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Development and validation of ultra-high-performance liquid chromatography–tandem mass spectrometry methods for the simultaneous determination of beauvericin, enniatins (A, A1, B, B1) and cereulide in maize, wheat, pasta and rice

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ABSTRACT

Rapid and accurate UPLC–MS/MS methods for the simultaneous determination of beauvericin and the related enniatins (A, A1, B, B1), together with cereulide were successfully developed and validated in cereal and cereal-based food matrices such as wheat, maize, rice and pasta. Although these emerging foodborne toxins are of different microbial origin, the similar structural, toxicological and food safety features provided rationale for their concurrent detection in relevant food matrices. A Waters Acquity UPLC system coupled to a Waters Quattro Premier XE™ Mass Spectrometer operating in ESI+ mode was employed. Sample pretreatment involved a fast and simple liquid extraction of the target toxins without any further clean-up step. For all toxins the sample preparation resulted in acceptable extraction recoveries with values of 85–105% for wheat, 87–106% for maize, 84–106% for rice and 85–105% for pasta. The efficient extraction protocol, together with a fast chromatographic separation of 7 min allowed substantial saving costs and time showing its robustness and performance. The validation of the developed method was performed based on Commission Decision 2002/657/EC. The obtained limits of detection ranged from 0.1 to 1.0 $\mu g\,kg^{-1}$ and the limits of quantification from 0.3 to 2.9 $\mu g\,kg^{-1}$ for the targeted toxins in the selected matrices. The obtained sensitivities allow detection of relevant toxicological concentrations. All relative standard deviations for repeatability (intra-day) and intermediate precision (inter-day) were lower than 20%. Trueness, expressed as the apparent recovery varied from 80 to 107%. The highly sensitive and repeatable validated method was applied to 57 naturally contaminated samples allowing detection of sub-clinical doses of the toxins.

1. Introduction

Contamination of food and feed with toxins is one of the main concerns in the food industry. Both bacteria and fungi are capable of producing microbial metabolites in food and feed under the appropriate environmental conditions. These toxins can enter the food chain directly through contaminated food or indirect through the presence of contaminants in food of animal origin derived from animals, which were fed with contaminated grains. Even though several pre-and post-harvest efforts such as sorting, kernel and hand sorting are made in order to prevent and control bacteria and fungi, the produced toxins can remain active even after very harsh treatments [1]. In addition, the toxins are stable under the most common conditions used in food processing and can consequently be found in the prepared products [2,3]. Contamination with toxins of fungal and bacterial origin may lead to acute poisoning or have long-term negative consequences on the health of both human and animals [4]. Besides the health risk, contaminated food and feed causes financial losses with enormous economic impact all over the world. Therefore, an assessment of the presence and impact

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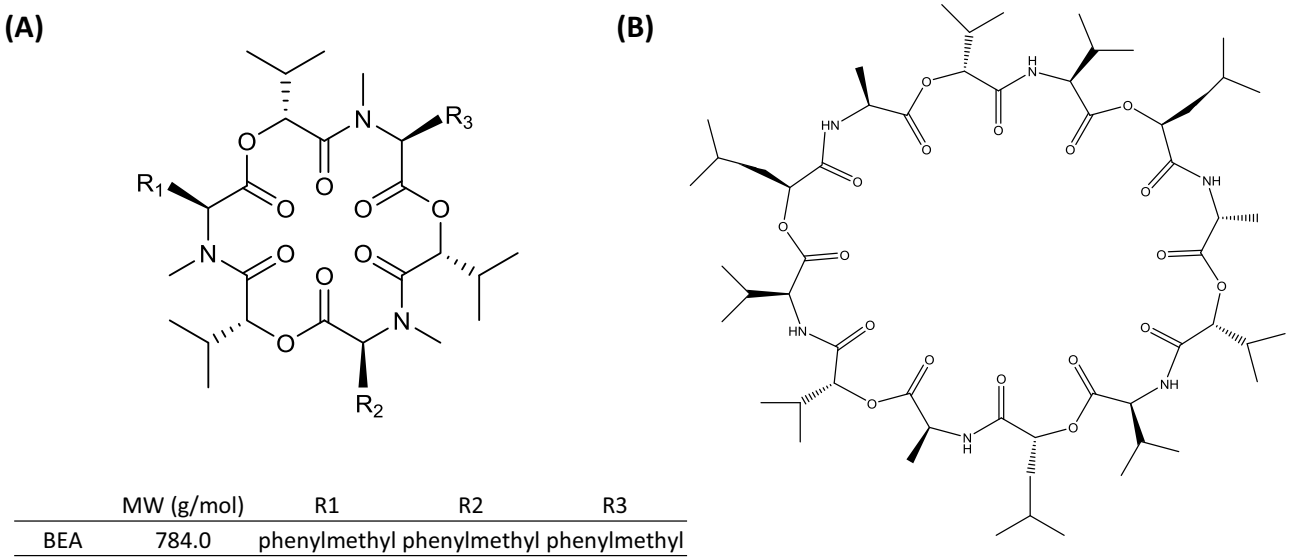
of these harmful toxins is imperative and starts with developing methods for their detection and quantification.

