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Research Paper / Makale

Quantitative Determination of Nicotine in Smokeless Tobacco (Maras Powder) By Liquid Chromatography-Tandem Mass Spectrometry with Electrospray Ionization

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Abstract: A sensitive, fast, low-cost and simple determination method was developed by using liquid chromatography-electrospray ionization tandem mass spectrometry for the determination of the nicotine in smokeless tobacco (Maras powder). For chromatographic separation, a Hypersil Gold (2.1 mm x 200 mm, 1.9 µm) column at 30°C with 0.2% acetic acid in water and acetonitrile mobile phases at a flow rate of 0.3 $mLmin^{-1}$ were used. Analysis time was 10 minutes. Tandem mass spectrometric detection was used in positive electrospray ionization in SRM mode. The developed method was linear from 1 to 10 mgL^{-1} for nicotine and the linearity coefficient in this range was R^2=0.999. The LOD and LOQ was determined as 0.057 mgL^{-1} and 0.191 mgL^{-1}, respectively. Additionally, the developed method was successfully applied to determine of nicotine in Maras powder samples.

Keywords: Nicotine, smokeless tobacco, maras powder, liquid chromatography-mass spectrometry, electrospray ionization

Dumansız Tütünde (Maraş otu) Nikotinin Elektrosprey Iyonizasyon Sıvı Kromatografisi-Tandem Kütle Spektrometrisi ile Kantitatif Tayini

Öz: Dumansız tütünde (Maraş otu) nikotin tayini için sıvı kromatografi-elektrosprey iyonizasyon tandem kütle spektrometrisi kullanılarak hassas, hızlı, düşük maliyetli ve basit bir tayin yöntemi geliştirildi. Kromatografik ayırma için, 30°C'de bir Hypersil Gold (2.1 mm x 200 mm, 1.9 um) kolonu ile 0.3 $mLdak^{-1}$ akış hızında %0.2 asetik asitli su-asetonitril mobil fazları kullanıldı. Analiz süresi 10 dakikaydı. Tandem kütle spektrometrik tayininde SRM modunda pozitif elektrosprey iyonizasyon kullanıldı. Geliştirilen yöntem, nikotin için 1 ila 10 mgL^{-1} arasında doğrusaldı ve bu aralıkta doğrusallık katsayısı R^2=0.999 idi. LOD ve LOQ sırasıyla 0.057 mgL^{-1} ve 0.191 mgL^{-1} olarak belirlendi. Ayrıca geliştirilen yöntem Maraş otu örneklerinde nikotin tayini için başarıyla uygulandı.

Anahtar Kelimeler: Nikotin, dumansız tütün, maraş otu, sıvı kromatografi-kütle spektrometrisi, elektrosprey iyonizasyon

1. Introduction

Smoking and the use of different tobacco products, which are one of the leading health problems in our country, are actually one of the most important health problems in the world. [1]. Tobacco is one of the bad habits for people around the world. It has many varieties grown in different countries and marketed under different names such as cigarettes, spiral tobacco products, pipe, cigar, hookah, snuff and chewing tobacco. Tobacco can also be used in its smokeless forms, of which there are different types around the world. Smokeless tobacco preferred by people to reduce smoking or quit

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smoking. Using smokeless tobacco is increasing day by day because of its low price and easy availability. Smokeless tobacco products have different names in the countries like Southeast Asia, India (Gutkin A), Bangladesh (bits) and Turkey (Maras powder) in the world.

In our country, it is known as Maras powder around Kahramanmaraş and Gaziantep. It obtained from tobacco leaf and used by taking it into the oral mucosa [2]. Maras powder is obtained from the leaves of the Nicotiana Rustica plant. After being powdered, they are mixed with oak or walnut ash. They kept in copper cauldrons by slightly moistened [3]. The mixing ratio between mad tobacco plant and ash in the mixture is approximately half. The ash added during the production of Maras powder. It makes environment alkaline so alkaloids absorbed better by oral mucosa [4,5]. This mixture is consumed by taking an average teaspoon of powder in the palm and sometimes wrapped in cigarette paper and placed between the lower lip (sometimes upper lip, sometimes cheek mucosa) and the chin. It is kept in the mouth for 5-10 minutes and then spit out. Depending on the level of habit, this process can be repeated throughout the day, and some people may even sleep with Maras powder in their mouth.

