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Development and Validation of a New Simple Analytical Method for the Analysis of Nicotine in Mushroom using LC-MS/MS

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ABSTRACT

Nicotine is a natural alkaloid found in members of the plant family *Solanaceae*, with considerable toxic effects on the nervous system of mammals. In accordance with the Eu regulations, Egypt has banned the use of plant protection products containing nicotine as pesticide. A simple, rapid and sensitive method for determination of nicotine in mushroom using reversed phase liquid chromatography coupled with tandem mass spectrometer (LC-MS/MS) was developed. The samples were extracted using acidified water/methanol (1:1 v/v), followed by centrifugation and analysis using LC-MS/MS system in a total run time of 15 minutes. Samples were quantified by multi-level calibration curve in the range of 0.005-0.5 µg/mL with $R^2 > 0.9998$. The obtained results were corrected due to matrix effect using one point matrix matched calibration level at 0.05µg/ml. The method has been validated according to EU guidelines; SANTE 2016. The recovery percentages were 71.0 % to 98.0 % with CV was less than 7.0 % for all validation levels 0.05 and 0.1 mg/kg including the LOQ level (0.01 mg/kg). The developed method was applied to 20 commercial mushrooms samples to ensure the applicability of the proposed protocol. The results revealed that all samples were below the quantitation limits.

Key words: Nicotine, toxic effects, Eu regulations, Validation

Introduction

Nicotine ((S)-3-(1-methylpyrrolidin-2-yl) pyridine) is a highly toxic alkaloid found naturally in members of the family *Solanaceae*, especially tobacco (concentration range, 2–8%). all other plants of this family, such as tomato, tomato leaf, and eggplant have nicotine at levels around 180 ng/g. Other plant from (*Solanaceae* family) such as green pepper and potato show levels around 50–60ng/g (Moldoveanu *et al.*, 2016). Nicotine is one of the most widely used botanical insecticide due to it is easy and cheap extraction from natural resources. It can be obtained cheaply from the tobacco industry as a byproduct. Although, the Environmental Protection Agency (EPA) outlawed the use of nicotine insecticide products in the US In January 1, 2014, its use in Third Countries may continue (European Food Safety Authority, 2009).

Nicotine is a volatile compound ($P_{vap,}$ 5.6 Pa at 25 °C) with high polarity (log P = 0.93 at 25 °C/unionized). Moreover, nicotine is a basic alkaloid with two pK_a s; 3.1 and 8.2 (Chamberlain *et al.,* 1996). The different forms of nicotine at different pH are presented in Fig 1

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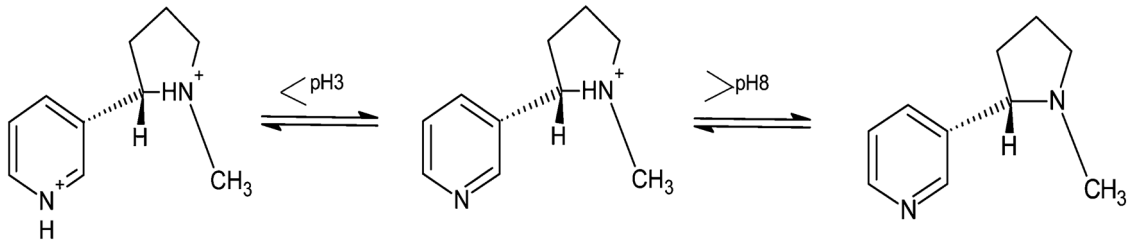


Fig. 1: Effects of pH on nicotine protonated forms

Significant amounts of nicotine have recently been detected in mushroom samples. The concentrations of nicotine in dried mushrooms were in the range 0.22 mg/kg to 5.87 mg/kg. The origin of nicotine in these products has not clarified yet. Potential source could be cross-contamination during the drying procedure or harvesting, illegal use as a pesticide or natural occurrence in the mushrooms themselves. Nicotine is acutely toxic by all routes of exposure (oral, dermal, and inhalation). Consistently with its action as agonist at the nicotinic receptors, it targets the peripheral and central nervous systems causing for example dizziness, salivation, increased heart rate and blood pressure (European Food Safety Authority, 2009).

Temporary maximum residue limits (MRLs) for nicotine in food matrix were set by European food safety authority (EFSA) (European Food Safety Authority, 2009). For mushroom, a safe level of nicotine of 0.036 mg/kg for fresh wild mushrooms was established, corresponding to 0.32 mg/ kg for dried wild mushrooms. Afterwards, The European Food Safety Authority proposed a maximum residue limit (MRL) of 0.04 mg/kg for fresh mushrooms, 1.2 mg/kg dried mushrooms with the exception of dried ceps dried ceps 2.3 mg/kg (commission regulation (EU) No 1276/ 2011).

