DETERMINATION OF PATULIN IN APPLE PUREE USING LC-MS WITH TRIPLE QUADRUPOLE DETECTOR

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Patulin is a high-hazardous mycotoxin formed in apples affected by the fungus. This mycotoxin, when ingested, has the irreversible effect of deteriorating health. Most often, patulin enters the human body through processed apple products or baby food. The countries of the European Union have decided to reduce the level of the maximum residue level of patulin in apples to the lowest that can be measured by analytical instruments. Therefore, the aim of this work is to develop a rapid and sensitive method of patulin determination for the lowest level detection allowed by European law.

In this work, patulin was artificially added to apple puree at the level of 10 μg/kg for further detection. The analysis was performed on an LC-MS/MS system with electrospray ionization type and a column based on modified silica. The mobile phase used in the analysis was ultrapure water and acetonitrile with the addition of formic acid. Measurement of the quantity of PAT in the sample was performed using MRM transitions. Subsequently, there was a problem of significant inclusion of the matrix on the spectrum, but the problem was solved by using cartridges for cleaning of matrix impurities. As a result, we achieved a successful and accurate result at level of 10 μg/kg. A calibration curve was felicitously constructed for five different concentrations. The most important parameters of validation of the method use were determined – reproducibility, repeatability, recovery and linearity. The final result of this work is the development and implementation of a rapid, cost-effective and suitable method for determining patulin at the level of the maximum residue level.

Keywords: *apple puree, liquid chromatography, mass spectrometry, mycotoxin, patulin*

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1. Introduction

Patulin (in further PAT) is a mycotoxin that is a byproduct of fungi such as Penicillium, Aspergillus and Byssochlamys. PAT is mostly formed in apples and apple juice, but sometimes it can be found in pears, plums, apricots, strawberries and grapes [1]. PAT is produced in the so-called "brown rot" of apples. The main stimulators of PAT formation are improper storage and transportation of apples, which leads to their damage and the subsequent process of attachment and reproduction of fungi [2].

When processing apples into apple juice or puree, they go through several stages, such as homogenization, pulping, pasteurization, clarification, concentration. These processes can reduce the concentration of PAT in the fruit, but they do not completely neutralize or even significantly reduce the amount of PAT. All this is due to the high thermal stability of this mycotoxin in an acidic environment. Therefore, with a high initial content of PAT

in apples, the subsequent stages of processing of apple products will be powerless.

The only way is appropriate agricultural methods before, during and after the harvest. These methods may include treatment with fungicides and biocontrol agents, careful harvesting, necessary treatment in case of fruit falling and low temperatures for the harvested crop [3].

Patulin, whose full name (according to IUPAC) is 4-hydroxy-4H-furo [3,2-c] pyran-2 (6H) -one, is a heterocyclic lactone. It has a molecular weight of 154.12 g/mol and it is low volatile. It is heat-resistant and stable in aqueous solutions at temperatures between 105-125 ℃ and at pH = 3.5-5.5. In a more alkaline environment, the decomposition of mycotoxin is observed [4]. The structural formula of patulin is shown in Figure 1.

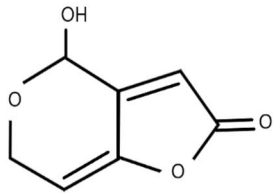

Fig. 1. *Structural formula of Patulin*

The lethal dose of LD50 for mice ranges from 25 to 55 mg/kg body weight. For chickens, turkeys, ducks and other poultry, this figure is little bit higher - 170 mg/kg body weight. Toxicity of PAT sharply increases at intravenous dose. The most common signs of mycotoxin intoxication are agitation, convulsions, pulmonary congestion, edema, and distension of the gastrointestinal tract [5].

Contact with PAT in mammals can lead to ulcers, internal bleeding in the stomach and intestines and inflammation. Significant neurotoxicosis was observed in some livestock species. The kidneys are very sensitive to PAT and when it enter the body, there is a high probability of their hemorrhage. In experiments on mice, there were also frequent cases confirming the carcinogenicity of PAT - at the maximum dose of patulin, the formation of tumors and sarcomas was observed [6].

Due to the danger of mycotoxin, the World Health Organization and Food and Drug Administration (FDA) of USA and the European Union (EU) have proposed to reduce the maximum residue levels of PAT in apples and apple products as follows: fruit, fruit juice, concentrated apple juice, nectar, cider and other fermented beverages - 50 µg/kg; solid apples, compotes, purees, intended for direct consumption - 25 µg/kg; apple juice, compotes, purees for infants and young children - 10 µg/kg. [7-8]. In accordance to this values, it will be very appropriate to develop and further use high-sensitive methods for PAT determination with high accuracy.

