Journal of Food Science and Engineering 1 (2011) 27-34

Rapid Analysis of Nicotine in Mushrooms Using QuEChERS Extraction and Liquid Chromatography Tandem Mass Spectrometry

A. Santilio and R. Dommarco

Department of Environmental and Primary Prevention, Pesticide Unit, National Institute of Health, Rome 00161, Italy

Received: March 23, 2011 / Published: June 15, 2011.

Abstract: A procedure based on the QuEChERS methodology and Liquid Chromatography Tandem Mass Spectrometry (LC/MS/MS) is described, for the determination of Nicotine in mushrooms. QuEChERS methodology was used to determine Nicotine in dried and fresh mushrooms under basic conditions with primary secondary amino sorbent (PSA) clean up. The chromatography was performed on C18 reversed phase column using a gradient of acetonitrile and ammonium formiate 1mM pH = 3.4 as mobile phase at a flow rate of 0.3 mL min^{-1}. Nicotine was determined by using Nicotine-d3 as internal standard. Limit of quantification (LOQ) was 0.01 mg kg^{-1} for both fresh and dried mushrooms. Calibration curve was linear over the concentration range of 0.01-2.3 mg mL^{-1}, with $r^2 > 0.99$. As for recoveries in dried mushrooms, spiking levels of 0.32 mg kg^{-1} and 2 mg kg^{-1} were considered whereas for the fresh mushrooms the recoveries were determined at 0.036 mg kg^{-1} and 0.36 mg kg^{-1}. Satisfactory results were obtained for both matrices and the recoveries proved to range from 105% to 135%, with a standard deviation in the range 17-20. The method was applied to the analysis of Nicotine to assess the levels of nicotine in fresh and dried mushrooms.

Key words: QuEChERS, Nicotine, mushrooms, pesticides, residues, liquid chromatography tandem mass spectrometry (LC/MS/MS).

1. Introduction

Nicotine ((S)-3-(1-methylpyrrolidin-2-yl)pyridine) is the predominant component of the crude alkaloid extract, its structure formula is showed in Fig. 1; it is used as insecticide to control of aphids, thrips, whitefly and other insects on glasshouse ornamentals and on crops including fruit, vines and vegetables [1]. In European Countries, the use of plant protection products containing Nicotine is phased out on June 2010 but its use in Third Countries may continue and may lead to residues of Nicotine in food [2].

During the years 2008/2009, Nicotine was detected in dried wild mushrooms (mainly *Boletus edulis*), at the levels higher than 0.01 mg kg^{-1} on a fresh weight basis. The level of 0.01 mg kg^{-1} was the maximum residue level (MRL) established by the article 18.1.b of Regulation (EC) No 396/2005) [3].

As consequence of the high levels of nicotine, the European Commission asked to the EFSA to establish a safe nicotine level for consumers on the basis of the residue levels from monitoring plan and of the toxicological properties of the Nicotine. According to the EFSA opinion [2], a safe level of nicotine of 0.036 mg kg^{-1} for fresh wild mushrooms was established, corresponding to 0.32 mg kg^{-1} for dried wild mushrooms. Afterwards, the European Commission set a temporary MRL of 0.04 mg kg^{-1} for fresh wild mushrooms [4].

As a result of the European Commission request to monitor the level of nicotine on dried mushrooms, the Italian National Reference Laboratory for Single Residue Methods (NRL-SRM) studied the performance of a simple and fast method using QuEChERS methodology and liquid chromatography

Corresponding author: A. Santilio, researcher, research field: pesticide residues. E-mail: angela.santilio@iss.it.

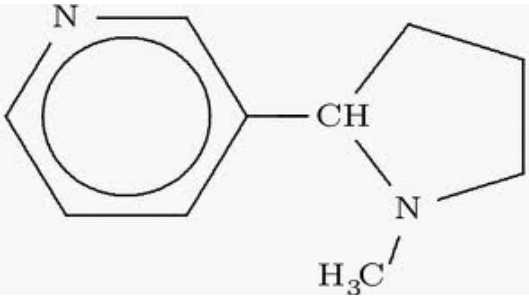

Fig. 1 Structure formula of nicotine.

tandem mass spectrometry (LC/MS/MS) to determine the nicotine levels in both dried and fresh mushrooms.