Several methods for identification and quantification of nicotine in plant (Moldoveanu, Serban *et al.*, 2016) and biological samples (Maskarinec *et al.,* 1978; Iwai *et al*., 2013) have been published, including both LC–MS/MS (Thräne *et al.,* 2015) and GC–MS/MS (Siegmund *et al.,* 1999) methods. In addition, the widely used and applicable protocol for sample preparation; QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) has been modified and developed for nicotine analyses in mushrooms (Lozano *et al.*, 2012). This approach was adapted in various analyte and matrices including nicotine in mushroom (Shendy *et al.,* 2016; Müller *et al.,* 2011; Attallah *et al.,* 2017). In general, the modified QuEChERS involves an extraction step with an organic solvent (acetonitrile), pH adjustment and a cleanup step followed by acidification, evaporation and reconstitution prior to instrumental analysis. Several drawbacks in the Modified QuEChERS method such as pH adjustments via initial basification and late acidification steps which consider critical and time consuming and the use of primary and secondary amine (PSA) as dispersive solid phase extraction (d-SPE) which is inefficient as clean-up for matrices of high protein contents such as mushroom.

The current study aimed to introduce simple, rapid, sensitive method with reduced cost for nicotine determination in mushroom using LC-MS/MS. For the use in routine analysis, the proposed method has been validated on fresh mushroom samples based on the EU guidelines (SANTE/EU, 2015) and Eurachem guideline (Magnusson, 2014). The validation parameters; linearity, LOQ, repeatability and intermediate precision were carried out. The Applicability of the method has been investigated using spiked samples and real samples.

Materials and Methods

Samples collection and homogenization:

Twenty mushroom samples were purchased from local markets in Egypt and then analyzed using the proposed method. The samples were further processed by chopping them into small pieces then complete homogenization was accomplished using the electric mill.

Chemicals and reagents:

Nicotine standard (99.3% purity) was obtained from Dr. Ehrenstorfer (Augsburg, Germany). Acetone and methanol of HPLC grade were purchased from Sigma-Aldrich (USA). Deionized water (DIW) was obtained using a MilliQ UF-Plus system (Millipore, Germany) with a resistivity of at least 18.2 M.cm at 25 ^{0}C and TOC below 5 ppb. Formic acid (FA) and ammonium hydroxide with purity ≥ 98% and 33%, respectively were purchased from Riedel-de Haen (Germany).Ammonium formate buffer in (Water: Methanol, 9:1 v/v) and pH adjusted at 3. Extraction solvent (DIW (2%FA): Methanol, 1:1).

Apparatus:

Geno/Grinder SPEX® sample preparation device (United Kingdom). Centrifuge Heraeus Megafuge40 was obtaioned from Thermo scientific (USA).The pH meter is from Thermo Electron Orion Star pH. Analytical Balance (Mettler Toledo AG 204: 0.1 mg to 210 gm range) and Precision Balance (Mettler Toledo GG 4002-S: Delta range, 0.5 gm. to 4100 gm range).

Standard preparation:

Nicotine stock solution of 1000µg/mL was prepared by dissolving 0.0101gm in 10mL volumetric flask using acetonitrile and kept in the freezer at -20±2^{0}C. Intermediate solution of 10µg/mL was prepared in DIW by transferring 1mL of the stock solution into 100 mL volumetric flask. Calibration mixtures ranging from 0.005-0.5µg/mL were prepared via serial dilution of the intermediate solution in the injection solvent (DIW (1%FA): Methanol, 2:1). Both of the intermediate and calibration mixture solutions were stored in the refrigerator at 4±2^{0}C.

Instrumentation and conditions:

HPLC 1200 series was purchased from Agilent technologies (USA). A 4000 Qtrap mass spectrometer with triple stage quadruple/linear ion trap from AB Sciex (USA). C18 column ZORBAX Eclipse XDB 4.6 x 150mm with 5.0µm particle size was obtained from Agilent technologies (USA). The mobile phase reservoir (A) was 10 mM Ammonium formate buffer (pH 3) and reservoir (B) was Methanol. Separation procedures were achieved using gradient elution at the flow rate of 200µL/min and a column temperature of 40°C. Injection volume of 5µL was directly injected into LC-MS/MS. Nicotine retention time was 7.5 minutes with total run time of 15.0 minutes. The chromatographic separation was achieved using gradient elution program as summarized in Table 1.