The aim of this work is to develop a method for PAT determination in apples and apple products using HPLC system with triple quadrupole mass spectrometer.

2. Material and methods

All samples of solid apples were collected in Cherkasy district, Ukraine, harvest from 2021. Apple variety name is Goldi.

The PAT analytical standard was purchased from Romer Lab’s (Tulln, Austria), a liquid standard sample in acetonitrile. All standard and working solutions of PAT were prepared in acetonitrile and stored in the freezer at -18 ℃. The solvent used for mobile phase preparation - acetonitrile was ordered from Honeywell (Seelze, Germany), LC-MS

grade. Formic acid as an additive component for mobile phase was purchased from Honeywell (Seelze, Germany), LC-MS grade. Ultrapure water was made in our laboratory using the Millipore installation. Ethyl acetate solution ordered from Sigma-Aldrich (Saint Louis, USA), HPLC grade was used for extraction. Also QueChERs cartridges for sample's cleaning were chosen from Supelco, Superclean PSA/MgSO4/Discovery DSC-18 (55439-U) (Merck, Germany).

All chromatographic measurements were performed on Agilent Infinity II 1290/6460 with a triple-quadrupole mass spectrometric detector with electrospray ionization (USA). MassHunter was used as program for measurement's monitoring and result's calculation. Separation was performed using an Agilent InfinityLab Poroshell SB-C18-120 2.1x100 mm column. pore size - 2.7 microns (USA). Thermostat temperature - 40 °C. Mobile phases used - A: water with 0.1% FA; B: ACN with 0.1% FA. Gradient for phase B: 0 min - 10%; 2.40 min - 42%; 6.00 min - 51%; 6.20 min - 90%; 7.20 - 90%; 8.00 min - 10%. The flow is 0.4 ml/min. PAT parameters for the mass spectrometer are shown in Table 1.

Table 1. *Mass spectrometric Patulin's parameters*

Mycotoxin's name	Precursor ion, (m/z)	Daughter ions, (m/z)	Collision energies, eV	Ionizationmode
Patulin	153	109.1	3	ESI $^-$ (negative)
		81.2	10	ESI $^-$ (negative)

The apple samples were gritted into apple puree. 10 g of homogenized puree (± 0.1 g) was weight into a 50 ml centrifugal tube. The samples were spiked with a standard of PAT solution at 10 μg/kg (0.01 mg/kg) and mixed. 10 ml of ethyl acetate was added into each test tube and shake vigorously for 5 min on a shaker. The samples were placed in a centrifuge at 10,000 rpm for 10 min. After centrifugation, it was decided to clean the samples using cleaning cartridges QuECheRs, as previous studies have shown that apple puree makes a significant matrix contribution, which can lead to false-positive or false-negative results. After purification, the sample is filtered through a 0.2 μm PTFE syringe filter directly into a chromatographic vial.

After full extraction procedure all used glassy flasks which were contacted with patulin solution should be washed and rinced with 9% hydrogen peroxide solution.

The validation of the method was performed in accordance with Commission Decision 2002/657 / EC (7, p. (). Linearity was measured at five points from 10 μg/kg to 100 μg/kg. The linearity index was R^2 = 0.9934, which is satisfactory. Other parameters – repeatability, reproducibility and recovery were in optimal ranges, so developer

method was successfully validated and started to be used in routine analyses.

3. Results and discussion

This article presents a simple method for determination of PAT in apple puree. The method is based on simple and fast extraction of the sample with ethyl acetate. The method allows to obtain high recovery value of extraction and reproducibility. A 81.2 m/z daughter ion was selected for quantification in

the batch. The abundance's ratio of these two MRM transitions was used to conclusively identify PAT.

To investigate the matrix effect, extraction and chromatographic measurement of pure apple puree without spiking as a blank were performed. The puree sample with addition of PAT at the level of 10 µg/kg without cleaning and cleaned sample with PAT addition at the level of 10 µg/kg are shown in Figure 2,3, respectively.

As can be seen from Figures 2, the matrix contribution of apple puree is significant and almost completely covers the peak of spiked patulin. In addition, the peak was tailed.