It has been reported that Nicotine has a wide distribution in various edible vegetables belonging to the nightshades family (Solanaceae) such as potatoes, tomatoes or eggplants (aubergines) [5-8]. The function of Nicotine in these vegetable is still not fully known and it is assumed that the Nicotine as a natural defense against fungi, bacteria, insects and animals. In the above-mentioned publications, the authors have described complicated methodologies to eliminate interfering compounds, including liquid-liquid extraction and clean up. The determination of Nicotine has been usually performed by gas chromatography/mass spectrometry [5-8] and only few authors have used the liquid chromatography /mass spectrometry and tandem mass spectrometry [9-15]. Some authors determined the Nicotine content in pharmaceutical formulations using liquid-liquid extraction followed by liquid chromatography [16] or ultrasonic extraction and gas chromatography [17]. In 2002, a case of contamination of ground beef adulterated with a Nicotine sulfate has been reported in Ref. [18] and the Nicotine was determined by HPLC/UV and by GC/MS for confirmation.

Before now, no study has been reported for the determination of Nicotine in mushrooms because of no evidence of the contamination was detected. Only recently the presence of Nicotine in mushrooms has been pointed out and in Ref. [19] the author described a study of determination of Nicotine in mushrooms by LC/MS/MS. The cause of Nicotine contamination in dried and fresh mushrooms has not yet been established as a naturally occurring compound.

The aim of the present paper was to study the performance of the QuEChERS methodology applied to the analysis of Nicotine in dried and fresh mushrooms. The QuEChERS methodology introduced by Anastassiades [20, 21] well fit to the analysis of Nicotine in mushrooms. The methodology is rapid and allows the analysis of several samples in short time, moreover it is well arranged with the liquid chromatography tandem mass spectrometry. Special emphasis was put on the use of the Nicotine-d3 as appropriate internal standard to compensate the lack of fully quantitative extraction for the analysis.

2. Materials and Methods

2.1 Reagents

The reference standard of Nicotine was purchased from Sigma Aldrich (Milan, Italy) which was from 99.8% certified purity. The reference standard of Nicotine d3 was purchased from Toronto Research Chemicals Inc. (TCR) (North York, Canada) which was from 99% isotopic certified purity.

Individual stock standard solutions were prepared at 1 mg mL^{-1} in acetonitrile and stored in darkness at +4 ℃. The working standard solutions were prepared at concentration of 10 μg mL^{-1} and stored in darkness at +4 ℃.

Acetonitrile (HPLC) grade was obtained from Carlo Erba (Milan, Italy). Water purified with Elix/Milli-Q water purification system (Millipore, Bedford, MA, USA) was used.

Ammonium Formiate (HPLC grade) was obtained from Fluka (Switzerland).

Anhydrous magnesium sulfate (97% pure) was supplied from Across Organics (New Jersey, USA). Sodium chloride was obtained from Merck (Darmstadt, Germany) and purified from interfering substances by heating for 3 h at 600 ℃ and then for 12 h at 120 ℃ in muffle furnace and in oven respectively. Primary secondary amine sorbent (Bondesil-PSA), 40 μm was obtained from Varian (Palo Alto, CA, USA).

Anotop 25 LC (0.2 μm pore size) was purchased from Whatman (Maidstone, England).

2.2 Apparatus

A Buchi Mixer B-400 (Buchi Labortechnik AG Fawil, Switzerland) was used to mince the sample.

A vortex mixer IKA MS 3 Basic (Wilmington, USA) was used to mix the samples for the extraction. A centrifuge ALC 4237R (Milan, Italy) was used to centrifuge the samples after extraction.

A Varian System ProStar (HPLC) (Valnut Creek, CA), equipped with a Varian 1200L Quadrupole MS/MS detector was employed. The liquid chromatograph was equipped with a Varian 410 autosampler (Valnut Creek, CA). A Valco valve of injection with a loop of 100 μL was used. The injection mode was partial loopfill. The injection volume was 5 μL, and to avoid carry-over, the autosampler was flushed with acetonitrile before sample injection.

Chromatographic analysis was performed on 150 × 2.1 mm Zorbax Eclipse XDB-C18 column (Agilent Technologies, USA) with a 3.5 μm pore size. Acetonitrile (B) and ammonium formiate at pH = 3.0 (A) were used as mobile phase at flow rate of 0.3 mL min^{-1}. The linear gradient started at 100% A to 40% A (12 minutes) the column was held to 40% A for 1 minute and then increased to 100% A (3 minutes) and was held 2 minutes before return to the initial mobile phase composition. The total run-time was 15 minutes.