Table 1: Gradient elution scheme for nicotine separation from mushroom samples

Time (min)	Mobile Phase Buffer % (A)	Methanol % (B)
0 .00	90	10
3.00	90	10
3.10	89	11
5.00	30	70
10.00	30	70
10.10	90	10
15.00	90	10

The detection process of the studied compound was carried out using electrospray ionization (ESI) in the positive ion mode. The instrumentation parameters were applied based on the manufacturer's recommendations as shown in Table 2. Multiple reactions monitoring mode (MRM) was employed for nicotine confirmation and quantitation. According to EU guidelines (SANTE/EU, 2015), at least Two MRMs are essential for compound identification. In the current study, one quantifier (163→132, *m/z*) and three qualifiers (163→130, 163→117and 163→84 *m/z*) were then used.

Table 2: 4000 Qtrap mass spectrometer operating conditions

Temperature (^{0}C)	400
curtain gas (psi)	30
collision gas	5
ion spray voltage (V)	5500
ion source gas 1 (psi)	35
ion source gas 2 (psi)	35

Sample preparation:

The homogenized mushroom sample was weighed (10 g) into a 50 mL teflon centrifuge tubes. For spiked samples preparation, adequate quantity of the nicotine standard was added to 10 g homogenized sample to give a concentration of 0.1µg/mL. The spiked sample was mixed by vortex mixer for 30 seconds and left to stand for 10 minutes prior to extraction to favor the diffusion of Nicotine into the matrix. A 10mL of extraction solvent was added to the sample. The sample was then shaken using automatic shaker for 5 min at 700 rpm. Further centrifugation was carried out for 10 min at 4500 rpm under cooling conditions (-4.0 ^{0}C). Appropriate volume of the obtained supernatant was filtered through 0.45µm acrodisc and transferred to vial. An exact volume of 5 µL was injected into LC-MS/MS system.

Stability of nicotine stock solution:

The stability of the stock solution was tested by measuring the difference in the average response of old and new standard and insured that it's within the recommended criteria EU guidelines (SANTE/EU, 2015).

Calibration and validation:

A serial dilution to the intermediate solution (10µg/mL) was carried to freshly prepare calibration levels with concentrations ranging from 0.005-0.50 µg/mL with each run. The levels of calibration were then injected into the LC-MS/MS and the calibration curve was constructed. Afterwards quantification of the analyzed samples (real samples, blank reagent, spiked samples and one point matrix matched level) was carried out using the linear regression equation. The validation of the proposed method has been investigated in compliance with the requirements of the EU guidelines (SANTE/EU, 2015) and Eurachem guideline (Magnusson, 2014). In the current work, validation requirements including linearity, accuracy, precision, intermediate precision, limit of quantification (LOQ) and limit of detection (LOD) were performed.

Results and Discussion

On the basis of the studied compounds physicochemical characteristics, a well-planned protocol was developed and employed. Therefore, sample preparation optimization and chromatographic separation efficiency along with enhanced mass spectrometric sensitivities were tested as will be discussed later.

Nicotine mass spectrometric optimization:

In order to achieve maximum sensitivity for the studied compound, initial investigation for the precursor ion, product ions, declustering potential (DP), entrance potential (EP), collision energies (CE) and cell exiting potential (CXP) was performed by the Analyst® software automatic optimization. According to AB-SCIEX® recommendations, direct infusion of suitable aliquot from the nicotine (M.Wt, 162.2 g/mole) standard solution (0.1µg/mL) to the ESI-MS/MS system in positive ion mode was carried out. The major ionization of nicotine was found to have mass to charge ratio (m/z) of 163 which is corresponding to $(M + H)^+$. The obtained ion was then considered the precursor ion (Fig. 2). Automatic variation to the fragmentation parameters was carried out in order to scan the

Product ions' profile of the precursor ion (Fig. 3). The obtained results exhibited that the most sensitive and characteristic transitions were achieved under the conditions described in Table 3. For correct identification and quantitation of the compound, at least Two MRMs are required according to SANTE guideline (SANTE/EU, 2015). In the current study, the most sensitive transition (m/z, 163→132) was used as the quantifier while the remaining transitions were used as qualifiers (Table 3).

