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Overcoming the challenges in the analysis of nicotine in edible mushroom: Utilizing the dissociation constant effect

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Abstract

Nicotine is an alkaloid that can be naturally found in several edible plants of certain families such as the nightshade family. While nicotine has been also reported in other edible plants such as mushrooms without any clear scientific consensus about their origin. Hence, this study aimed to develop a method for the determination of nicotine in mushrooms. Nicotine is a very analytically challenging compound with two pkas and three forms (double ionized, single ionized, and non-ionized). Therefore, this study aimed to utilize nicotine's unique characteristic for extraction optimization and chromatographic separation. The optimum conditions were chosen based on matrix effect, spike recovery and chromatographic separation. The final method involved extraction using methanol under alkaline conditions (5% ammonium hydroxide). Alkaline condition in injection solvent was optimum for separation in reversed liquid chromatography. Additionally, the spiking recoveries were comparable with the acidic conditions, with the matrix effect being in favor of the alkaline conditions. The developed protocol was validated in accordance with the method validation criteria stated in the document SANTE/11312/2021 (V2) in terms of linearity, matrix effect, trueness, precision, and limit of quantification. A small-scale survey of six samples were done on mushrooms from the Egyptian market, all samples were found to be under permissible limits.

Introduction

Nicotine ((S)-3-(1-methylpyrrolidin-2-yl) pyridine) is the most abundant alkaloid in cultivated tobacco where it occurs in concentrations ranging between 2 and 8% [1]. Nicotine is present in other nightshade family (Solanaceae) plants such as peppers and potatoes at lower concentrations [2, 3]. Nicotine can be also found in other edible plants such as cauliflower [4].

Two decades ago, nicotine in mushroom was of no big concern, and a default maximum residue limit (MRL) for this toxic alkaloid was set at the available method for analysis limit of quantification (0.01 mg/kg) by the European Union (EU) regulation (EC) No 396/2005. Nevertheless, this MRL was set to test after multiple samples were reported to be exceeding this MRL in 2008 and 2009 by several countries such as Germany and Slovenia [3, 5–7]. According to the received data, 99% of samples tested in the EU from the 2008 production did not comply with the 2005 MRL [1]. Several possible reasons for this incident were suggested, and till now one or more of these factors are suggested to affect the presence of the presence of nicotine in mushroom. First of all, nicotine has neurotoxic effect on insects, and has long been used as a commercial insecticide in agriculture. However, its use was banned in several countries such as the EU due to its adverse effect on human health and impact on biodiversity [1, 8, 9]. Improper storage can also be a factor of nicotine's concentration increase in mushroom, as both nicotine precursors, ornithine and arginine, were identified in wild mushrooms [10, 11]. In addition, cross contamination due to bad practice during the storage/drying and packaging process (smokers handling the mushrooms, storage in rooms that have been disinfected with nicotine, simultaneous drying of tobacco and mushrooms in the same room) [6, 12]. *Boletus* (Seps) mushrooms are the species with the highest nicotine levels among mushrooms, followed by truffles and chanterelles [1]. Hence, the EU set MRLs for fresh mushrooms at 0.01 mg/kg and 0.02 mg/kg for cultivated and wild fresh mushrooms respectively. Furthermore, an MRL of 2.3 mg/kg for nicotine in Ceps and 1.2 mg/kg for dried wild mushrooms other than ceps. These MRLs are temporary and subject to change pending information provided by the continuous monitoring of this trace food contaminant [13]. Although, the MRLs has been set at more than 100 times higher for dried wild mushrooms, some incidents of noncompliance are still observed in recent years [7]. Hence, this contaminant is still of need for close monitoring at trace levels.

As shown in Fig. 1, nicotine is a basic alkaloid with two pKa at 3.1 and 8.2. Hence, the polarity of this compound is susceptible to change with the adjusted pH. Basic pH modifiers have been repeatedly used in combination with the Quick, Easy, Cheap, Effect, Rugged and Safe (QuEChERS) extraction method [5, 6, 8, 14]. This in terms convert the nicotine to its most nonpolar form and making it easy for the acetonitrile (extraction solvent) to have good extraction performance. In comparison, the Quick, Polar, Pesticides (QuPPe) method that uses methanol (more polar in comparison with acetonitrile) acidified with formic acid is being used for multipurpose extraction of highly polar analytes, recommended by the EU reference laboratory for single residue methods, including nicotine [15]. Also, this unique chemical property case has allowed the determination of nicotine in liquid chromatography tandem mass spectrometry (LC-MS/MS) using columns suitable for polar compounds such as

Hydrophilic Interaction Liquid Chromatography (HILIC) [14], and slightly less polar stationary phases such as phenylhexyl columns [16].