The detection was achieved using mass spectrometry equipped with electrospray ionization interface (ESI) operating in positive ion mode. Typical source parameters were as follow: Ionization voltage was 1400 V; Capillary voltage was 40 V; The solvent evaporation in the source was assisted by a drying gas (heater synthetic at 250 ℃ at 30 psi). Data analyses were assured by software Varian MS Workstation version 6.8.

2.3 Samples

Twelve samples of mushrooms were collected from local markets in different area of Rome.

The fresh mushrooms (*agaricus bisporus*) were chopped up by Buchi mixer to obtain an homogeneous sample.

The dried mushrooms (*botules edulis*) were added to cold water (+4 ℃) and were minced by Buchi mixer to obtain a homogenate. The cold water was added in the proportion to the weight of the sample (10 mL of water to 2 g of dried sample).

2.4 Extraction and Clean Up Procedure

For fresh mushrooms, 10 g of sample were added to a PTFE tube of 50 mL. For dried mushrooms, 5 g of homogenate was added to a PTFE tube of 50 mL, corresponding to about 2 g of dried mushrooms. 100 μL of internal standard Nicotine d3 at concentration of 10 μg mL^{-1} was added to the sample.

10 mL of acetonitrile were added to the sample and it was mixed for 1 minute.

200 μL of NaOH 5N was added and the pH was checked, after 4 g of $MgSO_4$ and 1 g of NaCl were added and the tube was mixed for 1 minute. Then the sample was mixed at 3000 rpm for 5 minutes.

The extract (7 mL) was transferred in a PTFE tube of 15 mL containing 150 mg of PSA and 900 g of $MgSO_4$. The sample was mixed for 30 seconds and was passed in a centrifuge at 3,000 rpm for 5 minutes. The supernatant was transferred in a tube of 15 mL and the pH was adjusted at 3-4 with 75 μL of 5% formic acid. An aliquot was filtered on Anotop 0.2 μm and injected in LC/MS/MS.

2.5 Validation Experiments

The validation of the method was carried out taking into consideration the criteria of the document SANCO/10684/2009 [22]. The following parameters were determined during the validation of the analytical method: accuracy, precision, linear dynamic range and limit of quantification (LOQ).

The accuracy and precision of the method were tested by recovery experiments using fresh and dried

mushrooms which turned out to be free from Nicotine. A standard solution at concentration of 10 μg mL^{-1} was used to fortify the samples. Ten g homogenized sample was added with an adequate quantity of standard to give a concentration of 0.01 mg kg^{-1}, 0.02 mg kg^{-1}, 0.32 mg kg^{-1} and 2.0 mg kg^{-1} for dried mushrooms and of 0.01 mg kg^{-1}, 0.02 mg kg^{-1}, 0.036 mg kg^{-1} and 0.36 mg kg^{-1}or fresh mushrooms. The spiked samples were mixed by vortex for a few seconds and left to stand for 10 minutes before extraction to favor the diffusion of Nicotine into the matrix. Then the samples were prepared according to the determination procedure described above. Recoveries were determined in five replicates at each spiked level and for both studied matrices.

External calibration with internal standard Nicotine d3 was used to quantify the Nicotine. Matrix matched calibration solution containing Nicotine d3 (Internal Standard) was employed to minimize errors related to matrix-induced signal enhancement effects.

The linear dynamic range was studied by plotting 5-point calibration curves. Each point of the linearity curve was obtained as average of two consecutive injections. Five calibration solutions at concentration of 0.01 μg mL^{-1}, 0.036 μg mL^{-1}, 0.36 μg mL^{-1}, 1.0 μg mL^{-1} and 2.3 μg mL^{-1} were prepared in acetonitrile.

The limit of quantification (LOQ) was set at 0.01 mg kg^{-1} for both fresh and dried mushrooms and was established as the minimum concentration of the nicotine that can be quantified with acceptable accuracy and precision.

3. Results and Discussion

In this paper we described the validation of an analytical procedure for the quantification of Nicotine in mushrooms by QuEChERS methodology and LC/MS/MS determination. The quality criteria described in the Document SANCO/10684/2009 were used to judge whether the validation was successful or not. The method described here fit the criteria established in the SANCO document.