Table 3: Nicotine mass spectrometric optimization conditions

Compound	Precursor ion (m/z)	Product ion (m/z)	DP (V)	EP (V)	CE (V)	CXP (V)
Nicotine	163	132	66	10	29	22
		130	66	10	21	22
		117	66	10	37	18
		84	66	10	25	14

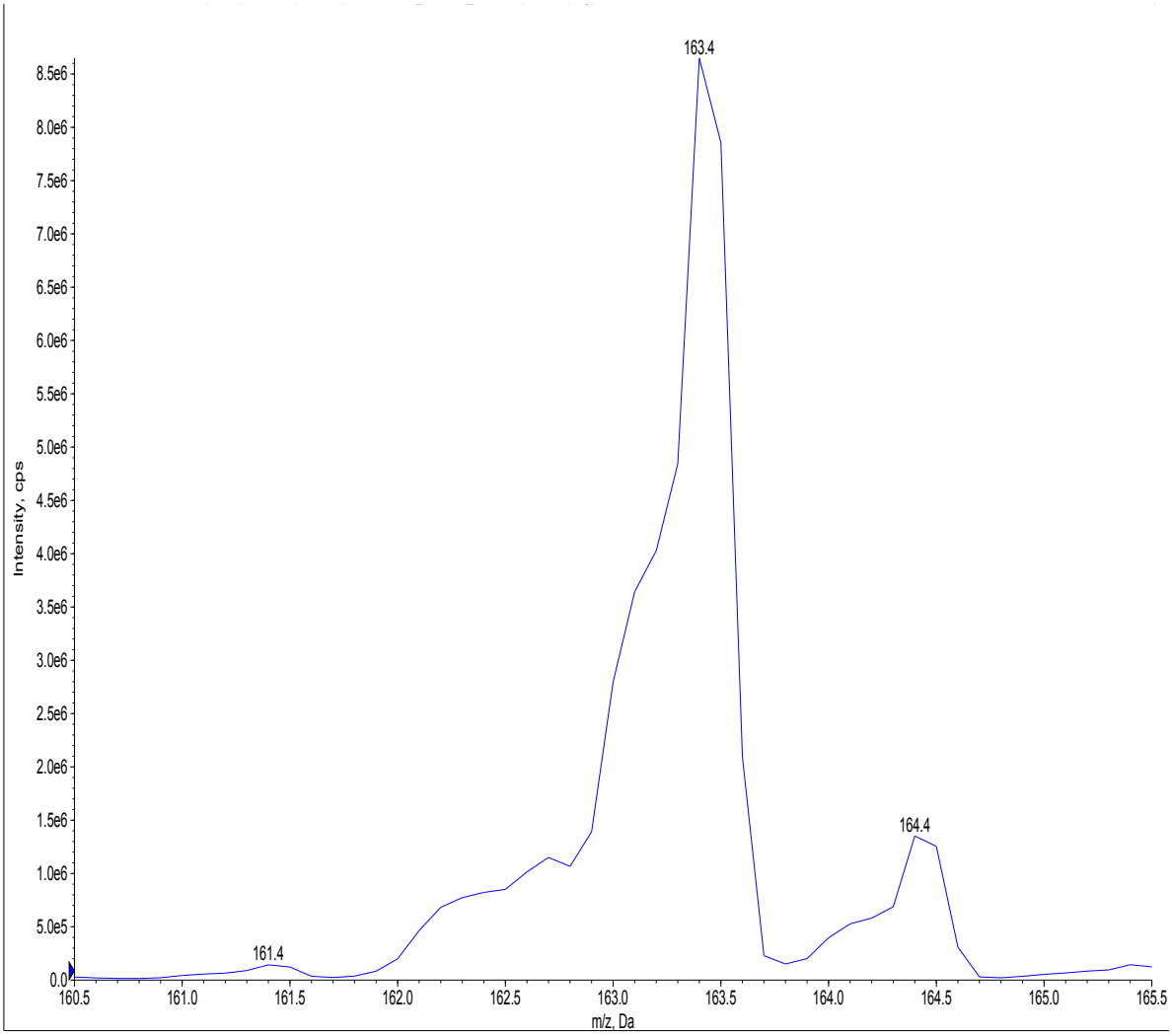


Fig. 2: Nicotine Precursor ion profile

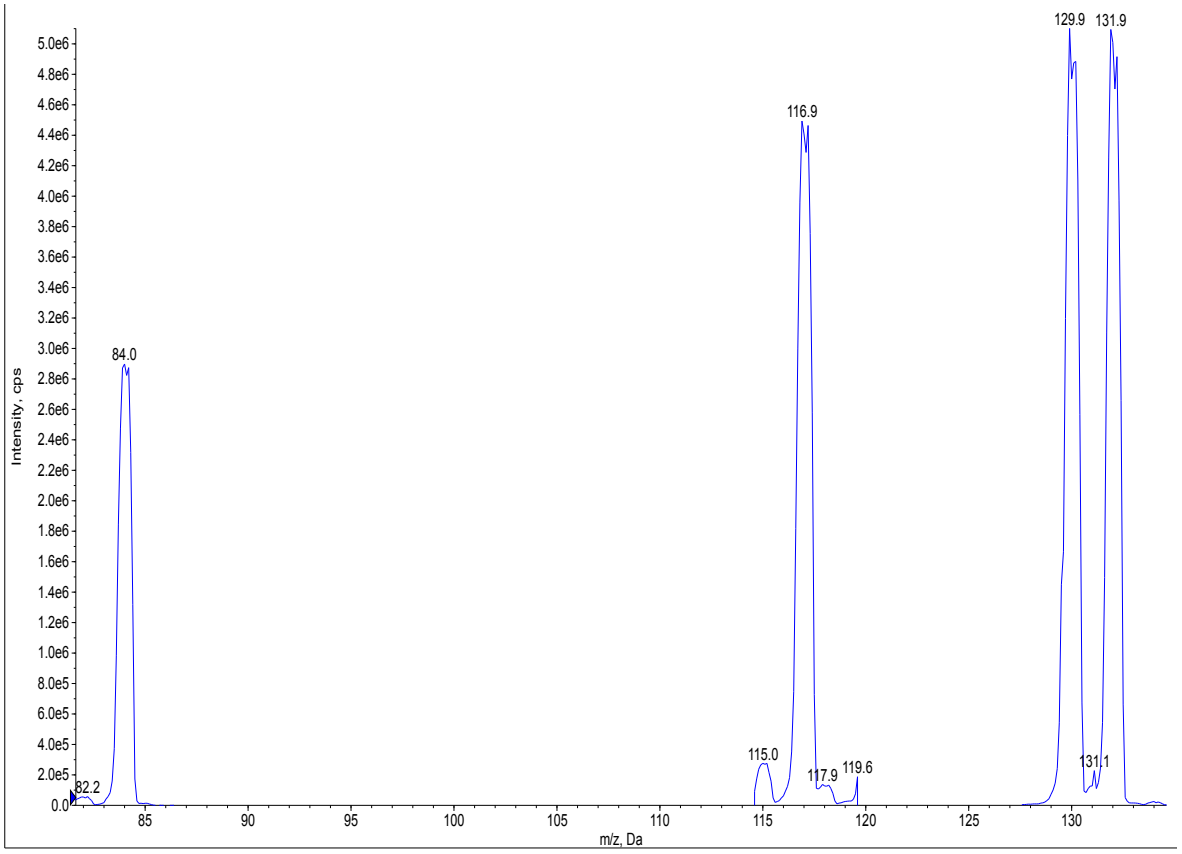


Fig. 3: Nicotine Product ions' profil

Nicotine chromatographic optimization:

Owing to the complex composition of the mushrooms' samples; high contents of protein and carbohydrates in addition to trace amounts of minerals, fats and vitamins, development of an efficient chromatographic method is crucial. Therefore, a well-suited chromatographic gradient for the analyses of nicotine in mushroom was developed. Particular goals of the optimization were the elution of nicotine within a reasonable period of time to be suitable for routine analysis of a large number of samples. A further objective was to obtain a sufficient retention of nicotine in order to avoid possible interferences with highly polar matrix components. Thus, different mobile phase compositions were studied. This include ammonium formate buffer at two different pH (3 and 4), methanol and acetonitrile. The obtained results showed that both of methanol and ammonium formate (pH 3) achieved highly symmetric peaks along with enhanced sensitivities. On the other hand, broad peak shape along with reduced sensitivity was observed in case of acetonitrile and ammonium formate (pH 4), respectively. As a result, a gradient elution using the conditions previously described in our proposed method of analysis was applied. The typical quantifier and qualifier chromatograms for nicotine in mushroom samples spiked at 0.1µg/mL are presented in Fig 4 and Fig 5.