Maras powder can be used with cigarettes or can be used alone. In both uses, the aim is to reduce or completely quit smoking. Nowadays, the idea that smoking can be quitted more easily by using smokeless tobacco. People thinks that Maras powder is less dangerous than smoking. That’s why, uses of Maras powder has increased. Besides, smokeless tobacco has commonly use in workplaces, vehicles and areas where smoking is prohibited. Smokers usually don’t want to smoke again after they start using Maras powder due to its high nicotine content.

It has been reported that there is no significant difference between mad tobacco (Nicotina Rustika L) plant from which Maras powder is made and cigarette tobacco (Nicotina Tobacum L) in terms of the alkaloid composition they contain. Nicotiana Rustica is a rainforest plant belonging to the Solanaceae family. It is known as Aztec tobacco [6] or strong tobacco [7]. Nicotiana tabacum (common tobacco) is containing nicotine nine times more than common Nicotiana species [8]. More specifically, Nicotiana Rustica leaves have a nicotine content as high as 9%. Nicotiana tabacum leaves contain about 1% to 3% nicotine [9].

It is thought that Maras powder is not as harmful as cigarettes. However, when we look at the literature, there are many studies that reveal the harmful effects of products known as "smokeless tobacco", although not as much as cigarettes [10-12].It has been reported that Maras powder is as least as harmful to the cardiovascular system in those who use Maras powder [13,14]. It has also been shown that Maras powder is unreliable and causes many systemic diseases such as genetic damages and has negative effects on cardiovascular risks such as smoking. These harmful effects are seen in the use of tobacco as well as in chewing in the mouth [15- 17]. It has been reported that chronic stimulation of lymphoid tissues in the oral mucosa may be associated with the increased rate of gingivitis, leukoplakia and oral cancer due to ingestion of Maras powder. It has also been reported to have a stronger addiction potential than smoking, due to its higher nicotine concentration and long-term use. It is known that the consumption of Maras powder increases oxidative stress and thus accelerates the atherosclerotic process [18,19]. The use of Maras powder has been reported to accelerate atherosclerosis by increasing oxidative stress, as well as having negative effects on many tissues and organ systems [20- 22]. As a result, it should not be forgotten that the systemic effects of Maras powder use are as harmful to the cardiovascular system as smoking. Studies are needed to reveal whether Maras powder has an effect on other systems in the body [23].

In a study, it was found that the use of Maraş powder acutely increased heart rate, systolic blood pressure and diastolic blood pressure [24]. The effects of Maraş powder on respiratory function were investigated [25]. As a result of the examinations, it was observed that Maras powder, a type

of smokeless tobacco, didn't effect the respiratory tract as it was not inhaled during its use. However, it should not be forgotten that the damages to the cardiovascular system are at least as much as smoking. Studies have been conducted to examine the depression and anxiety states of people who use cigarettes and Maras powder and those who do not consume tobacco products. [26]. When the data obtained at the end of the study were evaluated, it was shown that depression and anxiety scores of people who used Maras powder and cigarettes were significantly higher than those who did not use tobacco products. Laurian Vlase calculated nicotine in tobacco products using LC-MS/MS in a study they conducted [27].

When the literature is examined, the effects of Maras powder on health are generally examined. Studies on the quantitative determination of Maras powder are scarcely. In the study, a method for the quantitative determination of Maras powder was developed and successfully applied to the samples.

2. Experimental Methods

2.1. Materials

2.1.1. Reagents

Nicotine and acetic acid were bought from Sigma Aldrich (Germany). Acetonitrile 98 % was bought from Merck (Darmstadt, Germany). All chemicals were used without further purification as they were of analytical grade.