The recovery rates and precision were studied at two fortification levels: 0.036 mg kg^{-1} and 0.36 mg kg^{-1} for fresh mushrooms and 0.32 mg kg^{-1} and 2.0 mg kg^{-1} for dried mushrooms, considering five replicates for each spiking level. The recoveries were expressed as percent ratios between the mass of Nicotine after extraction and cleanup steps and the mass of Nicotine added to fortify the sample investigated. We also compared our results with Ref. [18] which describes the QuEChERS methodology applied to the analysis of mushrooms. A good agreement between the results was obtained, even though higher fortification levels were investigated in that case.

The response of the processed spiked matrix was considered with respect to the response of a matrix -matched standard at the same fortification level. The use of the matrix-matched standard minimized the matrix effect relate errors. Therefore, the Nicotine isotope d3 and d4 were used as internal standard in the analytical determination on vegetables [6] and in human mecomiun [12], also by other authors. In this work, the quantification was carried out using the Nicotine methyl-d3 as internal standard (IS), which improved the recovery rates and minimize the dilution errors.

The elution of the Nicotine by LC/MS/MS technique is reported on different stationary phases as C18 or C8 reversed-phase column. In either case, the elution is completed in less than 10 minutes: The nicotine elution on C18 column is achieved in less than 5 minutes, whereas 8 minutes are required on the C8 phase.

In this study, the Nicotine elution was performed on a Zorbax Eclipse XDB-C18 column in 4 minutes and the identification of the compound was carried out by retention time and ion transitions as reported in Table 1.

A typical MRM chromatograms of matrix matched calibration solution at 0.036 mg/kg is shown in Fig. 2. Fig. 2 reports the chromatograms with ion transitions for Nicotine and Nicotine d3. The nicotine ion transition 163 > 130 m/z was used for the quantitative determination.

Table 1 Retention time, ion transitions and collision energy of Nicotine and Nicotine d3 by LC/MS/MS.

Compounds	Retention time (min)	Transition		
		Precursor ion (m/z)	Product ion (m/z)	Collision energy (eV)
Nicotine	4.278	163.1	130.1	29
		163.1	132.2	23
Nicotine methyl d3	4.273	166.2	132.1	23