Sample Extraction:

The objective of the current study was to develop a simple extraction procedure suitable for routine analysis with simplified work flow and still delivers excellent extraction efficiency, without the need of pH adjustment, clean up procedure or evaporation. These extra steps are time consuming with high cost and extra possibility for more errors. Despite the fact that nicotine is volatile and has two pK_a s (pk_{a1} = 3.1 pk_{a2} = 8.2) which complicated it is analysis. Efficient extraction was achieved by using extraction solvent (DIW (2%FA): Methanol, 1:1). The role of each constituent will be discussed next**.**

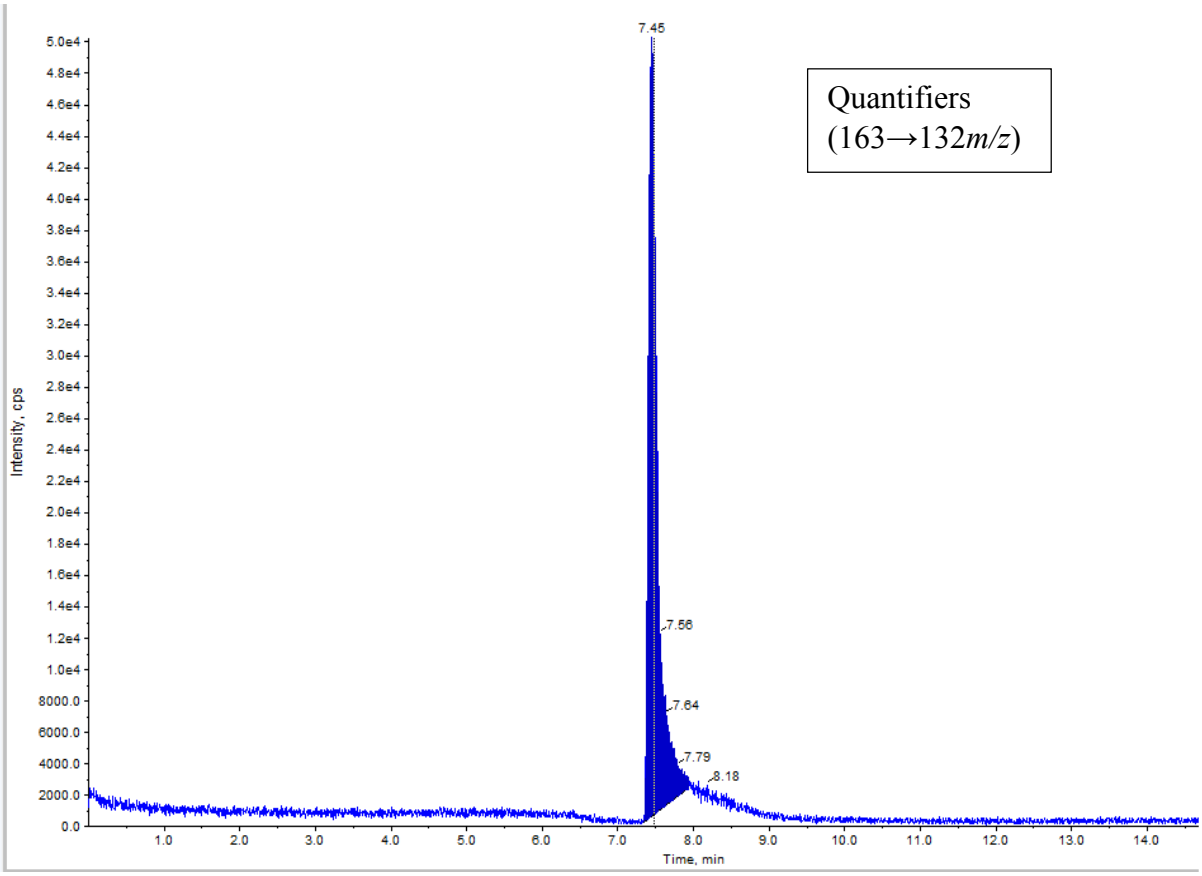


Fig. 4: Nicotine quantifier of 0.1µg/mL concentration.