Mycotoxins are toxic secondary metabolites produced by several fungi, mainly *Aspergillus* spp., *Penicillium* spp. and *Fusarium* spp. [5]. Acute effects (short-term) as well as chronic effects (long-term) have been reported after exposure to these toxic fungal metabolites. Mycotoxins are common contaminants of many grains like wheat, barley, maize, and rice. The most prevalent mycotoxins such as zearalenone, aflatoxins, ochratoxin A, trichothecenes, deoxynivalenol have been frequently studied. Unfortunately, there is limited data on the toxicity and occurrence of the so-called 'emerging' mycotoxins. These mycotoxins are neither routinely determined, nor legislatively regulated. Examples are beauvericin (BEA) and the related enniatins A, A1, B, B1 (ENNs), both produced by several *Fusarium* species. Their presence has been reported in cereals from several countries and in human biological fluids [6–8]. Recently EFSA published an opinion on the presence of ENNs and BEA in food and feed, but the lack of relevant toxicity data prevented a risk assessment [9].

In addition to mycotoxins, bacterial toxins are of global concern, mainly related to foodborne illnesses. The latest report of EFSA on zoonoses, zoonotic agents and foodborne outbreaks revealed that bacterial toxins encounter for 16.1% of all reported foodborne outbreaks caused by microbial contamination. This figure shows an increase of 60% over a period of 5 years [10]. Foodborne bacterial pathogens that are well known as toxin producers are *Staphylococcus aureus*, *Clostridium botulinum, Clostridium perfringens* and *Bacillus cereus*. Of multiple toxins produced by these pathogens the most resistant is the emetic toxin cereulide. *Bacillus cereus* is a gram-positive spore-forming pathogen that causes two types of food poisoning syndromes: an emetic (vomiting) intoxication and a diarrheal infection. The emetic syndrome, which is inducted by the toxin cereulide results in vomiting a few hours after ingestion of the contaminated food [11]. Although *B. cereus* can be present in various food products, most reported food poisoning cases were associated with rice and pasta dishes. This emetic toxin is often related to acute food poisoning, occasionally even with a fatal outcome [12,13]. Cereulide is characterized by its resistance to extreme pH and heat conditions, and resistance to digestion enzymes like pepsin and trypsin [2]. Consequently, it survives food processing and preparation and retains activity during gastrointestinal passage [2,14]. This illustrates the high importance of a rapid identification and detection of the emetic toxin.

BEA, ENNs and CER are all cyclic depsipeptides with ionophoric properties. Their apolar nature gives them the ability to incorporate into lipid bilayers of cell membranes. Hereby they create cation selective channels that increase the permeability for cations, resulting in disturbances of the physiological cation level in the cell [15,16]. CER is a cyclic dodecadepsipeptide (twelve-membered) while BEA (and ENNs) are smaller cyclic hexadepsipeptides (six-membered) [17,18]. The chemical structures of beauvericin and enniatins and cereulide are depicted in Fig. 1. Both the bacterial toxin CER and the fungal toxin BEA (and the related ENNs) are regarded as emerging health hazards and their striking similarities should allow a common approach towards the development of a detection technique. The possible co-occurrence of the different toxic compounds in one matrix implies a potential risk for additive, synergic or antagonist toxic effects. Considering the risks to human and animal health, the determination of the occurrence of these medium-sized cyclic depsipeptides in food and feed is imperative. Their potential presence at low levels is of special relevance to food safety [19,20].

The risk associated with the presence of these toxins initiated the search for more sensitive analytical methods applicable in various matrices. Santini et al. published a review that summarizes techniques used for extraction and quantification of beauvericin and fusaproliferin in food matrices [21]. It became clear that in the search for low detection levels, mass spectrometry has been increasingly used to achieve this goal. The commonly exerted steps regarding the sample preparation are extraction with solvents sometimes followed by an extra clean-up with different types of columns and/or a filtration step. Over the past few year, several methods have been developed for BEA and/or ENNs using mainly acetonitrile, chloroform, methanol or a mixture with water as extraction solvent [22–26]. Alternately, Ambrosino et al. optimized a sample preparation involving supercritical fluid extraction (SFE)



	MW (g/mol)	R1	R2	R3
BEA	784.0	phenylmethyl	phenylmethyl	phenylmethyl
ENN A	681.9	sec-butyl	sec-butyl	sec-butyl
ENN A1	667.9	sec-butyl	sec-butyl	iso-propyl
ENN B	639.8	iso-propyl	iso-propyl	iso-propyl
ENN B1	653.9	iso-propyl	iso-propyl	sec-butyl

Fig. 1. The chemical structures of beauvericin and enniatins (A) and cereulide (B).