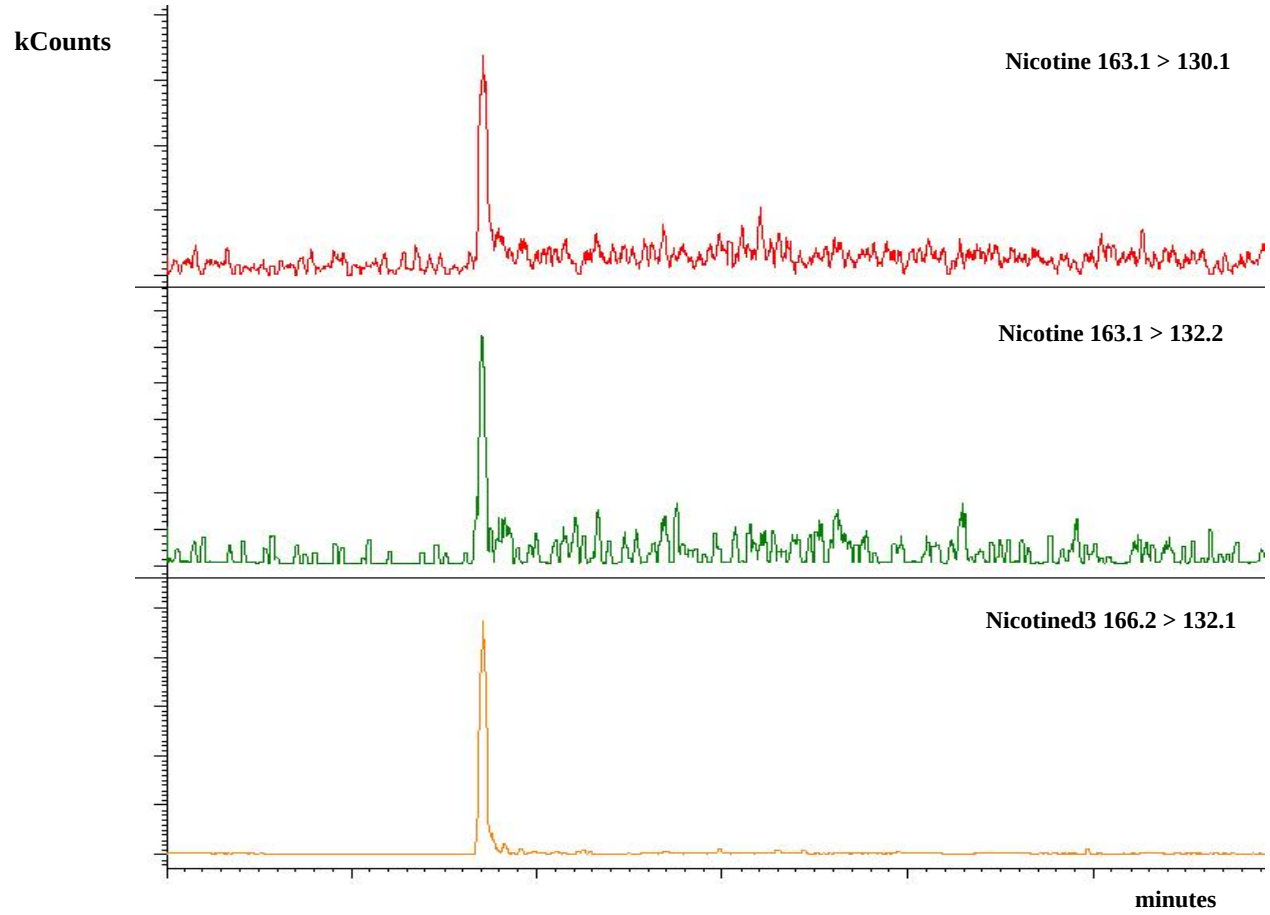

Fig. 2 Multiple Reaction Monitoring (MRM) chromatograms obtained on Zorbax Eclipse XDB-C18 column (150 × 2.1 mm; 3.5 μm) of a matrix-matched calibration solution of Nicotine at 0.036 μg/mL and Nicotine d3 at 0.10 μg/mL. Nicotine ion transitions 163.1 > 130.1 and 163.1 > 132.2 m/z; Nicotine d3 ion transition 166.2 > 132.1 m/z.

The mean recoveries for each matrix ranged from 120% to 135% for dried mushrooms and from 105% to 112% for fresh mushrooms. Table 2 shows the recoveries and the standard deviation for both matrix and for each fortification level. The precision of the method was investigated and expressed as relative standard deviation (RSD%). Good results were obtained for both matrices and the values are ranged between 10% and 17% for dried mushrooms and RSD% of 15% and 16% were obtained for fresh mushrooms for both spiked levels.

A linear relationship between the detector response and the Nicotine concentration was observed. The calibration curve was obtained plotting the ratio Nicotine/internal standard concentration against the ratio Nicotine/internal standard peak area. The Nicotine showed linearity in electrospray ionization (EI) positive mode over a concentration range of 0.01-2.3 μg mL^{-1}. A linear curve with correlation coefficient R^2, higher than 0.99, was obtained as shown in Fig. 3.

Table 2 Mean recoveries (n = 5), standard deviation and %RSD for Nicotine on dried and fresh mushrooms.

Spiking level (mg/kg)		Nicotine	
		% Recovery (n = 5) ±S.D.	%RSD
Dried mushrooms	0.01	178 ± 27	15%
	0.02	171 ± 12	7%
	0.32	135 ± 13	10%
	2.0	120 ± 20	17%
Fresh mushrooms	0.01	126 ± 23	18%
	0.02	97 ± 28	29%
	0.036	112 ± 17	15%
	0.36	105 ± 17	16%