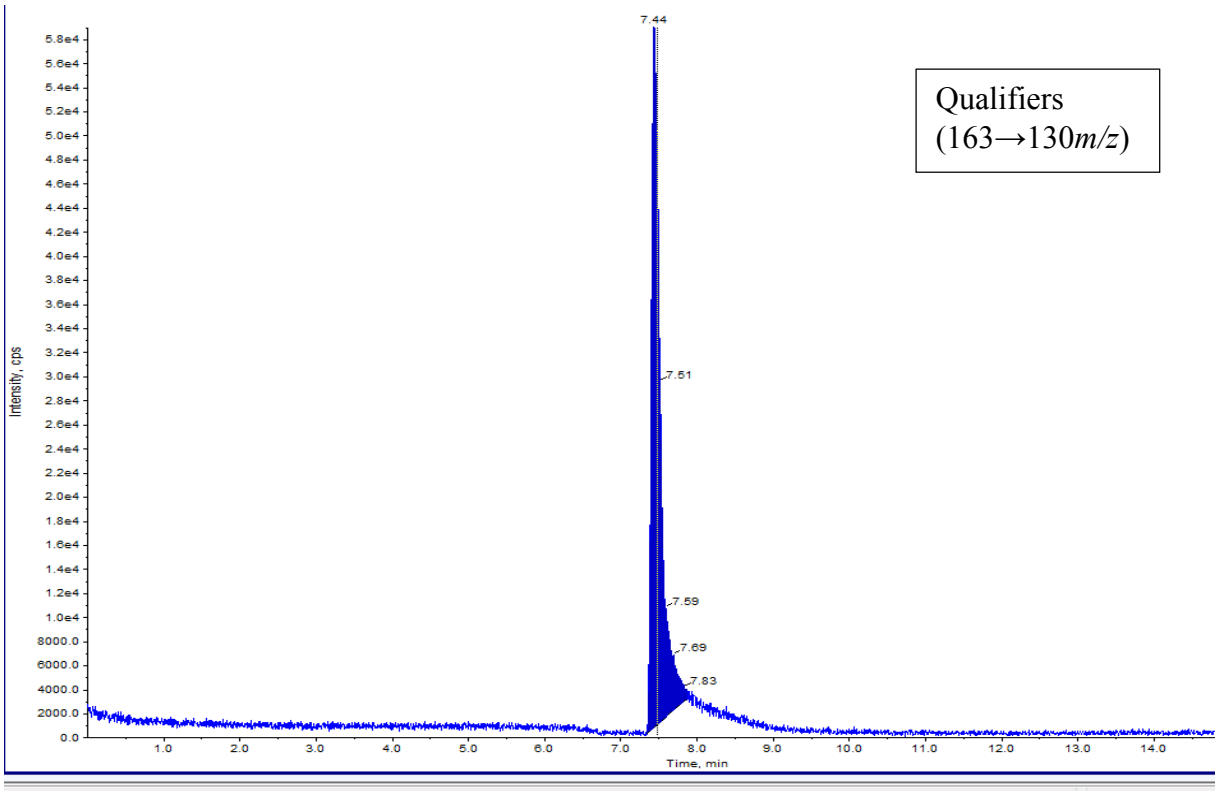


Fig. 4: Nicotine qualifier of 0.1µg/mL concentration.

Extraction solvents composition and selection:

Choosing water as extraction solvent due to high solubility of nicotine in water (S=1,000 g/ L at 20 °C) although, water alone has some disadvantage such as high solubility of sugars, starch, coloring agent and protein and can be easily extracted by water. Furthermore Water serves as excellent growth media for molds, yeast, algae and bacteria and the freezing point of water is 0 ^{0}C .All of this disadvantage can be solved by addition of methanol which prevent water from freezing which allow the use of centrifuge at -4 ^{0}C which help with precipitation of protein and other suspended particle and lower the solubility of water soluble mushroom extract and dissolve organic suspended particle which can cause irreducible result and plug the needle of the HPLC. Also prevent formation of molds, yeast, algae and bacteria. The additions of formic acid help with protein precipitation which is one of the major constituent of mushroom. Also the change of pH due to FA addition shift nicotine to more polar structure which increase its solubility in water hence better extraction efficiency and become less volatile . Several ratio of water to methanol was tested in order to obtain high recovery, sensitivity and good peak shape. The best ratio was found to be (DIW: Methanol, 1:1).

Nicotine stability studies:

The degradation of standards in stock solution in any analytical method can be one major source of errors and must be controlled. Stability test has been done according to *SANTE/11945/2015* to evaluate the stability of nicotine in stock solution. One pack of Nicotine certified standard with purity 99.3% was purchased from Dr. Ehrenstorfer (Augsburg, Germany) and was used for the entire period of stability test. Nicotine stock solution of 1000µg/mL was prepared in acetonitrile from the Nicotine certified standard and stored at -20±2. New stock solution was freshly prepared after 3, 6 and 12 months with the exact preparation methodology as the old stock solution. The freshly prepared stock is considered to be the reference standard. Afterwards a series dilution of the old and new stock to 10µg/mL then to 0.1µg/mL was done and injected alternatively 5 times into LC_MS/MS. The difference in the average response of old and new standard was calculated according to equation (1).

$$difference\ in\ \% = \left(\frac{Average\ response\ of\ OLD\ /\ STORED\ solution}{Average\ response\ of\ NEW\ /\ REFERENCE\ solution} \times 100\right) - 100$$