Therefore, this study aimed to evaluate the optimum conditions for the determination of nicotine in mushroom, taking into consideration several factors such as pH adjustment in extraction and LC-MS/MS determination. The method was validated in accordance with the EU Analytical Quality Control and Method Validation Procedures for Pesticide Residues Analysis in Food and Feed guidelines (SANTE/11312/2021 (V2)). A small-scale monitoring was done on both fresh and dried wild mushrooms sold in Egyptian market.

Materials and methods

Chemicals, reagents and standard solutions

Nicotine standard (99.3% purity) was purchased from Dr. Ehrenstorfer (Germany). Acetonitrile, acetone and methanol of HPLC grade were purchased from Sigma-Aldrich (USA). Formic acid (FA) and ammonium hydroxide with purity ≥ 98% and 33%, respectively, were purchased from Riedel-de Haen (Germany). Deionized water (DIW) was obtained using a MilliQ UF-Plus system (Millipore, Germany). All solvents used were analyzed for nicotine concentration to avoid contamination and interference.

Nicotine stock solution of 1000 µg/mL was prepared using acetonitrile and kept in the freezer at -20 ± 2^0C. Two 10 µg/mL working solution were prepared in DIW and acetone from stock solution to be used in the preparation of calibration preparation and spiked samples preparation respectively. Calibration mixtures ranging from 0.005 - 0.5µg/mL were prepared via serial dilution of the intermediate solution in the injection solvent (Methanol (5% ammonia): DIW, 1:1). Both of the intermediate and calibration mixture solutions were stored in the refrigerator at 4 ± 2^0C.

LC-MS/MS conditions

LC–MS/MS was performed using an Agilent (USA) 1200 Series HPLC instrument coupled to an API 4000 Qtrap MS/MS from Applied Biosystems (USA). The separation was performed on an Agilent C18 column ZORBAX Eclipse XDB 4.6 × 150 mm, 5.0 µm particle size. Mobile phase reservoir (A) was 10 mM Ammonium format buffer at pH=3 ± 0.05, and reservoir (B) was Methanol.

Separation procedures were achieved using gradient elution as shown in table 1 at the flow rate of 200µL/min and a column temperature of 40°C. An injection volume of 5µL was directly injected into LC-MS/MS.

The detection process of the studied compound was carried out using electrospray ionization (ESI) in the positive ion mode. The instrumentation parameters were decided based on the manufacturer's recommendations. The Ion spray voltage was 5500 V, the temperature source was set at 400 C, and the curtain gas has a value of 30 psi.

Multiple reactions monitoring mode (MRM) was employed for nicotine confirmation and quantitation. Optimization of the targeted MRMs were done by the direct infusion of 0.1 µg/mL nicotine standard prepared in methanol:buffer (1:1) directly into the MS. Based on sensitivity and selectivity (163/132, m/z) was chosen to be the quantifier and three other MRMs (163/130, 163/117and 163/84 m/z) were chosen as confirmatory qualifiers.

Sample homogenization

Sample extraction

The pre-homogenized fresh mushroom sample was analyzed to ensure it was free from nicotine (blank sample). Then (500 gm) of the sample was sprayed with 5 ml nicotine standard (10 µg/ml) dissolved in acetone (easily evaporated solvent) and then homogenized further using an electric mill at high speed to give an expected value of 0.1 µg/ml. Five replicates of the contaminated sample were analyzed using the same method to ensure homogenization. This contaminated sample has been used in method development and optimization.