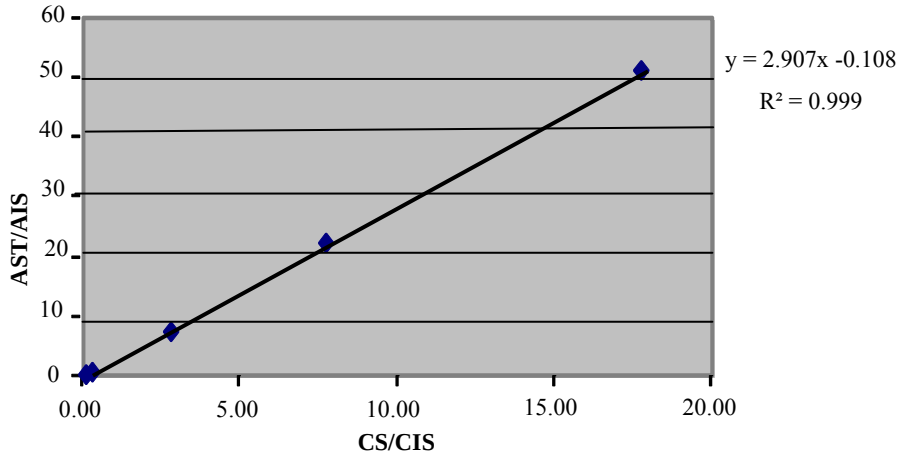

Fig. 3 Calibration curve of nicotine.

The limit of quantification (LOQ) was assessed as the minimum concentration of the nicotine that can be quantified with acceptable accuracy and precision. For this purpose, two recovery tests were performed at 0.01 and 0.02 mg kg^{-1} levels, for both matrices. Table 2 showed the recovery results and it is possible to establish the level of 0.01 mg kg^{-1} as the LOQ value for both fresh and dried mushrooms.

This value is consistent with the LOQ reported from Cavalieri et al. [18] for fresh mushrooms while for dried mushrooms we obtained a LOQ value lower than of 0.06 mg kg^{-1}[18].

The studied method is easy to apply for the analyses of more samples simultaneous and as far it was used to analyze sample of mushrooms from local market of Rome.

Seven samples of dried mushroom were collected from supermarkets. The studied mushrooms come from *Boletus edulis* and its related group (*Boletus Areus, Boletus Pinicola, Boletus Reticulatos*). The levels of Nicotine were ranged from 0.02 mg kg^{-1} to 0.30 mg kg^{-1} dried weight basis, corresponding to 0.01-0.03 mg kg^{-1} fresh weight basis as reported in Table 3.

A total ion chromatogram (TIC) of a dried mushroom sample and a TIC corresponding Nicotine standard solution are shown in Fig. 4.

Five fresh mushroom (*agaricus bisporus*) samples were collected from market and supermarket and no Nicotine level above 0.01 mg kg^{-1} (LOQ) was detected as shown in Table 3.

From the results of this study, the nicotine levels for all analyzed samples are below the EU MRLs of 0.04 mg kg^{-1} on fresh weight basis and no evidence of risk for consumers can be observed.

Table 3 Nicotine levels on dried and fresh mushrooms from Roman market.

Sample ID	Sample	Nicotine level on dried weight basis (mg kg^{-1})	Nicotine level on fresh weight basis (mg kg^{-1})
D1	Died mushrooms (*Boletus edulis* and related group)	0.30	0.03
D2	Died mushrooms (*Boletus edulis* and related group)	0.06	0.01
D3	Died mushrooms (*Boletus edulis* and related group)	0.02	0.002
D4	Dried mushrooms (*Boletus edulis* and related group)	0.14	0.02
D5	Dried mushrooms (*Boletus edulis* and related group)	0.25	0.03
D6	Dried mushrooms (*Boletus edulis* and related group)	0.21	0.02
D7	Dried mushrooms (*Boletus edulis* and related group)	0.10	0.01
F1	Fresh mushrooms (*agaricus bisporus*)		< 0.01
F2	Fresh mushrooms (*agaricus bisporus*)		< 0.01
F3	Fresh mushrooms (*agaricus bisporus*)		< 0.01
F4	Fresh mushrooms (*agaricus bisporus*)		< 0.01
F5	Fresh mushrooms (*agaricus bisporus*)		< 0.01