2.1.2. Sample Preparation

200 mg of Maras powder samples were weighed and extracted with 10 mL of solvent (6 mL of 1% acetic acid water and 4 mL of acetonitrile) with the aid of an ultrasonic bath. Obtained extract was filtered through a 0.45 μm syringe filter. It was diluted in another tube at a ratio of 1/200. The diluted extract was given directly to LC-MS/MS (liquid chromatography-tandem mass spectrometer) for analysis.

2.2. Chromatographic Conditions

The study was carried out using TSQ Quantum Access Max model Thermo Scientific brand LC-MS/MS device. For liquid chromatography-tandem mass spectrometric analysis, LC-MS/MS device was used at Hitit University Scientific-Technical Application and Research Center (HÜBTUAM) and analyzes were made there.

Table 1. Sample information and chromatographic conditions

Name of the Product	Maras Powder		
Product Material	Solid		
Solvent Program	Minute	% A (0.2% acetic acid-water)	% B (acetonitrile)
	0	25	75
	10	25	75
Solvent Flow Rate	0.3 mL/min		
Column Oven Temperature	30 ^{o}C		
Column Features	Hypersil Gold 2.1x200 mm 1.9 μm column		
Injection Volume	20 μL		
Analysis Time	10 min		
Retention Time	1.5 min		

Chromatographic analysis was performed using the Thermo Scientific Dionex Ultimate 3000 LC pump and autosampler which has a 20 mL loop. Analytical column: Hypersil Gold 2.1x200 mm 1.9 µm particle size, (Thermo Fisher, USA). The mobile phase was A 0.2 % acetic acid-water mixture and the mobile phase B was acetonitrile. The flow rate was 0.3 mL/min. Injection volume was 20 µL. Analysis time was 10 minutes. Sample information and chromatographic conditions are summarized in Table 1.

2.3. MS/MS Analysis Conditions

The mass analyzer operated with an ESI source positive ion mode. Cappilary temperature:300 ^{o}C, vaporizer temperature:300 ^{o}C, sheat gas pressure (Arb):35, aux gas pressure (Arb): 20, sprey voltage (V) positive polarity:4000, sprey voltage (V), negative polarity:2500, discharge current (µA):4,0 and collision gas (Ar):1.5 m Torr. The precursor to product ion transitions of m/z 162.92>(117.20/130.10) were used for nicotine quantification. MS/MS device conditions are given in Table 2.

Table 2. MS/MS device conditions

Ion Source (Polarity)	ESI (+)
Scan Type	SRM
Cappilary Temprature	300 ^{o}C
Vaporizer Temprature	300 ^{o}C
Sheat Gas Pressure (Arbitrary Units)	35
Aux Gas Pressure (Arbitrary Units)	10
Sprey Voltage (V) (Positive Polarity)	4000
Sprey Voltage (V) (Negative Polarity)	2500
Discharge Current (µa)	4.0
Collision Gas (Ar):	1.5 mTorr
Scan Width:	0.002 Da

3. Results and Discussion

3.1. Standard Solutions and Calibration Curve

The stock solution of the nicotine standard was prepared in a ratio of 6: 4 (1% acetic acid water and acetonitrile) to 10 ppm. To draw the calibration curve, standard solutions (1 ppm, 2 ppm, 4 ppm, 6 ppm, 8 ppm) were prepared from 10 ppm nicotine stock solution and their chromatograms were obtained. The calibration curve was drawn using the obtained chromatograms. The method was calibrated with six standard solutions in the range of 1-10 ppm depending on the expected concentrations of the samples. The analytical method is calibrated before each measurement series and control samples are measured and checked. The calibration curve of nicotine standards is given in Figure 1.

The mass spectrometer was operated in selected reaction monitoring (SRM) mode. The identification of ions was done in carried out using LC coupled to ESI-MS/MS in positive ionization mode. MS/MS conditions for nicotine were optimized for nicotine determination.