The extraction procedure was based on the dilute and shoot QuPPe method [15]. Basically, 10 ± 0.1 g of the sample was weighed in the tube for fresh mushrooms, and 5 ± 0.05 g for dried mushrooms. This was followed by the addition of methanol (1% formic acid) and shaking for 1 minute then centrifuged for 5 minutes. In our case, 1% and 5% formic acid were tested, in addition ammonium hydroxide 5% was also tested. The aliquot was then filtered using a 0.45 µm syringe filter into the LC-MS/MS vial to be injected.

Method validation

The developed protocol was validated in accordance with the method validation criteria stated in the document SANTE/11312/2021 (V2). The matrix effect was measured by comparing a single point matrix matched solution with the calibration used as described in [17,18]. Different spiking levels were done to calculate the following three parameters: The trueness, expressed as average recovery for each tested level; the precision, expressed as the RSD of each tested level; and the Limit of Quantification (LOQ), expressed as the lowest validated spike level with acceptable criteria for trueness and precision. The linearity was expressed as the correlation coefficient (R2) and the deviation of back-calculated concentration from true concentration.

Results and discussion

Extraction and chromatographic methods optimization

As explained in the introduction, nicotine is a basic alkaloid with two pKa at 3.1 and 8.2. Meaning, depending on the adjusted pH nicotine may take one of three forms; double protonated, single protonated, or non-ionized form as shown in figure one. Hence, due to that unique fact, the chromatographic separation and extraction methods optimization cant be separated in a one factor at a time design of experiment and should be done simultaneously, as the final extract solvent will be the injection solvent for the LC-MS/MS determination, that has already been a proved factor that can affect the sensitivity [19], peak shape [20], and retention time [21].

For the chromatographic separation, as a part of the method development policy in the laboratory, a C18 column is always prioritized in an effort to do all the routine analysis methods on the same type of separation columns to save time and money. This is extra challenging since the optimal conditions for the determination of nicotine through the ESI is at pH>3.1 to increase the chance of nicotine being ionized. Using this pH for the mobile phase goes hand in hand with using a polar separation column such as HILIC [5]. On the other hand, using basic mobile phase (pH >8), completely converts nicotine to its non-polar form which leads to higher retention in reversed phase columns in general. Nonetheless, this strategy may lead to decreased ionization efficacy in the ESI and significantly longer retention times on C18 columns. Hence, a mobile phase at pH 3 was used with a gradient mobile phase as shown in table 1.

The null hypothesis of this step was that the retention times of nicotine will change using acidic and basic conditions in the injection solvent due to the fact that nicotine in non-ionized form will be retained more on the non-polar C18 column, presumably in the beginning of the column prior to the complete solvation of the nicotine and its transformation to its polar form due to the pH of the mobile phase. Hence, nicotine at 0.1 µg/mL was injected in both acidified methanol (1% and 5% formic acid), and alkaline methanol (1% and 5% ammonium hydroxide). Interestingly enough, nicotine appeared at retention time 8.5 min for both the acidified methanol injections, and 10.5 min for the 5% ammonium hydroxide. As for the 1%

ammonium hydroxide injection, both peaks appeared, indicating that the amount of ammonium hydroxide was not enough to completely convert the nicotine to the non-ionized form as shown in figure 2.

The next step was to compare the alkaline methanol extraction with the conventional acidified methanol QuPPe. Table 2 shows that all three conditions gave comparable results in terms of spiked sample recovery. However, the matrix effect injections indicated suppression in the peak area of samples extracted with the acidified conditions in comparison with the ammonium hydroxide QuPPe extraction approach as shown in figure 3. Scan injections for both extraction methods indicates that a huge matrix peak co-elutes with the nicotine peak in acidic conditions, while clearly separated from the ammonium hydroxide peak as shown in figure 4. This believed to be because of the high polarity of methanol which can co-extract a high amount of polar matrix, this co-eluted polar matrix can compete with the nicotine to be charged in the ESI, which in terms cause signal suppression [22–24]. A clear separation between the polar matrix and the nicotine in basic injection solvent most likely happens due to that nicotine is introduced to the C18 column in its most non-polar form which as explained earlier increased its retaining. This is not the case for acidic injection conditions where both the co-extracted matrix and nicotine introduced to the column which lead to their co-elution.

Method validation

Calibration linearity and matrix effect

The linearity of the calibration curve of nicotine was established by plotting the detector response area versus the concentration of the analytical solutions at five concentration levels ranging from 0.005 to 0.5 µg/mL.

