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Article

Development and Validation of a Reable LC-MS/MS Method for the Nicotine Residues Determination in Mushrooms

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Abstract: The purpose of this study was to develop and validate a specific and sensitive liquid chromatography tandem mass-spectrometry (LC-MS/MS) method for the quantification of the nicotine residues in mushrooms according to SANTE/12682/2019. During the validation process the spiked mushroom samples were extracted using a QuEChERS AOAC method for the extraction (in the alkaline conditions, pH 10-11) and clean-up. The Quick Polar Pesticide Method (QuPPe) was used for the chromatography analyses and quantification, followed by the LC-MS/MS.

Keywords: nicotine residues; mushrooms; LC-MS/MS.

1. Introduction

Nicotine is the main alkaloid in tobacco (*Nicotiana tabacum*) and other tobacco species where it occurs at the concentrations ranging from 2% to 8%; it is synthesized in the tobacco root from ornithine and/or arginine by way of putrescine [1]. This substance naturally occurs at low levels in various plants belonging to the *Solanaceae* family, such as tomatoes, sweet peppers, potatoes (< 10 µg/kg) and eggplant (< 100 µg/kg) [1-3].

The origin of nicotine in mushrooms has not been clarified yet. A potential source of nicotine could be cross-contamination during the drying/packaging process or harvesting (via contact with smokers' hands); intentional use as a pesticide in the growing areas or during storage; natural occurrence in the mushrooms themselves, including the natural formation during the drying process. All routes of the exposure, i.e. oral, dermal or inhalation, could be toxic due to nicotine. Consistently with its action as agonist at the nicotinic receptors, it targets the peripheral and central nervous systems causing dizziness, salivation, increased heart rate and blood pressure [3, 4]. During the ingestion the high levels of nicotine could be very toxic and consuming greater than 40-60 mg

(the amount contained in 4 cigarettes) is potentially lethal for adults. It should be kept in mind that the smoke of one cigarette contains ca. 0.9-1.7 mg of nicotine.

The maximum residue limit (MRL) for nicotine in food matrix was set by EC/396/2005 [5]. The MRL of nicotine by default is set at 0.01 mg/kg for all the commodities.

The analysis of this analyte presents specific problems that are of great interest for the analysts. The nicotine is a volatile compound (Pvap, 5.6 Pa at 25 °C) with a high polarity (log P = 0.93 at 25 °C/unionized). This compound contains two basic moieties; a tertiary amine group (pKa=8.2) and a pyridine group (pKa=3.1) (Figure 1). It is therefore predominantly protonated at pH between 3.1 and 8.2 and double protonated at pH below 3.1 [6].

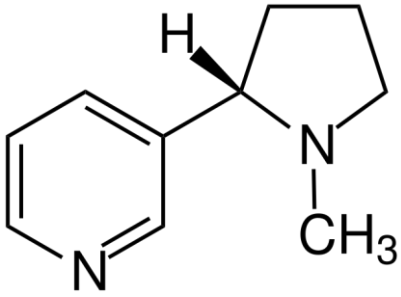

Figure 1. Nicotine chemical structure

One of the most reliable modern analytical methods is liquid chromatography tandem-mass spectrometry (LC–MS/MS), having advantages of both an accurate identification and a quantification of nicotine in this matrix [7].

In this paper a nicotine residues method has been developed for the determination of this analyte in mushrooms. The extraction was done by the modified QuEChERS method established by the EU Reference Laboratories for Residues of Pesticide (CRL-SRM version, SRM-44/(V2)/10.03.2021), followed by liquid chromatography coupled with tandem quadrupole mass spectrometry (LC-MS/MS). The detection process of the studied compound was carried out using electrospray ionization (ESI) in the positive ion mode.

2. Materials and Methods

Chemicals and reagents. Acetonitrile (MeCN) and methanol (HPLC grade) were purchased from J.T.Baker, NaOH and formic acid (FA) were analytical grade (Fluka). The QuEChERS kits were purchased from Agilent Technologies, US (QuEChERS orig.Meth. Bond Elute Part No: 5982-755 and and Dispersive SPE 15 m, Fruit and Veg. Bond Elute Part No: 5982-5056). The nicotine standard was purchased from Dr.Ehrenstorfer (Augsburg, Germany). The stock solution was prepared in acetonitrile at nearest 1.0 mg/mL. The working standard solutions were prepared in acetonitrile at 10 and 1 μg/mL. These solutions were used for the validation.