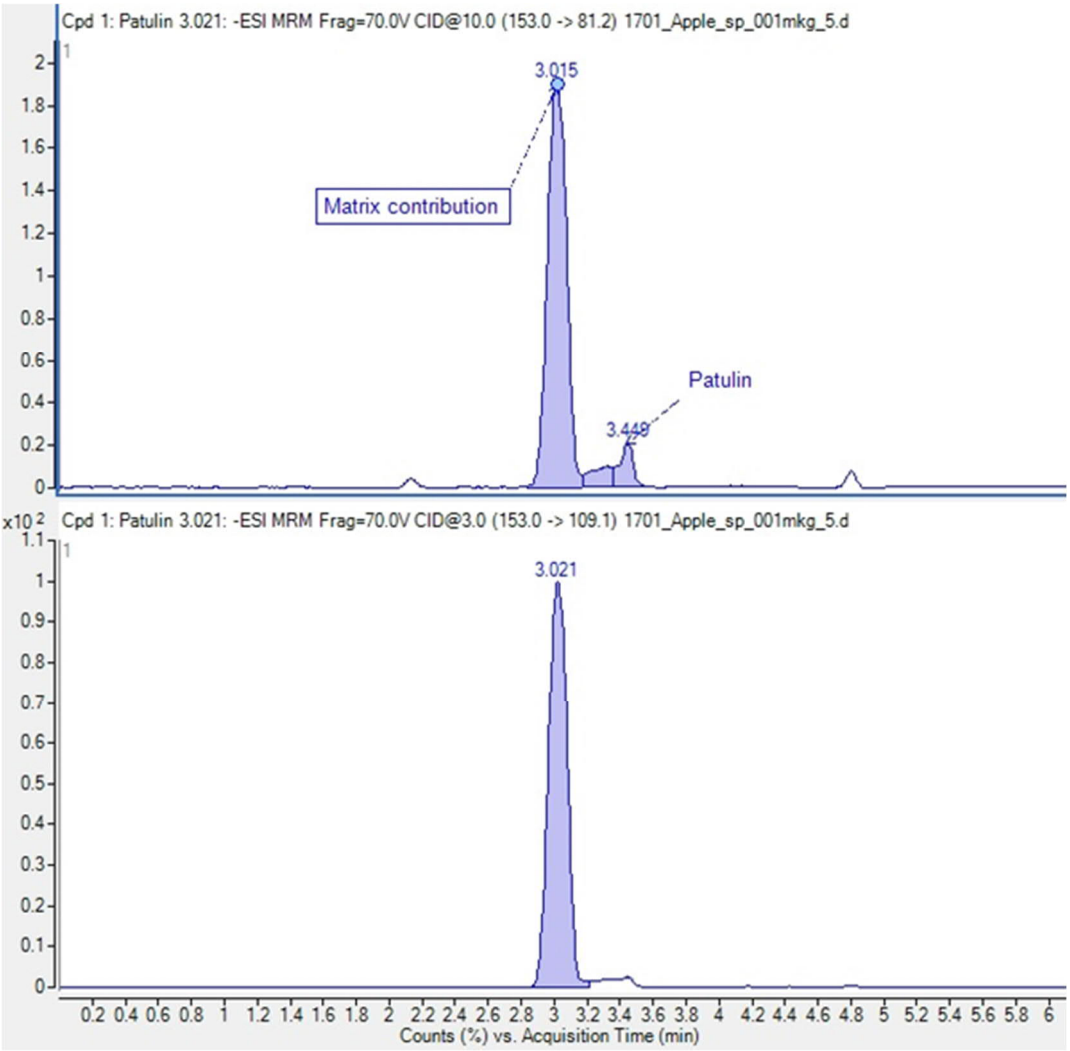

Fig. 2. *Uncleaned spiked sample at the level 10 µg/kg*

After cleaning on QueChERs cartridges (Fig. 3.) there is a significantly improved baseline and a much clearer and more visible patulin peak is identified (in accordance to current scale). The peak is symmetrical, smooth and not tailed. At the first MRM transition (81.2), which was chosen as the quantification, there is a significant reduction in the matrix effect

During the construction of the calibration curve, the linearity shown in Figure 4 was obtained. The calibration curve consisted of 5 points: 10, 25, 50, 75 and 100 µg/kg, respectively