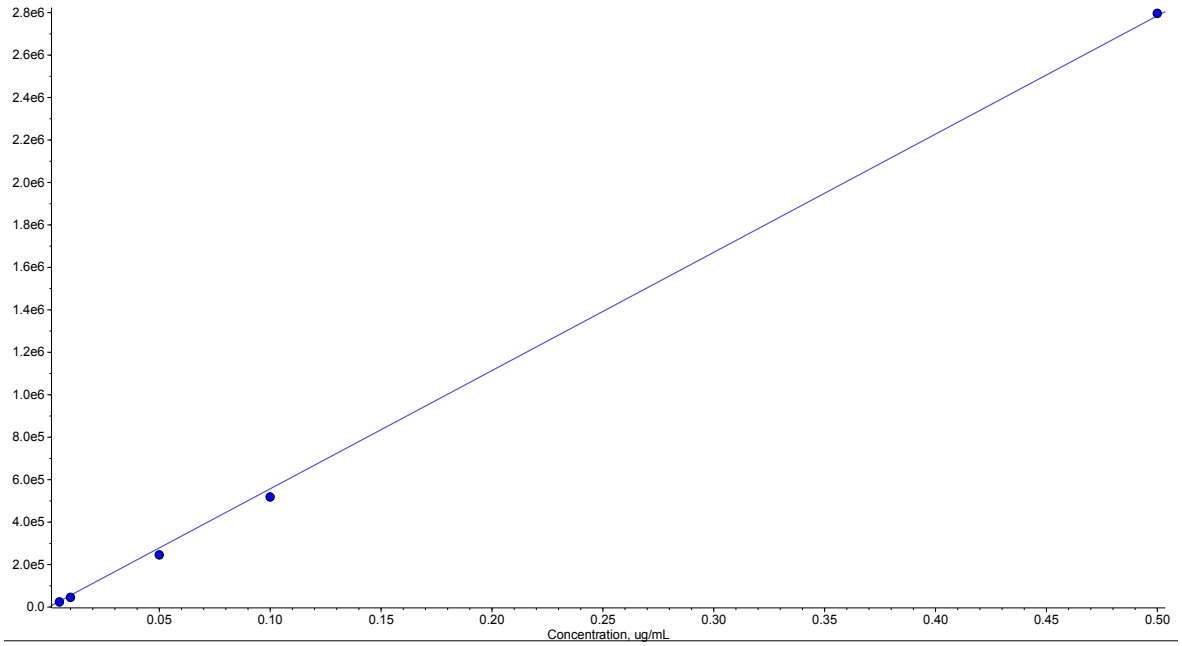
The result shows that even after 1 year the difference in concentration was < 5% which is in the acceptable range of ±20% set by (SANTE/EU, 2015).

Method validation:

The developed method was validated in accordance with the method validation criteria stated in the document EU guidelines (SANTE/EU, 2015)

Linearity of calibration curves:

The linearity of the calibration curve of nicotine was established by plotting the detector response area ratio versus the concentration of the analytical solutions at five concentration levels ranging from 0.005 to 0.5µg/ml. A calibration curve was obtained by plotting the peak area vs. concentration. The analyte showed linear behavior (Fig 4) in the studied concentration levels with correlation coefficient (R^2) 0f 0.9998. Each of the concentrations was injected five times, and the CV % of the five repeated injections was < 5% for each concentration which is in the acceptable range of ±20% according to SANTE guideline (SANTE/EU, 2015).



Quantification limit (LOQ):

The lowest validated level (0.01 mg/kg) was set as the LOQ in accordance with the (SANTE/EU, 2015).

Trueness and precision:

Trueness and precision were obtained by analyses of 5 replicate spiked mushroom samples at 3 different levels (0.01, 0.05 and 0.1 mg/kg). Recoveries ranged from 87% to 98% for 0.05 and 0.1 mg/kg levels and 71% to 85% for 0.01 mg/kg. The Trueness was based on the mean recoveries and the precision based on the associated relative standard deviations (CV %)and Recoveries, means are shown in Table 5.

Spiking level (mg/kg)	Recovery % (n=5)					Mean	STD	CV%
0.01	85	75	77	76	71	77	5	7
0.05	98	93	93	93	98	95	3	3
0.1	98	87	88	95	95	93	5	5

Conclusion

A sensitive and simple method for the determination of nicotine residue in mushroom was developed and validated. The proposed method is fast, easy, cheap, and sensitive .the developed procedure does not require solid phase extraction clean up, or dilution prior to injection while it permits the detection of nicotine residues of 0.01mg/kg. The method can be used for the routine determination of nicotine residue in mushroom samples.

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