Method validation. The performance characteristics of the method were performed by single-laboratory validation employing analyses with nicotine spiked mushroom samples. The linearity, LOD, LOQ, matrix effect, recovery and precision were determined according to SANTE/12682/2019 [8].

The evaluation of the linearity range was checked for five fortification levels (0.01, 0.025, 0.05, 0.1 and 0.2 μg/mL) in the mobile phase and matrix.

The recovery, a characteristic of the extraction efficiency, was obtained by spiking samples at two concentrations (0.01 and 0.1 mg/kg) in six replicates. The precision of the method (expressed as the Relative Standard Deviation (%RSD)) was calculated within the testing of the recovery. Durin the validation process „recovery calibration" was done, within the preparation of the calibration standards (0.01 and 0.10 μg/mL), for getting the analytical curves in the solvent, as well as for spiking the samples.

The limit of detection LOD (or detection limit, DL) is the lowest possible concentration at which the method can detect (but not quantify) the analyte within the matrix with a certain degree of confidence. It is also defined as the lowest concentration that can be separated from a background noise with some reliability. The limit of quantitation LOQ (or sometimes also referred as the quantification limit, QL) is the lowest possible concentration of the analyte that can be quantified by the method in a reliable way.

Samples extraction and clean-up procedure. The spiked mushroom samples were extracted using the modified QuEChERS method according to Anastassiades et al. [9] (Figure 2).

