Determination of patulin in apple juice by liquid chromatography-electrospray tandem mass spectrometry

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Abstract. Patulin is a mycotoxin produced by several fungi, (Penicillium, Aspergillus, Byssochlamys). The main sources of patulin intake in human diet are apples, apple juice and apple nectar, and for this reason, apple based foods are monitored for the presence of this mycotoxin. Commission Regulation EC No 1881/2006 lays down maximum residue limits (MRLs) of 50 µg/kg in apple juice and cider, 25 µg/kg in solid apple products, and 10 µg/kg in products for infants and young children. In Serbia, maximum permitted amounts of patulin in fruit juices, reconstituted concentrated fruit juices and fruit nectars, as well as in solid apple products, including apple compote and apple puree, intended for direct human consumption are prescribed in the Regulation on maximum concentrations of certain contaminants in foodstuffs. This paper presents the LC-MS/MS method for quantitative determination of patulin in apple juice. Criteria for method validation were taken from Commission Decision 2002/657/EC. Linearity was confirmed in the concentration ranges of 0-100 µg/kg, with the limit of detection (LoD) of 9.85 µg/kg. The performance of the method was successfully verified by participating in a proficiency study.

1. Introduction
Patulin, with the formula shown in Figure 1, is a secondary metabolic product of moulds such as Penicillium, Aspergillus and Byssochlamys species [1]. Penicillium and Byssochlamys are the most studied species, while Penicillium expansum is known as the major producer of patulin. Patulin is a common contaminant of fruit and vegetable based products, most notably apples, and it is particularly associated with apples exhibiting “brown rot” or other rotting characteristics. Fruits that are damaged or improperly stored are susceptible to the growth of patulin producing moulds [2, 3]. Both patulin and the moulds which produce it, especially Byssochlamys strains, are heat stable, so the normal pasteurization treatment is not sufficient to decompose it [4]. Several studies have shown toxic, mutagenic, carcinogenic and teratogenic properties of patulin [5]. Fliege and Metzler extensively studied patulin’s chemical and biochemical properties, emphasizing its electrophilic properties [6, 7]. Patulin exerts toxicity through covalent binding to the sulfhydryl groups on proteins and glutathione [6, 7]. In vivo toxicity assessment shows damage to vital organs and systems including the liver, kidney, intestinal tissues and immune system [8, 9].

Patulin occurrence in the food commodities poses a serious threat and necessitates effective methods to remove it from food products such as removal of decayed and trimming of mouldy portions of rotten fruits [10]. It also creates a demand to improve handling and food processing techniques. Because apple juice is such a popular beverage and the possibility for life-long exposure exists, the permissible levels of patulin are regulated. Commission Regulation EC No 1881/2006 lays down maximum residue limits (MRLs) of 50 µg/kg in apple juice and cider, 25 µg/kg in solid apple
products and 10 µg/kg in products for infants and young children [11]. In Serbia, maximum permitted amounts of patulin in fruit juices (50 µg/kg), reconstituted concentrated fruit juices and fruit nectars (50 µg/kg), as well as in solid apple products, including apple compote and apple puree (25 µg/kg), intended for direct human consumption are prescribed in Article 2, Annex 1 of the Regulation on maximum concentrations of certain contaminants in food [12].

Since patulin is a regulated compound and has negative impact on food safety, it is necessary to develop the method for its reliable quantitative determination for the purpose of official controls. Therefore, the aim of this work was the development of a sensitive, simple and rapid method for the determination of patulin in apple juice by reverse phase liquid chromatography tandem mass spectrometry.

2. Materials and Methods
Patulin (CAS No. 149-29-1) analytical standard was purchased from Toronto Research Chemical (Toronto, Ontario, Canada). Water, methanol, ammonium acetate, ethyl acetate were all HPLC grade and purchased from Sigma-Aldrich (St. Louis, USA). Nylon filters, pore size 0.22µm were purchased from AMTAST (Lakeland, Florida, USA). Stock solution of patulin, c = 1.00 mg/mL was prepared in methanol, and working solution, c = 10.0 ng/µL, was prepared by diluting stock solution with methanol. All solutions were stored at -20ºC.

Mass spectrometric analysis of patulin was carried out on Shimadzu mass spectrometer LCMS-8040 coupled to a Shimadzu UHPLC instrument (Shimadzu, Europa, Duisburg, Germany). The instrument was controlled by LabSolution software. Separation was carried out using Kinetex 50 x 2.1 mm 2.6µ C-18 100Å analytical column with UltraGuard cartridge (Phenomenex, Torens, CA, USA). The oven temperature was set to 40ºC. The mobile phase consisted of 40mM ammonium acetate in water (mobile phase A) and 40mM ammonium acetate in methanol (mobile phase B) flowing at a rate of 0.30 mL/min in gradient mode. Electropray ionization (ESI) was used in negative mode, with the following parameters: probe voltage 4kV, temperatures of block heat (BH), desolvation line (DL) and interface were 400 ºC, 250 ºC and 350 ºC respectively, nebulizing and drying gas flow were 3 and 15 L/min respectively. Argon was used as the collision gas. The precursor and product ions and collision energies for patulin are presented in Table 1.

