most often associated with *Penicillium expansum* and is found frequently in apple products. PAT was originally described as an antibiotic and exhibits strong antibiotic activity against different Gram-positive and Gram-negative bacteria including *Mycobacterium tuberculosis*. It was tested unsuccessfully as an antibiotic candidate and was found too toxic to humans and animals (Puel et al., 2010).

PAT mainly induces gastrointestinal disorders including ulceration, distension and bleeding (Puel et al., 2010; Wouters and Speijers, 1996). PAT provokes congestion and oedema of pulmonary, hepatic and gastrointestinal blood vessels and tissues (Puel et al., 2010; Wouters and Speijers, 1996).

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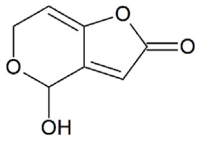

Fig. 1. Structural formula of PAT.

Glaser and Stopper (2012) investigated the mechanism of PAT-induced genotoxicity: chromosomal damage as micronucleus and nucleoplasmic bridge formation was detected.

In a limited experiment, subcutaneous injection of PAT produced local sarcomas in rats; consequently, PAT was only classified as Group 3 (*not classifiable as to its carcinogenicity to humans*) by the International Agency for Research on Cancer (IARC) *Monographs* (Ostry et al., 2017b).

The established Provisional Maximum Tolerable Daily Intake (PMTDI) at 0.4 μg/kg body weight (bw)/day for PAT is derived from the NOEL (no-observed-effect level) at 0.043 mg/kg bw/day divided by a safety factor of 100 (JECFA, 1995). Similarly, the Scientific Committee on Food (SCF) endorsed the PMTDI of 0.4 μg/kg bw/day for PAT (SCF, 2000).

CIT (PubChem CID: 54680783) (Fig. 2) is a polyketide mycotoxin produced worldwide in foodstuffs by several microfungi belonging to the genera *Penicillium, Monascus* and *Aspergillus* (Frisvad et al., 2007; Ostry et al., 2013; Vinas et al., 1993).

Data on CIT occurrence in foodstuffs are still limited and insufficient to reliably estimate CIT exposure in the human population (EFSA, 2012; Ostry et al., 2013). CIT was detected in grapes before storage (Aziz and Moussa, 2002). It was also found in other plant products such as beans, fruits, fruit and vegetable juices, herbs and spices (EFSA, 2012; Ostry et al., 2013).

CIT is nephrotoxic to animals: in repeat dose toxicity studies, the kidney was identified as the principal target organ for CIT, and significant species differences in the susceptibility to CIT have been observed (EFSA, 2012). In humans, CIT also affects the renal system (Ammar et al., 2000). In addition, CIT exhibits teratogenic, embryotoxic and reproductive and other significant toxic effects (de Oliveiri Filho, 2017; Vesela et al., 1983; Singh et al., 2007, 2008). Chronic CIT exposure has been associated with Balkan endemic nephropathy, although other etiologic agents including aristolochic acid and ochratoxin A were also discussed (Peraica et al., 2008; Pfohl-Leszkowicz et al., 2007).

CIT is not carcinogenic according to recent knowledge; CIT has been classified as Group 3 (*not classifiable as to its carcinogenicity to humans*) by the IARC *Monographs* due to *limited evidence of carcinogenicity* in experimental animals (Ostry et al., 2017b).

The European Food Safety Authority (EFSA) has assessed the toxicity of CIT and came to the conclusion that CIT is nephrotoxic, with a NOAEL (no-observed-adverse-effect level) of 20 μg/kg/bw/day that was identified from a 90-day study in male Wistar rats (EFSA, 2012). In the latter study, three concentrations had been administered, and no effects were observed at the highest dose tested of 20 μg/kg bw/day. Due to the limitations and uncertainties in the database, the derivation of a health-based guidance value was not considered appropriate, but a "*level of no concern for nephrotoxicity*" of 0.2 μg/kg bw/day was determined (EFSA, 2012).

The Netherlands National Institute for Public Health and the Environment (RIVM) has assessed the toxicity of CIT by setting a benchmark dose analysis (RIVM, 2017). The lowest benchmark dose level (BMDL) of 48 μg/kg bw/day obtained from the endpoint "*decreased crown rump length*" from the study by Singh et al. (2012) is considered as the appropriate point of departure for risk assessment. This BMDL is 2.4 times higher than the NOAEL determined by the EFSA in 2012.

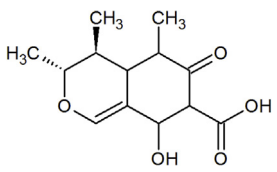

Fig. 2. Structural formula of CIT.

Table 1
Grape sampling in various vineyards.

Vineyard	No.	Grape variety	Type of grape variety
Petrovice – Kokusove hory	1	Green Veltliner	White
	2	Palava	White
Miroslav – Weinperky	3	Limberger	Red
	4	Palava	White
	5	Green Veltliner	White
	6	Pinot Gris	White
Podmoli – Sobes	7	Rhine Riesling	White
	8	Pinot Blanc	White
	9	Pinot Gris	White
	10	Welschriesling	White
	11	Pinot Noir	Red
Strachotice – Divci vrch	12	Pinot Gris	White
	13	Rhine Riesling	White
	14	Red Traminer	White
	15	Pinot Noir	Red
	16	Cabernet Sauvignon	Red
Havraniky – Stare vinice	17	Limberger	Red
	18	Pinot Noir	Red
	19	Zweigeltrebe	Red
	20	Rhine Riesling	White
	21	Sémillon	White
	22	Red Traminer	White
	23	Chenin blanc	White
	24	Sauvignon gris	White
	25	Viognier	White

The objective of this study was to obtain data on the possible dietary exposure sources of PAT and CIT contamination of the grape-wine chain in the Czech Republic. The mycobiota of grapes from several vineyards was examined. PAT and CIT producing abilities of the fungal isolates of *Penicillium expansum* were tested and the occurrence of PAT and CIT in grape must and wine were analysed.

2. Materials and methods

2.1. Grape sampling

Altogether, the study covered five various vineyards from the south of Moravia (the Znojmo wine region), and 25 grape samples of 17 different grape varieties were involved in this study (Table 1).

The distance between the vineyards in the Znojmo wine region was more than 10 km. The wine industry has a long tradition in the Znojmo wine region, with the first vineyard founded by Romans. The Znojmo wine region lies in the rain shadow and on the foothills of the Bohemia-Moravian Highland, whose stony soils are excellent for the cultivation of Rhine Riesling, Green Veltliner, and in certain parts and wine villages for red varieties such as Limberger and Pinot Noir.

One sample of grape varieties consisted of three subsamples of grapes, which were sampled in the left, middle and right parts of the vineyard. In total, 25 grape samples (ca. 300–350 g per sample according to grape variety) were collected in sterile plastic bags during harvesting at the beginning of October 2014, and transported to a mycological laboratory for immediate processing. Picked grapes were stored at 4 ± 1 °C and analysed by mycological methods within 24 h after harvest.