Table 3. SRM ions and energies of the nicotine standard

Parent	Product	Collision Energy	Polarity	Name
162.92	117.20	28.00	+	Nicotine
	130.10	20.00	+	

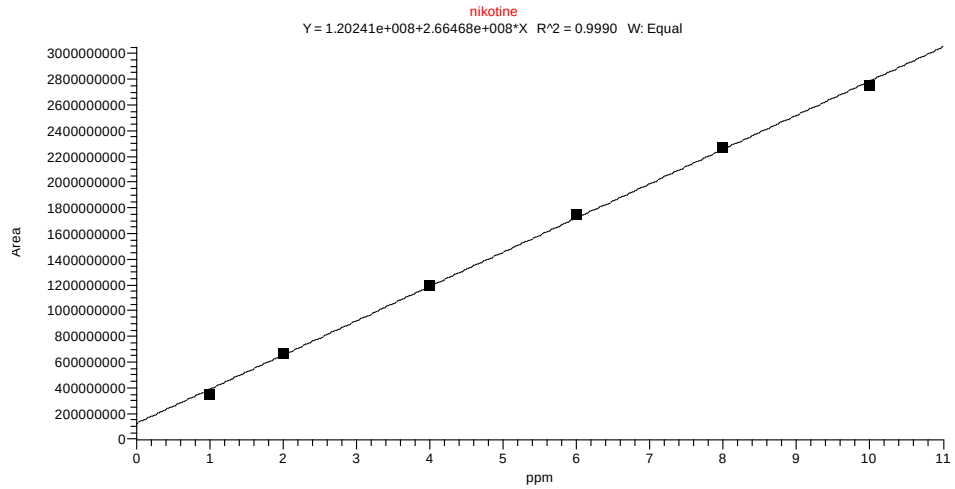

Figure 1. Calibration curve of nicotine standard

The collision energy was optimized for the formation of nicotine $(M^{+}H)^{+}$. The product ion spectra were taken at different collision energies and the ion characterized under positive ion ESI conditions. Table 2 shows the optimization conditions of the MS/MS device. In order to identify the analytes, the method was optimized in the device. Molecular ion (MH^{+}) of nicotine was found as m/z 162.92 and daughter ion of nicotine as m/z 117.20.and 130.10. Chromatogram and mass spectrum of the 2 ppm nicotine standard is given Figure 2. Information on SRM ions for nicotine and energies of the nicotine standard is given in Table 3.