The analyte showed linear behavior (Fig 4) in the studied concentration levels with correlation coefficient (R^2) 0f 0.9999. Each of the concentrations was injected five times, and the RSD% of the five repeated injections was < 2% for each concentration which is in the acceptable range of ±20% according to SANTE guideline.

By comparing the injection of nicotine in blank solvent vs matrix extract at concentration 0.05 µg/mL, it was found that the matrix effect was 14 % (within ± 20%). Hence, matrix compensation was not/needed. To make sure that matrix effect behaved at the same way at different concentrations, injections at 0.005, 0.01 and 0.1 µg/mL were done and gave comparable results.

Trueness, precision, and LOQ

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). Trueness was expressed as the mean recoveries of each spiking level, and precision as their associated relative standard deviation (RSD%). As shown in table 3, all mean recoveries and RSDs% were found to be within the accepted range (70-120% for mean recovery and <20% for RSD%).

The LOQ was set as the lowest validated level (0.01 mg/kg) with accepted trueness and precision.

Sample monitoring

Six (3 wild fresh, 3 wild dried) mushroom samples were collected from a local market in Giza, Egypt to monitor the levels of nicotine. All wild fresh samples didn't contain any residues of nicotine, while dried samples contained 0.9 mg/kg concentration for two samples and 1.1 mg/kg for one sample.

Conclusion

This study optimized a method for the determination of nicotine in mushrooms. The optimization was based on the pka of nicotine, taking advantage from both its double ionized and non-ionized form. The method was validated in accordance with the SANTE/11312/2021 (V2) guidelines. A small-scale survey showed that all samples analyzed comply with the limits in Egypt. However, further investigations should be done to truly assess the situation.

Declarations

Declaration of interest

The authors declare no competing financial interest.

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Author Contribution

All the authors contributed to the article. Mahmoud S. Elshabrawy : Project administration, conceptualization, software, formal analysis, methodology, validation, writing original draft preparation and Data analysis with interpretation. Mahmoud Hamdy Abdelwahed: reviewing, editing, resources. Aya M. Matloob: investigation, reviewing. Mostafa Soliman: reviewing, supervision.

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Data Availability

All data generated or analyzed during this study are included in this manuscript.

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Tables

Table 1 to 3 are available in the Supplementary Files section.

