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Analytical Methods

UPLC–MS/MS method for determination of selected pyrrolizidine alkaloids in feed

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ABSTRACT

Alkaloids known as secondary metabolites are grouped by typical structural characteristics into large families such as pyrrolizidine alkaloids (PAs) comprising more than 350 individual heterocyclic compounds. The PAs present a serious health risk to human and livestock; hence there is a need for methods that allow these dangerous plant toxins to be determined. In this study, a fast, reliable and sensitive approach is proposed to identify and quantify PAs in feed samples. PAs including monocrotaline, senkirkine, senecionine, seneciphylline and retrorsine were determined by ultra-performance liquid chromatography coupled with tandem mass spectrometry. Sample preparation was based on a modified QuEChERS approach. The mean recovery, precision, matrix effects and limits of quantification were assessed for three matrices within the method validation. The presented method was used to inspect 41 various feed samples, where the presence of PAs was expected. Roughages and feed for rabbits contained the highest levels of PAs, in general.

1. Introduction

Pyrrolizidine alkaloids (PAs) are naturally occurring plant toxins which are produced as secondary metabolites. They are not crucial for plant survival but their function is to defend the plant against herbivore and insect attacks (Boppre, 2011). It is estimated that approximately 3% of all flowering plants contain at least one PA (EFSA, 2011; Mulder, Beumer, Oosterink, & de Jong, 2009). In Europe, PAs occur in families of *Boraginaceae*, *Asteraceae* and *Fabaceae* (EFSA, 2011; Smith & Culvenor, 1981). The typical plant representatives are e.g., comfrey (*Symphytum officinale*) (Couet, Crews, & Hanley, 1996; Smith & Culvenor, 1981), ragwort (*Senecio jacobaea*) (Segall, 1978), and coltsfoot (*Tussilago farfara*) (Lebada et al., 2000). PAs represented by more than 350 individual heterocyclic compounds have a common basic structure composed of one of the four necine bases: platynecine, retronecine, heliotridine and otonecine.

PAs are suspected to be mutagenic and genotoxic carcinogens (EFSA, 2011). They cause intoxications characterised by hepatotoxicity which might result in hemorrhagic necrosis and veno-occlusion in the liver (Prakash, Pereira, Reilly, & Seawright, 1999; Zhou et al., 2010). Human exposure originates from directly consuming PA-containing teas, plant material and herbal dietary supplements (Avula, Wang, Wang, Smillie, & Khan, 2012; Cheng, Kirk, Vrieling, Mulder, & Klinkhamer, 2011; EFSA, 2011). Consumption is also possible via contaminated honey and animal product such as meat, milk and eggs. Nevertheless, cases of human poisoning resulting from honey and animal product exposure have not yet been reported (EFSA, 2011).

Due to the bitter taste of plants containing PAs, animals avoid their direct consumption. However contaminated feed such as silage can cause livestock losses, as PAs in their processed state become more palatable (EFSA, 2011). There are substantial differences in PA sensitivity between different animal species. Small ruminants such as sheep and goats and certain minor species such as rabbits are relatively more resistant to PA intoxication, whereas horses, pigs, cattle, poultry, rats and humans are generally considered to be more susceptible to PA toxicity (Cheeke, 1988; McLean, 1970; Swick, Cheeke, Goeger, & Buhler, 1982; WHO, 1988). These differences are believed to be partly due to the variations in the efficiency of liver enzymes in metabolizing alkaloids (Lanigan, 1971; Prakash et al., 1999).

Despite PA serious, well-known toxic effects and the fact that European Commission have regulated the contents of PA-containing plants and plant parts in feed (EC, 2002b), there are still no maximum residue limits (MRLs) for individual PAs in food or feed (Griffin, Danaher, Elliott, Glenn, & Furey, 2013). In 2011 the

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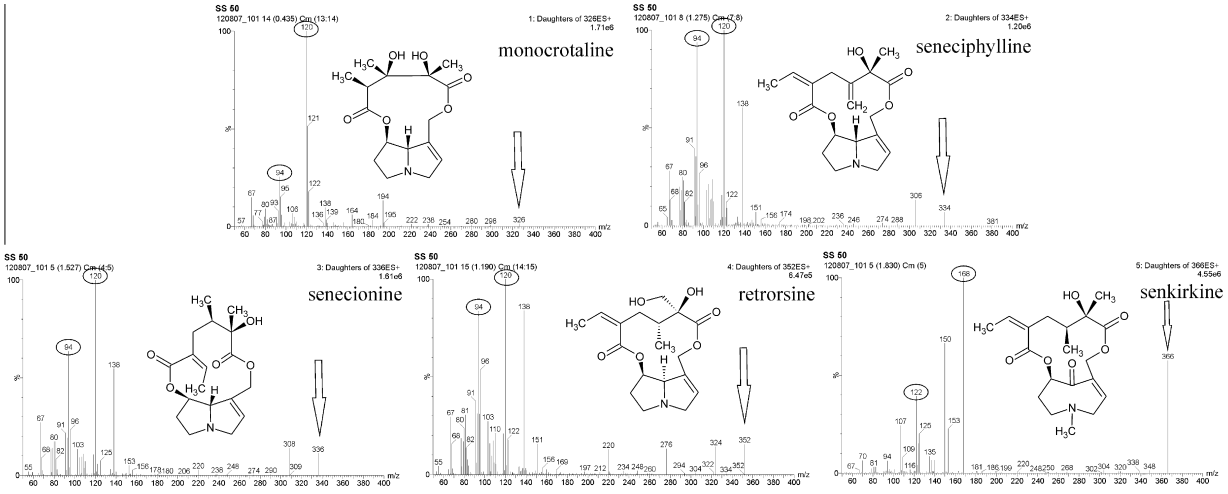

Fig. 1. The ESI$^+$ product spectra and chemical structures of five PAs. Monitored product ions and precursors are marked.