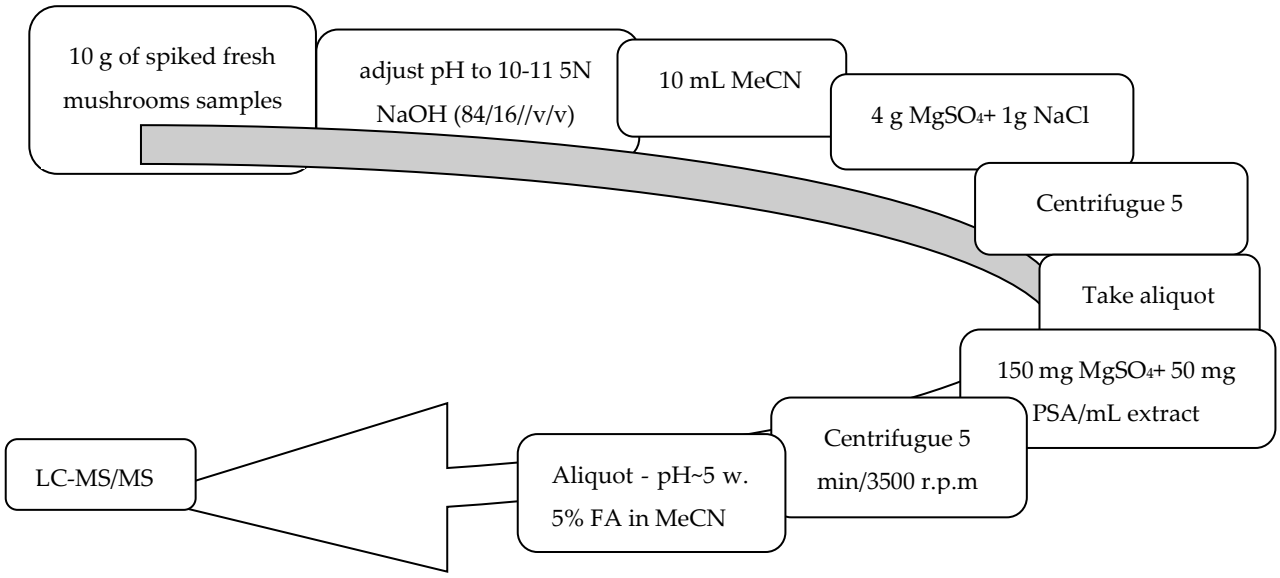

Figure 2. QuEChERS extraction

LC-MS/MS analysis. The chromatographic analysis was performed using Agilent 6470B LC/TQ coupled with 1290 Infinity II LC system (Agilent Technologies, US). The separation was performed using a (2.1mm x 100mm) 1.7μm particle size, Torus DEA 130A Column supplied by Waters. The oven temperature was set at 50ºC. The mobile phase consisted of 50 mM ammonium formate pH 2.9 (solvent A) and 0.9% formic acid in acetonitrile (solvent B), with the following gradient: 0 min-90% B; 4,5 min-40% B; 8.5 min 40% B; 20 min- 90% B; and then returned to the initial conditions in 5 min, with a total time of 25 min. The mobile phase flow rate was maintained at 0.2 mL/min and the injection volume was 10 μL. The MS source temperature was set at 250 ºC, nitrogen gas flow 13 L/min and nebulizer pressure 30 psi. The target ion transition with the highest intensity *m*/*z* 163.1→132.1 (primary ion transition) was used for the quantification, whereas the second target ion transition *m*/*z* 163.1→130.1 was used for the confirmation. The instrument uses MassHunter software version B.10.00 (Agilent Technologies, Inc. 2006-2018) for the quantification and confirmation.

3. Results and discussion

The LC-MS/MS (ESI+) fragmentation of the protonated molecular ion of the nicotine is given in Table 1. The fore multiple reaction monitoring (MRM) transitions were monitored for this analyte.

Table 1. MRM transitions, fragmentation and colision energies and retention time (Rt).

Pesticide	Molecular formula	M g/mol	Precursor ion, m/z	Product ion m/z	Frag (V)	CE (V)	Rt (min)
Nicotine	$C_{10}H_{14}N_2$	162.1	163.1	132.1	105	10	1.64
			163.1	130.1	105	28	
			163.1	117.1	105	40	
			163.1	84.1	105	20	

The LOD was determined as the lowest concentration giving a response of three times the average baseline. The ratio signal/noise in the obtained chromatograms for the LOD was calculated by MassHunter Qualitative Software and it was estimated to be 0.004 mg/kg. The obtained LOQ was 0.01 mg/kg and it was in accordance with the established MRL for nicotine in the food comodities (EC/396/2005) [5].

The calibration curve for the nicotine was constructed by plotting the peak area (y) versus the concentration (x) of the analyte which was expressed by the equation given as: y=558145.2*x+16798.6 with a correlation coefficient (r2) of 0.9995. The calibration curve was generated with concentrations ranging from 0.01 to 0.2 mg/kg, by "procedural calibration" (Figure 3).

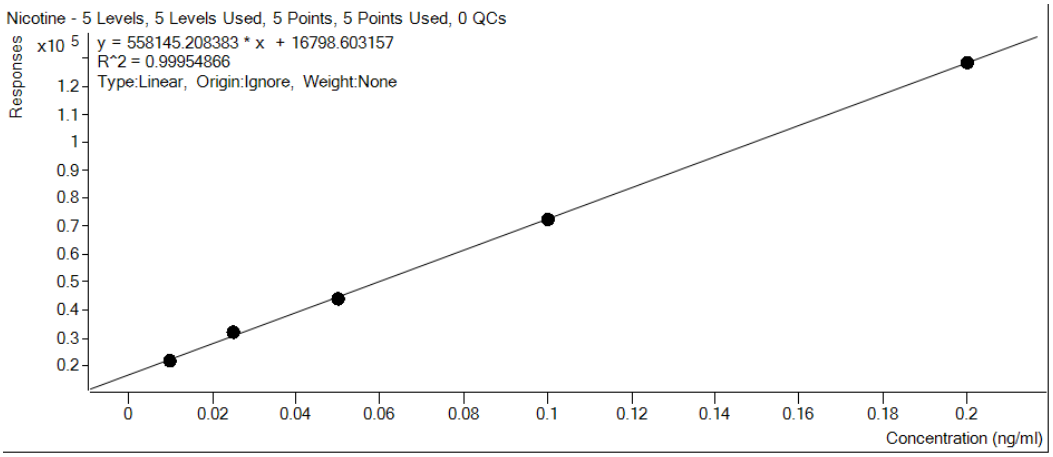

Figure 3. Calibration curve of the nicotine in mushroom extract.

Accuracy of methods. For the estimation of the accuracy, blank samples were spiked with the nicotine standard solution. The recovery of the method was determined using mushroom samples fortified at 0.01 and 0.1 mg/kg. The mean recovery (n=6) was 94.3% and the relative standard deviations were lower than 20% (Table 2).