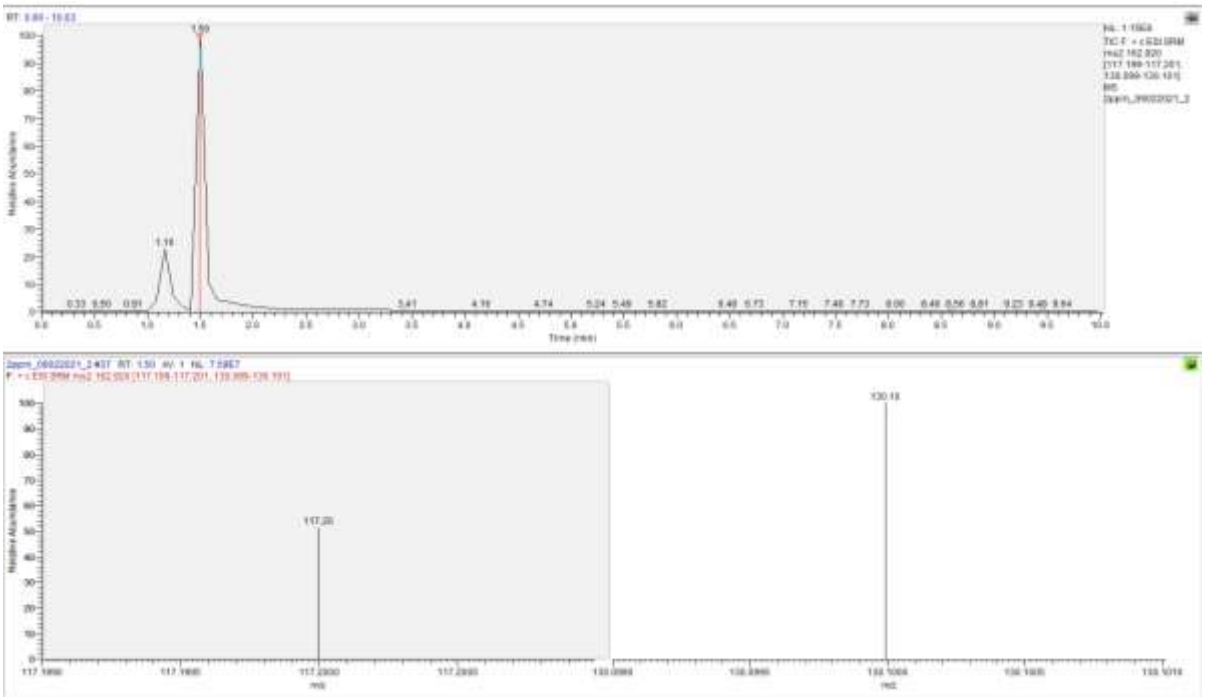

Figure 2. Chromatogram and mass spectrum of 2 ppm nicotine standard. Experimental conditions: mobile phase (gradient elution; A pump: 0.2 % acetic acid-water mixture (for 10 min); B pump: acetonitrile. (for 10 min); flow rate: 0.3 mL/min; injection volume: 20 µL; Hypersil Gold (2.1x200 mm 1.9 µm column)

For the nicotine determination, chromatographic conditions were chosen after evaluating several different mobile phase and column performance. Chromatographic test conditions for nicotine determination are given in Table 1.

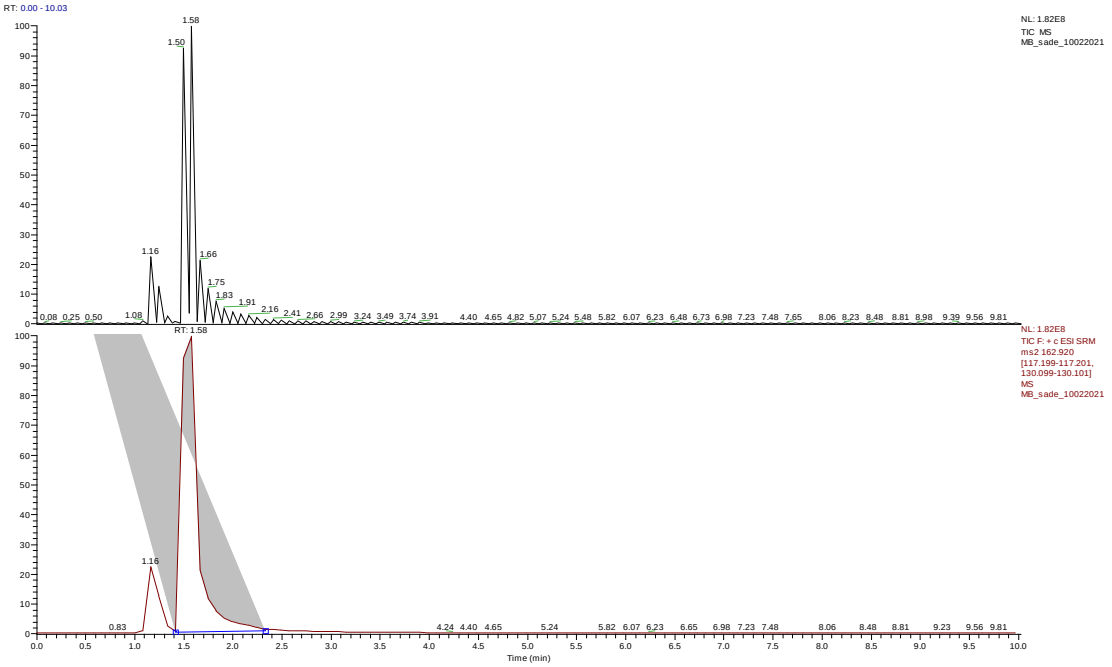

Figure 3. LC-MS/MS chromatogram of Maras powder sample. Chromatographic conditions: mobile phase (gradient elution; A pump: 0.2 % acetic acid-water mixture (for 10 min); B pump: acetonitrile. (for 10 min); flow rate: 0.3 mL/min; injection volume: 20 µL; Hypersil Gold (2.1x200 mm 1.9 µm column)