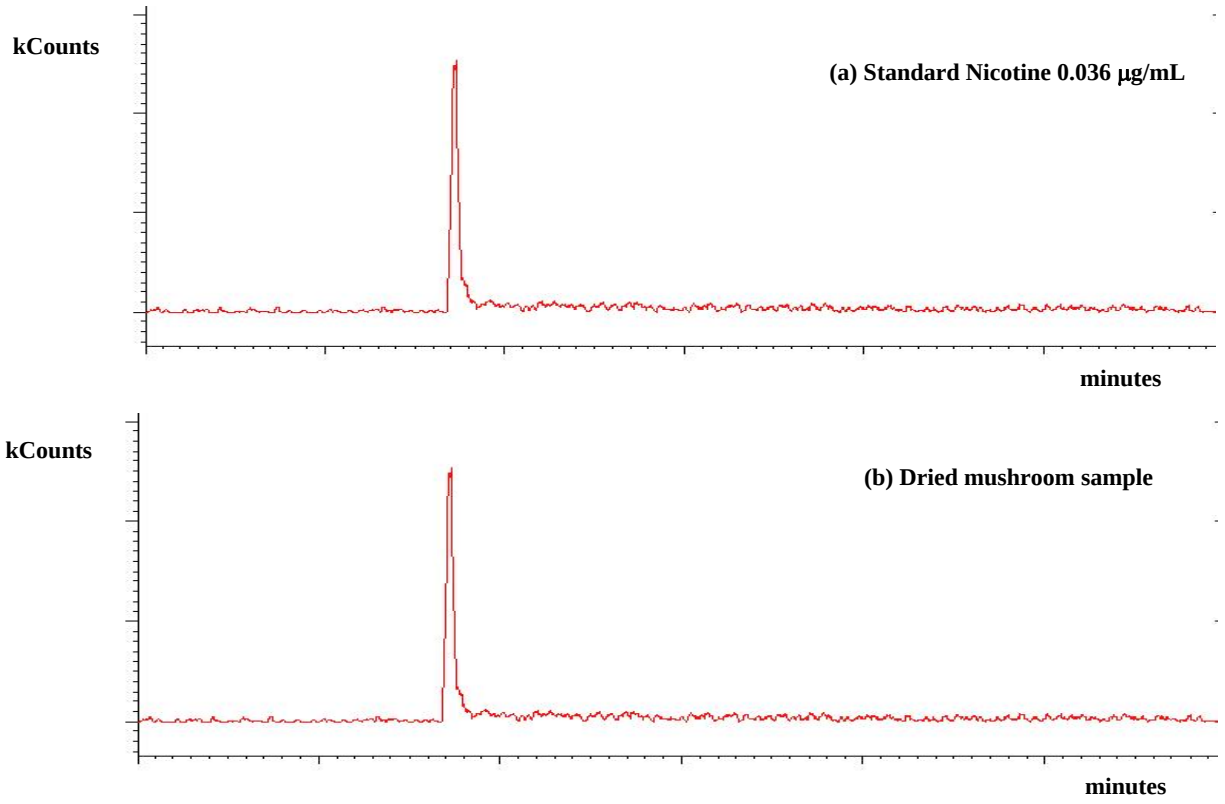

Fig. 4 A total ion chromatograms (TIC) obtained by injection of: (a) matrix-matched standard solution of Nicotine at 0.036 µg/mL and Nicotine d3 0.10 µg/mL and (b) dried mushrooms sample added with 100 µg/mL of a Nicotine d3 solution at 10 µg/mL.

References

[1] C.D.S. Tomlin, Pesticide Manual, 15th ed., British Crop Production Council (BCP), 2009, pp. 816-817.

[2] European Food Safety Agency (EFSA), Potential risks for public health due to the presence of nicotine in wild mushrooms, The EFSA Journal RN 286 (2009) 1-47.

[3] European Parliament, Regulation (EC) No 396/2005 of the European Parliament and of the Council of 23 February 2005 on maximum residue levels of pesticides in or on food and feed of plant and animal origin and amending Council Directive 91/414/EEC, Official Journal of the European Union L 70 (2005) 1-16.

[4] Commission Regulation (EU) No 765/2010 of 25 August 2010 amending Annexes II and III to Regulation (EC) No 396/2005 of the European Parliament and of the Council as regards maximum residue levels for chlorthalonil, clothianidin, difenoconazole, fenhexamid, flubendiamide, nicotine, spirotetramat, thiacloprid and thiamethoxam in or on certain product, Official Journal of the European Union L 226 (2010) 1-37.

[5] S.J. Sheen, Detection of Nicotine in foods and plants materials, J. of Food Science 53 (1988) 1572-1573.

[6] B. Siegmund, E. Leitner, W. Pfannhauser, Development of a simple sample preparation technique for gas chromatography-mass spectrometry determination of nicotine in edible nighshades (Solanacee), J. of Chromatography 840 (1999) 249-260.