Figures

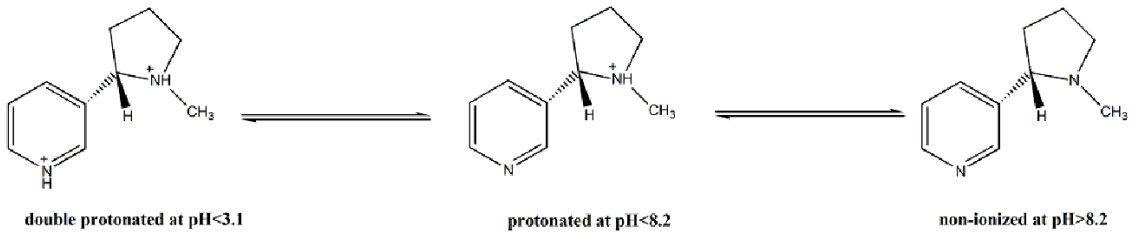


Figure 1 Effect of pH on nicotine structure

Figure 1

See image above for figure legend

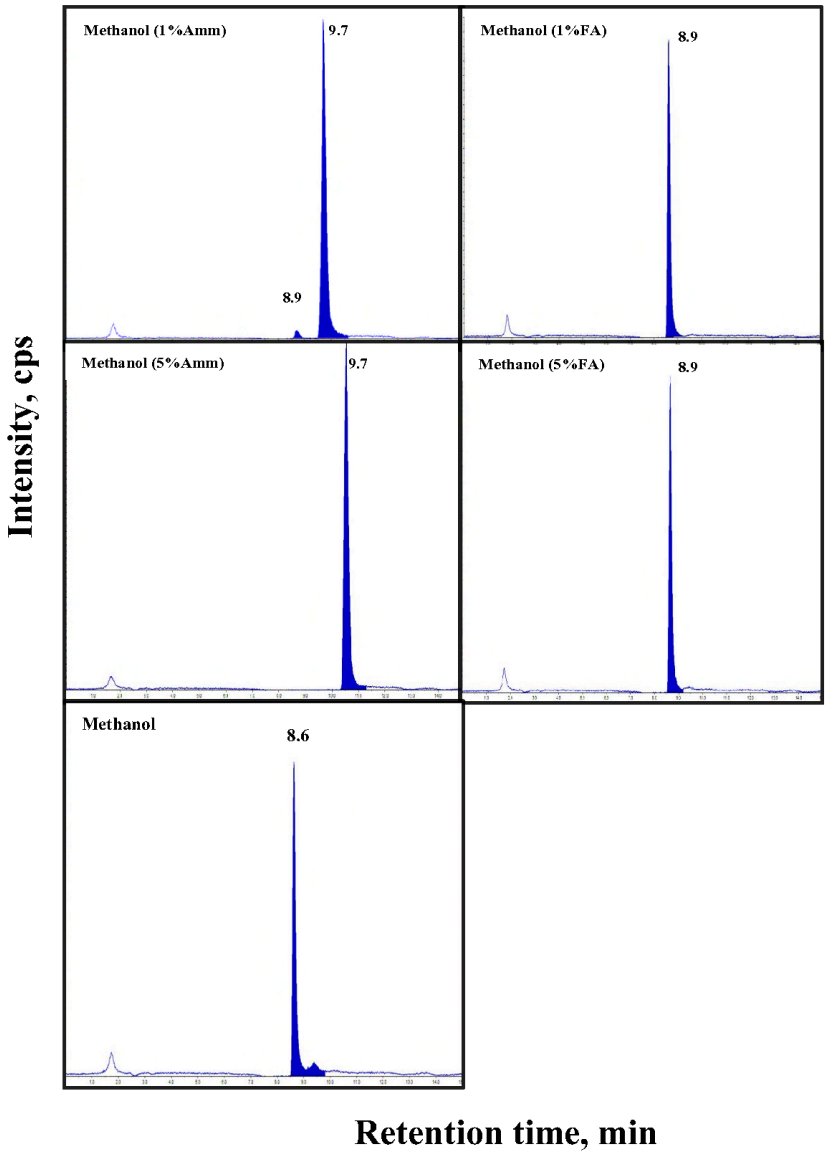


Figure 2 : Nicotine standard (0.05ppm) chromatogram at different extraction solvent