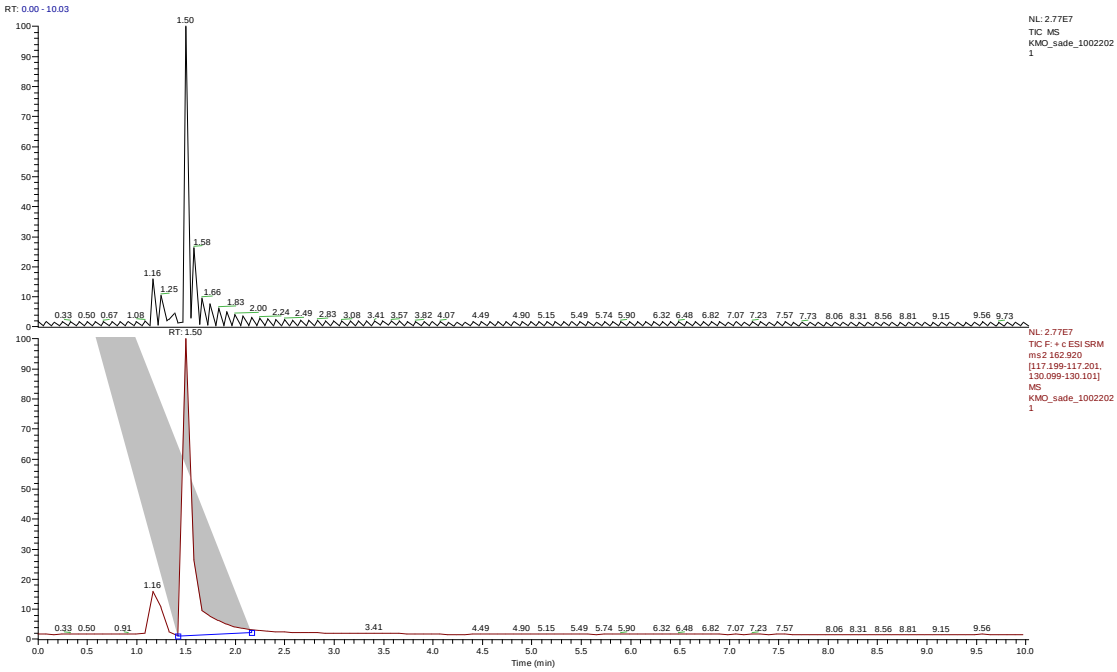

Figure 4. LC-MS/MS chromatogram of ashy Maras powder sample. Chromatographic conditions: mobile phase (gradient elution; A pump: 0.2 % acetic acid-water mixture (for 10 min); B pump: acetonitrile. (for 10 min); flow rate: 0.3 mL/min; injection volume: 20 µL; Hypersil Gold (2.1x200 mm 1.9 µm column)

Due to the specificity offered by SRM transitions, a relatively short gradient normal phase chromatography method has been developed. Optimum separation with beautiful peak shapes and good sensitivity for nicotine was achieved with a Hypersil Gold (2.1x200 mm 1.9 µm particle size column) and a mobile phase containing 0.2% acetic acid-water and acetonitrile. Chromatographic

elution of nicotine was achieved in 5 minutes, and the total chromatographic run was 10 minutes. The measurement was based on the most prominent SRM transition. Generally, for LC-MS/MS experiments, MS/MS provides sufficient precision to measure analytes precisely.

From the chromatograms exhibited in Figure 3 and Figure 4, the nicotine amounts (mg nicotine/kg Maras powder) in Maras powder and ashy Maras powder were calculated as 6635.60 ppm and 78062.22 ppm. Nicotine values calculated from the obtained chromatograms are given in Table 4.