Table 2. Recoveries and relative standard deviations (%RSD) of nicotine in spiking samples (n=6).

Spiking level (mg/kg)	$Rec_{1,2}$, %	$\%RSDr_{1,2}$	Average Rec, %	%RSDr	$\%RSD_R$
0.01	104.5	11.60	94.3	17.68	18.52
0.1	84.1	13.34			

Generally, the accuracy and precision results were satisfactory, according to the Document SANTE/12682/2019 [8].

The total ion chromatogram (TIC) with the MRM transitions of the nicotine in the mushrooms at the spiking level of 0.1 mg/kg was given in Figure 4.

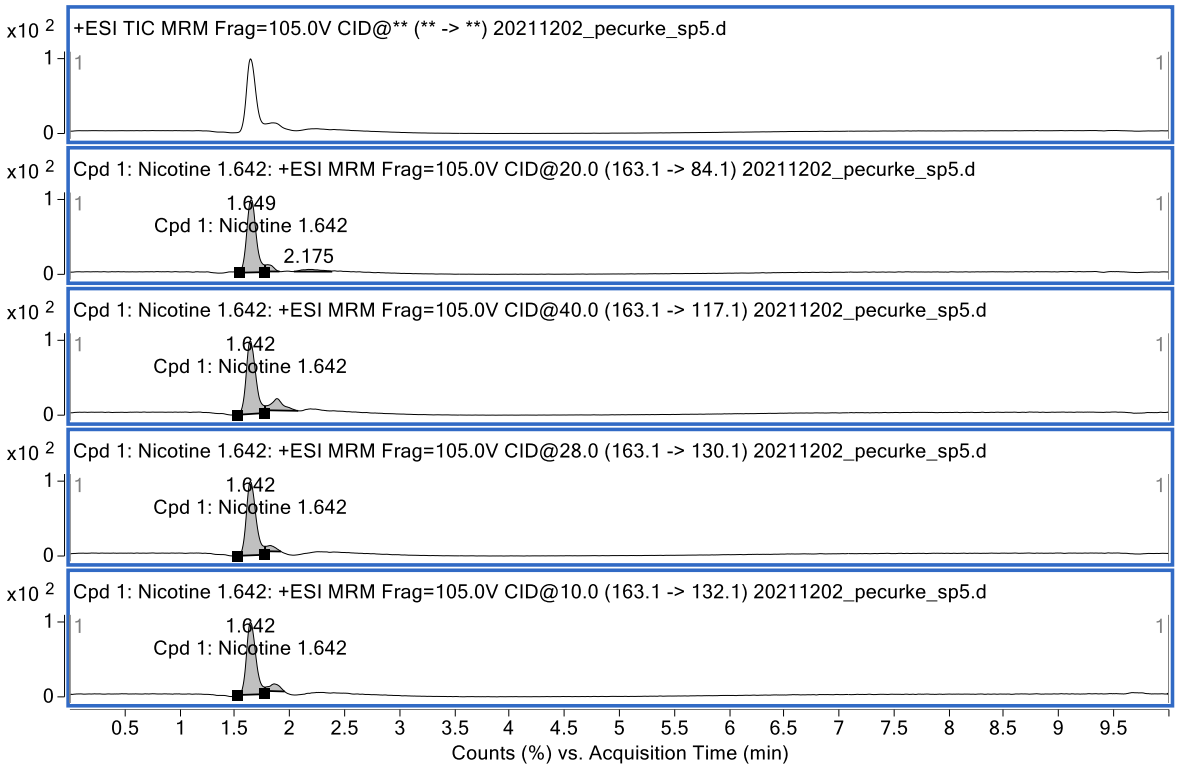

Figure 4. TIC and MRM chromatograms of the spiked mushroom sample (0.1 mg/kg).

5. Conclusions

The validated LC-MS/MS method presented in the article proves to be suitable for the accurate measurements of nicotine in mushrooms under the conditions given in this study.

According to the validation the LC-MS/MS method used in this research is selective, showing good linearity, expressed by the values of R^2>0.99, while the accuracy and precision results were satisfactory according to the SANTE/12682/2019.

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Conflicts of Interest: The authors declare no conflict of interest.

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