European Food Safety Authority (EFSA) published a scientific opinion on PAs in food and feed recommending the ongoing collection of analytical data on the occurrence of PAs in relevant commodities. The EFSA also pointed out the need for a larger and more diverse set of certified reference standards which are necessary to improve analytical methods (EFSA, 2011).

The methods currently available to detect PAs are usually dedicated to a certain commodity. In particular, methods based on LC–MS have been proven to be highly beneficial for the detection of PAs in honey (Crews, Startin, & Clarke, 1997; Dübecke, Beckh, & Lüllmann, 2011; Griffin et al., 2013; Martinello, Cristofoli, Gallina, & Mutinelli, 2014) or in complex matrices such as plant materials (Avula et al., 2012; Cheng et al., 2011; Zhang et al., 2008; Zhou et al., 2010). Another method using LC coupled to high resolution mass spectrometry has been published to determine PAs and other natural toxins in food and feed (Mol, Van Dam, Zomer, & Mulder, 2011). However, robust analytical methods allowing the detection and quantification of PAs in complex food and feed matrices are still missing.

The QuEChERS (Quick Easy Cheap Effective Rugged Safe) method is the most frequently used multiple-sample preparation approach originally developed for pesticide residue extraction (Anastassiades, Lehotay, Stajnbaher, & Schenck, 2003). Due to its dynamicity and simplicity this method can be applied to the analysis of other contaminants and residues, such as mycotoxins (Zachariasova et al., 2010), steroids (Klinsunthorn, Petsom, & Nhujak, 2011) and PAs (Kempf et al., 2011; Mol et al., 2011) in many different matrices.

The CONTAM Panel published a list of PAs which are of particular importance for food and feed (EFSA, 2011). In this study, we propose a fast, reliable and sensitive approach which allows the identification and quantification of PAs in feed samples using a non-selective modified QuEChERS method with no clean-up and ultra performance liquid chromatography coupled with tandem mass spectrometry (UPLC–MS/MS). Using this method we analysed 5 PAs (Fig. 1) from the EFSA list in 41 feed samples of different matrix composition. To our knowledge it is the first study which investigates PA contamination of feed in the Czech Republic.

2. Materials and methods

2.1. Chemicals and reagents

Methanol (LC–MS grade), ammonium formate (MS grade) and formic acid (LC–MS grade), formic acid (reagent grade) and acetonitrile (HPLC grade) were purchased from Sigma–Aldrich (Steinheim, Germany). Ultrapure water was produced by Milli-Q system (Bedford, MA, USA).

Individual evaporated PA standards of senkirkine, senecionine, monocrotaline, seneciphylline, and retrorsine were purchased from Biopure Referenzen substanzen GmbH (Vienna, Austria). All alkaloid standards were dissolved in methanol to obtain stock solutions at a concentration of 50 μg/mL and were stored at −20 °C. A stock standard mixture (1.25 μg/mL) prepared from the individual stock solution was diluted with either 50% acetonitrile or 50% extract solution to obtained solvent or matrix-matched calibration respectively, covering the levels from 0.125 ng/mL to 125 ng/mL.

2.2. Samples and pre-treatment

In total, 41 feed samples were collected within the official control program of the Central Institute for Supervising and Testing in Agriculture in the Czech Republic. These samples were ground and sieved to a particle size of 1 mm, homogenised and kept in dark and dry conditions at room temperature.

2.3. Sample preparation

The extraction procedure was based on the modified QuEChERS method (Anastassiades et al., 2003; Zachariasova et al., 2010). In brief, a homogenised sample (2.5 g) was weighed into a 50 mL polypropylene tube. Extraction with 10 mL acetonitrile and 10 mL 0.1% formic acid in water was performed using end-over-end shaking for 20 min. 4 g $MgSO_4$ and 1 g NaCl were added to the extraction mixture, the tube was shaken for 1 min and then centrifuged for 5 min at 5000 rpm. The 0.5 mL of upper organic phase was diluted with 0.5 mL of deionised water, mixed and filtered through a 0.2 μm nylon membrane filter before UPLC–MS/MS analysis. The final extract contained a 0.125 g sample equivalent per mL. No clean-up was performed.

2.4. UPLC–MS/MS determination

PA analysis was performed using a Waters Acquity UPLC system coupled to a Xevo TQ MS (Waters, USA) equipped with an electrospray interface (ESI). Chromatographic separation was performed using an Acquity UPLC BEH C18 column (50 mm × 2.1 mm × 1.7 μm) (Waters, USA). The column was kept at 40 °C whilst the autosampler was set at 15 °C. The injection volume was 2.5 μL. The flow rate of the mobile phase was 0.4 mL/min. The mobile phase consisted of water containing 0.1% formic acid