Figure 2

See image above for figure legend

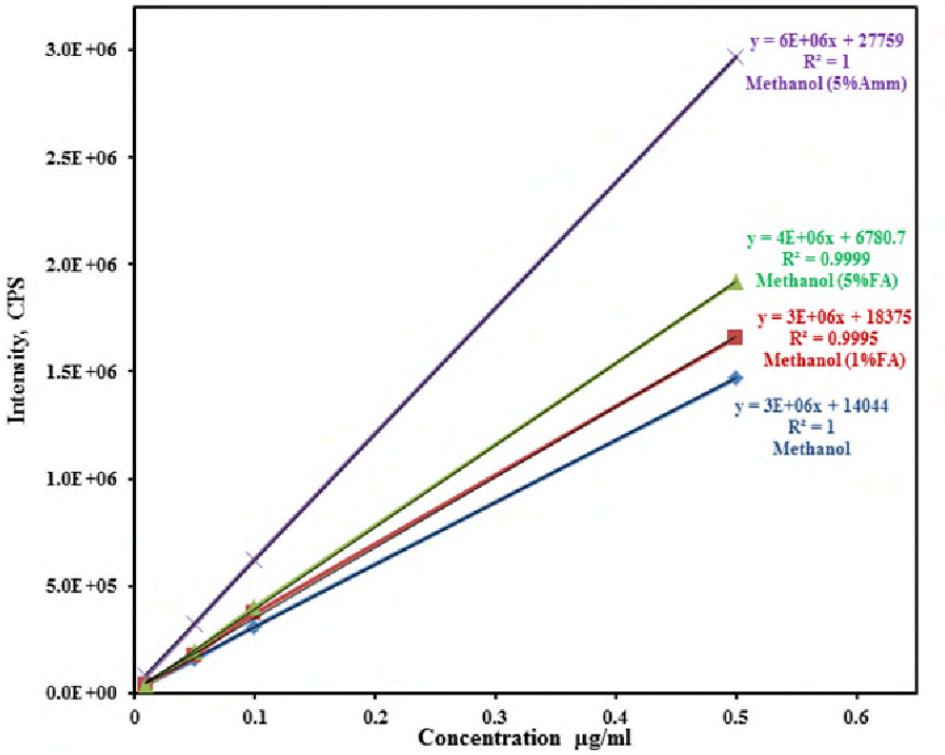


Figure 3 comparstion in signal supresstion between diffrenent extraction conditions and calibration solvent concn rang ()

Figure 3

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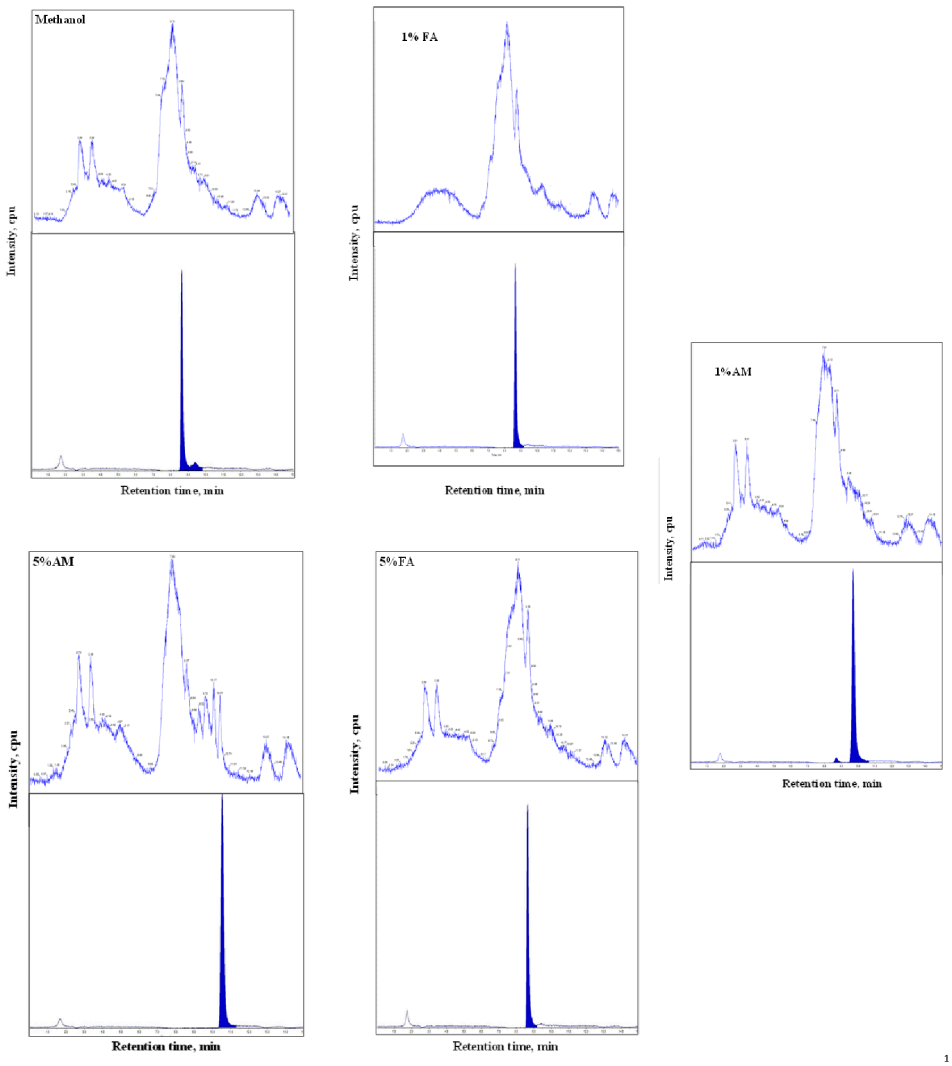


Figure 4 Nicotine standard and extracted co-matrix in scan and MRM mode

Figure 4

See image above for figure legend

Supplementary Files

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