| Compound | Precursor ion (m/z) | Product ions (m/z) | Collision energies (eV) | Ionization mode |
|----------|---------------------|--------------------|-------------------------|----------------|

Figure 1. Structural formula of patulin

Table 1. Mass spectrometry parameters for patulin
Apple juice sample (10 g) was weighed into polypropylene jars with caps. Ethyl acetate 20 mL was added, the jars were shaken and placed in an ultrasonic bath for 15 min. The extracted sample was centrifuged at 4000 rpm for 5 min (ProfiLab GmbH, Berlin, Germany) and supernatant transferred to clean flask and evaporated in stream of nitrogen at 50 ºC. Dry residue was dissolved in 1mL of methanol and filtered through nylon 0.22µm syringe filter into a HPLC vial. Quantification was carried out using matrix extracted calibration curves at four levels. With every analysis batch, blank apple juice was fortified at four different levels with working standard solution and submitted to the full extraction procedure.

Validation was performed in accordance to the Commission Decision 2002/657/EC [13]. The linearity of the method was evaluated on three different days over the range 0-100 µg/kg, and each calibration curve was constructed with five concentration levels (including zero) and was fitted to a linear equation. The linearity of curves measured as average regression coefficient (R²) was 0.9962, which was satisfactory. The acceptance criteria were that the average regression coefficient (R²) should be greater than 0.996. Other validation parameters (decision limit CCα, detection capability CCβ, accuracy, repeatability, reproducibility, measurement uncertainty) were determined based on the procedure given in the software ResVal for the validation of the analytical methods made in EURL RIKILT, Wageningen, The Netherlands. A total of four experiments were performed for four days. Blank sample was divided into identical sub-samples and fortified at different concentration levels. Twenty-one fortified samples, seven for each validation level at 25, 50 and 75 µg/kg, plus seven blank samples and five calibration level samples at 0, 10, 25, 50 and 100 µg/kg (ppb) were analysed on each day for three days. On fourth day, to determine ruggednes, two small changes – variables of the method (amount of ethyl acetate and time in ultrasonic bath) were varied.
After validation, the method was used in routine laboratory work and the performance of the method was verified by participating in proficiency testing organized by Progetto Trieste, Italy.

3. Results and Discussion
In this work we presented a simple, sensitive method for determination of patulin in apple juice. The extraction method is based on simple liquid extraction with ethyl acetate with high recovery rate. A good chromatographic separation was achieved with a reversed phase (C-18) column and in gradient elution using 40mM ammonium acetate in water (mobile phase A) and 40mM ammonium acetate in methanol (mobile phase B) at flow rate of 0.30 mL/min. We monitored molecules after proton loss [M-H] of 153.1 m/z and two fragments of 81.10 and 109.15 m/z respectively, as presented in Table 1. The most intense fragment, 109.15 m/z, was used for quantification. The ratio of abundance of these two fragments was used to conclusively identify patulin.

Typical chromatograms of blank and apple juice fortified with patulin are shown in Figure 2.
As can be seen no interference from the matrix, that might disturb the signal, was observed at the retention time of patulin, so method is considered very specific. The limits of detection and quantification were 9.85 µg/kg and 19.7 µg/kg respectively, showing that the developed method had sufficient sensitivity to detect patulin at the regulatory level (50 µg/kg). The decision limit $CC_\alpha$ and the detection capability $CC_\beta$ were 54.77 µg/kg and 59.55 µg/kg respectively. The other validation parameters are shown in Table 2. The results of accuracy, repeatability and within laboratory reproducibility expressed as relative standard deviation (RSD) were satisfactory. Extended measurement uncertainty was 11.1%.

### Table 2. Validation parameters

| Compound | Fortified level (µg/kg) | Accuracy (%) | RSD repeatability (%) | RSD reproducibility (%) |
|----------|-------------------------|--------------|------------------------|-------------------------|
| Patulin  | 25                      | 101          | 6.7                    | 7.0                     |
|          | 50                      | 97.8         | 5.8                    | 6.0                     |
|          | 75                      | 100.3        | 3.0                    | 3.1                     |

The performance of the method was verified by participating in proficiency testing organized by Progetto Trieste, Italy in 2020, PT code VF2054. A total of 17 laboratories participated in this study, and sample of apple juice was analysed. Z-score value obtained by our laboratory was 0.72.
4. Conclusion
The presented method for determination of patulin in apple juice is simple, rapid and has sufficient sensitivity to detect patulin at the regulatory level. After validation and successful participation in the proficiency testing, the method is suitable for routine laboratory work and efficient food control in accordance with European legislation. Patulin is heat stable, so the pasteurization process is not sufficient to decompose it, meaning apple juices are the major concern associated with patulin contamination. The need to use high-quality raw materials, that is, healthy apples without mouldly and rotten portions, as well as good hygiene practices and control measures, should also be emphasized.

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