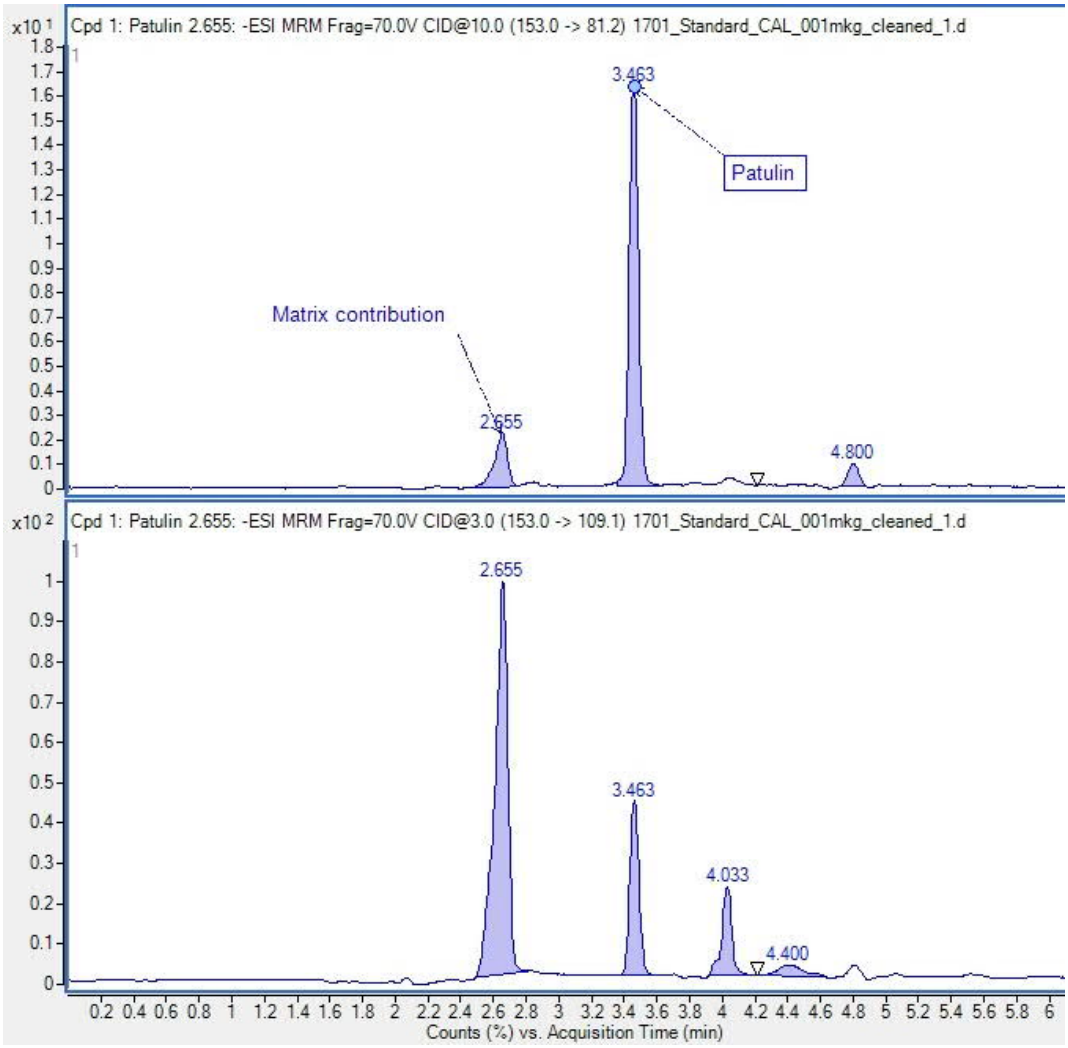

Fig. 3. *Cleaned spiked sampleat the level 10 µg/kg*

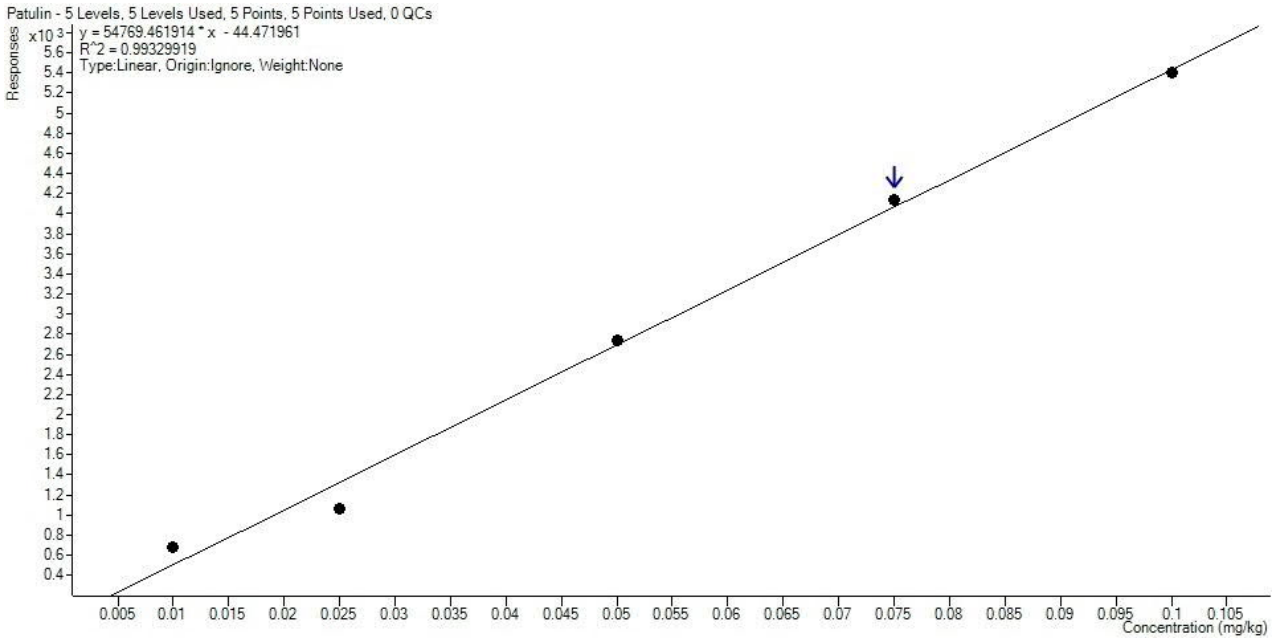

Fig.4. *PAT calibration curve linearity*

Validation of the method showed that the extraction method is quite effective, has satisfactory values of recovery, the results are reproducible in several attempts. The repeatability values between the parallels did not exceed the values allowed for PAT. According to Commission Regulation 401/2006 / EC (8, p.70/32), RSD must be less than 40%. The values of the recovery are also quite accurate, repeatable and do not exceed permitted in Commission Regulation 401/2006 / EC (8, p.70/32), i.e., kept within 50-120% for the spiked concentration of 10 µg/kg. The results of the method validation parameters are shown in Table 2. The obtained results show that this method can be used to determine the content of patulin in apples, apple products and baby food at the levels approved in Europe.

***Table 2**. Validation parameters for PAT*

Mycotoxin's name	Spiked level, µg/kg	Recovery value, %	RSD Repeatability, %	RSD Reproducibility, %	LOQ, µg/kg
Patulin	10	101 99	2.9 3.0	2.1	10

4. Conclusions

Patulin is a dangerous and thermostable mycotoxin that producing in spoiled apples by fungus. The main stages of processing of raw apples do not give significant degradation of PAT. Therefore, the question of controlling the patulin content in apples and apple products has arisen quite acute. The developed method is high-sensitive, effective and fast for PAT determination in apple puree according to regulatory documentation. After complete validation and several internal laboratory controls, it was decided that this method is suitable for routine tests to determine patulin in food products in accordance with European legislation.