[7] B. Siegmund, E. Leitner, W. Pfannhauser, Determination of the Nicotine content of various edible Nightshades (Solanaceae) and their products and estimation of the associated dietary Nicotine intake, J. Food Chem. 47 (1999) 3113-3120.

[8] C.O. Dasenbrock, L.A. Ciolino, C.L. Hatfield, D.S. Jackson, The determination of nicotine and sulphate in supermarket ground beef adulterated with black leaf 40, J. Forensic Sci. 50 (2005) 1134-1140.

[9] R.A. Davis, M.F. Stiles, J.D. de Bethizy, J.H. Reynolds, Dietary nicotine: a source of urinary cotinine, Food Chem. Toxicol. 29 (1991) 821-827.

[10] E.F. Domino, E. Hornbach, T. Demana, Relevance of nicotine content of common vegetables to the identification of passive tobacco smokers, Med. Sci. Res. 21 (1993) 571-572.

[11] A. Castro, N. Monji, Dietary nicotine and its significance in studies on tobacco smoking, Biomed Arch. 2 (1986) 91-97.

[12] T.R. Gray, D.M Shakleya, M.A. Huestis, Quantification of nicotine, cotinine, trans-3'-hydroxycotinine, nornicotine and norcotinine in human mecomium by liquid chromatography/tandem mass spectrometry, J. of Chromatography B 863 (2008) 107-114.

[13] M. Pellegrini, E. Marchei, S. Rossi, F. Vagnarelli, A. Durgbanishi, O. Garcia-Algar, O. Vall, S. Pichini, Liquid chromatography/electrospray ionization tandem mass spectrometry assay for the determination of nicotine and metabolites, caffeine and arecoline in breast milk, Rapid Commun. Mass Spectrom. 21 (2007) 2693-2703.

[14] S. Pichini, M. Pellegrini, R. Pacifici, E. Marchei, J. Murillo, C. Puig, O. Vall, O. Garcia-Algar, Quantification of arcoline (Areca nut alkaloid) in neonatal biological matrices by high-performance liquid chromatography/electrospray quadrupole mass spectrometry, Rapid Commun, Mass Spectrom. 17 (2003) 1958-1964.

[15] T.R. Gray, D.M. Shakleya, M.A. Huestis, A liquid chromatography tandem mass spectrometry method for the simultaneous quantification of 20 drugs of abuse and metabolites in human mecomium, Anal. Bioanal. Chem. 393 (2009) 1977-1990.

[16] Y. Zuo, L. Zhang, J. Wu, J. W. Frits, S. Medeiros, C. Rego, Ultrasonic extraction and capillary gas chromatography determination of nicotine in pharmaceutical formulations, Anal. Chim. Acta 526 (2004) 35-39.

[17] A.K. Dash, S.T. Wong, Liquid chromatographic method for the determination of nicotine in pharmaceutical formulations, J. of Chromaotgraphy A 749 (1996) 81-85.

[18] C. Cavalieri, L. Bolzoni, M. Bandini, Nicotine determination in mushrooms by LC/MS/MS with preliminary studies on the impact of drying on nicotine formation, Food Additives and Contaminants 27 (2010) 437-477.

[19] C.O. Dasenbrock, L.A. Ciolino, C.L. Hatfield, D.S. Jackson, The determination of Nicotine and sulphate in supermarket ground beef adulterated with black leaf 40, J. Forensic Sci. 50 (2005) 1134-1140.

[20] M. Anastassiades, S.J. Lehotay, D. Stajnbaher, F.J. Schenck, Fast and Easy Multiresidue method employing acetonitrile extraction/partitioning and dispersive solid-phase extraction for the determination of pesticide residue in produce, J. of AOAC International 86 (2003) 412-431.

[21] P. Paya, M. Anastassiades, D. Mack, I. Sigalova, B. Tasdelen, J. Oliva, A. Barba, Analysis of pesticide residues using the quick easy Cheap Effective Rugged and safe (QuEChERS) pesticide multiresidue method in combination with gas and liquid chromatography and tandem mass spectrometry detection, Anal. Bioanal. Chem. 389 (2007) 1697-1714.

[22] Health and Consumer Protection Directorate General (DG SANCO), Guidance document on method validation and quality control procedures for pesticide residue analysis in food and feed, Document No. SANCO/10684/2009, 01/01/2010, available online at: http://ec.europa.eu/food/plant/protection/resources/qualcontrol_en.pdf. (accessed at March 2011)