Table 4. Nicotine results in Maras powder and ashy Maras powder

	mg nicotine/kg Maras powder (ppm)	g nicotine/100 g Maras powder (%)
Maras powder	77322.77	7.73
ashy Maras powder	6551.25	0.65

3.2. Method Validation

Limit of detection and limit of quantification were calculated by injection of nicotine samples. Quantification limit and detection limit calculated as 0.191 mg L^{-1} and 0.057 mg L^{-1}. Standard deviation and relative standard deviation were calculated.

The linearity of the detector response for nicotine was evaluated by injecting a total of six calibration solutions at concentration levels 1, 2, 4, 6, 8 and 10 mg L^{-1} in LC-MS/MS. Linear relationships between the rates of peak area signals and the corresponding concentrations were observed.

The accuracy and precision of the method were examined. These were obtained with recovery studies carried out by samples spiked at levels of 2 and 5 mg L^{-1} following two replications. The reproducibility of the method was evaluated with the relative standard deviation (RSD,%) with the measurements made as a result of the recovery analysis. LOD, LOQ values and calibration curve data of nicotine standard are given in Table 5.

Table 5. LOD, LOQ values and calibration curve data of nicotine standard

	Nicotine
LOD (limit of detection)	0.057
LOQ (limit of quantification)	0.191
R^2 (regression coefficient)	0.999
Equation	Y = 1.20241e+008+2.66468e+008*X
SD (standard deviation)	0.019
RSD (relative standard deviation)	2.882

Table 6. Results of standard addition studies (ppm) (M.P.; Maras powder, A.M.P.; ashy Maras Powder)

	The result from the device	Theoretically expected result	Calculation of the theoretical result	Recovery %
M.P. raw result	7.732			
M.P._2ppm spike	8.185	8.186	(7.732x0.8 + 2ppm)	99.955
M.P._5ppm spike	8.907	8.866	(7.732x0.5 + 5ppm)	100.810
A.M.P. _raw result	0.655			
A.M.P._2ppm spike	2.521	2.524	(0.655x0.8 + 2ppm)	99.850
A.M.P._5ppm spike	5.232	5.328	(0.655x0.5 + 5ppm)	98.090

3.3. Standard Attachment Method

For the standard addition work, 800 µL of the diluted extract was taken and 200 µL of 10 ppm nicotine standard was added. Again for the standard addition work, 500 µL of the diluted extract was taken and 500 µL of 10 ppm nicotine standard was added. The prepared samples were analyzed with LC-MS/MS. Results of standard addition studies are given Table 6.

4. Conclusions

For the direct determination of nicotine in Maras powder and ashy Maras powder, a method based on liquid chromatography-tandem mass spectrometry (LC-MS/MS) using electrospray ionization in positive ion mode has been developed. With the developed method, the amount of nicotine in Maras powder and ashy Maras powder was analyzed over for ten minutes. The nicotine amounts (mg nicotine/kg Maras powder) in Maras powder and ashy Maras powder were calculated as 77322.77 ppm and 6551.25 ppm. The graph of the calibration curve obtained from the chromatograms of the standard solutions was found to be Y = 1.20241e + 008 + 2.66468e + 008 * X. From the calibration curve, the regression constant value was calculated as 0.999. Calibration was linear over the concentration ranges studied. Limit of detection and limit of quantitation were calculated as 0.057 and 0.191. Using the standard addition method, the accuracy of the method was evaluated. It was calculated based on the sample recovery. When the recovery results were evaluated, it was in the range of 98-101%, which means a high level of accuracy for the method. Developed method has many advantages such as be fast, sensitivity, selectivity and reproducible results. When the studies on nicotine in smokeless tobacco (Maras powder) in the literature are examined, the effects of nicotine on health were examined. Considering that there is no study using LC-MS/MS for the quantitative determination of nicotine in smokeless tobacco (Maraş powder), a new study has been added to the literature with the developed method.

Author Contributions

RA designed the study. RA made the sample analyzable. Services were purchased from the Hitit University Scientific-Technical Application and Research Center (HÜBTUAM) for the analyses. RA wrote the article by performing the experimental studies and necessary calculations.

The author has read and approved the final text.

Competing Interests

The authors declare that they have no competing interests.

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