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ВИЗНАЧЕННЯ ПАТУЛІНУ У ЯБЛУЧНОМУ ПЮРЕ ЗА ДОПОМОГОЮ LC-MS З ТРЬОХКВАДРУПОЛЬНИМ ДЕТЕКТОРОМ

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Патулін – високонебезпечний мікотоксин, що утворюється в яблуках, уражених грибком. Цей мікотоксин, потрапляючи всередину, має незворотний ефект погіршення здоров'я. Найчастіше патулін потрапляє в організм людини через продукти переробки яблук або дитяче харчування. Країни Європейського Союзу вирішили знизити рівень максимального залишкового вмісту патуліну в яблуках до найнижчого, який можна виміряти за допомогою аналітичних приладів. Тому метою даної роботи є розробка швидкого та чутливого методу визначення патуліну на найнижчому рівні, дозволеному європейським законодавством.

В даній роботі було взято в аналіз яблучне пюре із штучно внесеною добавкою патуліна на рівні 10 мкг/кг. Аналіз здійснювався на системі LC-MS/MS з типом іонізації – електроспрей та колонкою на основі модифікованого силікагелю. Мобільна фаза, що використовувалася при аналізі, була ультрачиста вода та ацетонітрил з добавкою мурашиної кислоти. Вимірювання змісту ПАТ у зразку проводилося з використанням MRM переходів. Згодом виникла проблема значного включення матриці на спектрі, проте проблема була вирішена за допомогою використання картриджів для очищення від матричних домішок. В результаті ми досягли вдалого та точного результату на рівні 10 мкг/кг. Вдалося успішно побудувати калібрувальну криву для п'яти різних концентрацій. Були визначені найважливіші параметри валідації використання методу – збіжність, відтворюваність, повернення та лінійність. Остаточним результатом цієї роботи є успішна розробка та впровадження швидкого, економічно-вигідного та підходящого методу визначення патуліну на рівні максимально-допустимої концентрації.

Ключові слова: *яблучне пюре, рідинна хроматографія, мас-спектрометрія, мікотоксин, патулін*