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Determination of Nicotine in Cigarette Tobacco Smuggled to Brazil by Modified QuEChERS Methodology

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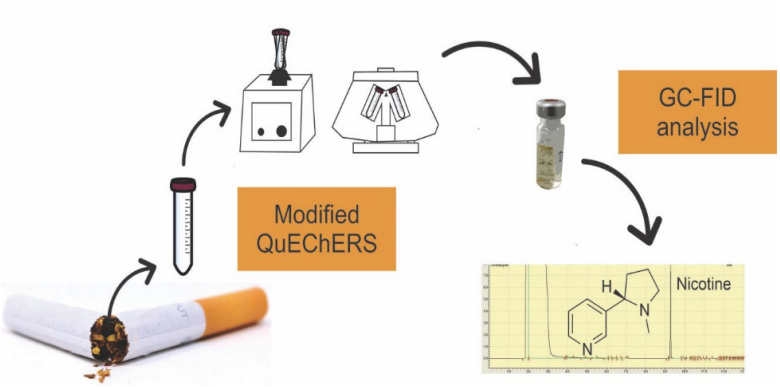
The study of cigarette authenticity in Brazil is important due to increasing consumption of contraband cigarettes. Nicotine concentration is an important parameter reflecting the quality of tobacco used in the production of these cigarettes. Simple methods for this determination, which produce reduced waste, are environmentally and industrially important. The nicotine concentration of smuggled cigarette tobacco was determined by the QuEChERS method, requiring some modifications, such as decreasing the volume of the extractor solvent, changes in pH, and removal of the sample hydration step. Quantification was performed by gas chromatography using a flame ionization detector. The Doehlert matrix design was used to optimize the method. The extraction recoveries ranged from 97.5% to 99.6%, with relative standard deviation (RSD) ≤ 2.5% and limits of detection and quantification of 0.6 mg L^{-1} and 2.5 mg L^{-1}, respectively. The method was sensitive and accurate for the detection and quantification of nicotine. The nicotine concentration in contraband cigarettes was found to be lower than that observed in legal cigarettes. The method was successfully applied to real samples of smuggled and legal cigarettes, providing a robust method for routine analysis and proving the need for more studies on quality control of smuggled cigarettes in Brazil.

Graphical abstract



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1. Introduction

The World Health Organization (WHO) estimates that 9–11% of cigarettes consumed worldwide are contraband [1]. In Brazil, the average of smuggled cigarettes in the market reached 57% in 2018 [2]. In addition, there was a 27% increase in seizures compared to 2017, with 276 million packages seized [3]. The lack of quality control of contraband cigarettes can be aggravating for public health. Cigarettes smuggled into Brazil have elevated concentration of heavy metals compared to legal cigarettes, with values up to eleven times higher for chromium (Cr), nickel (Ni), cadmium (Cd) and lead (Pb) [4]. Another study also showed the presence of fungi, insect parts, grass, and mites in smuggled cigarettes [5]. The tobacco leaf used in the production of cigarettes has more than 4000 chemical substances, with 0.3–5% corresponding to nicotine. Nicotine is a psychoactive drug, nitrogenous alkaloid, which acts directly in the central nervous system by binding to nicotinic neural receptors of acetylcholine, favoring the release of neurotransmitters as dopamine, serotonin, noradrenaline, and therefore, responsible for tobacco addiction [6].

Nicotine can exist in the monoprotonated, deprotonated, and free-base forms; the latter form is absorbed by the epithelial tissues of the body, and such forms depend on the pH of the matrix. Thus, in the tobacco production process, ammonia is added to the tobacco blend to increase the pH, and consequently, the amount of bioavailable nicotine [7].

The levels of nicotine in tobacco leaves directly depend on factors like production practices, climate, and soil fertility. Since nicotine is synthesized in the roots and transported to the leaves, its concentration influences the plant development. [8,9] Therefore, the nicotine content evaluation is an important parameter for the tobacco quality in cigarettes [10]. For cigarettes with intentionally reduced amounts of nicotine, it is necessary for the plants to be genetically modified; it takes 8–12 years of research for a certain variety to be commercialized [8,11].

Two methods are found for the extraction of nicotine from cigarette tobacco; one of them is recommended by the Cooperation Center for Scientific Research on Smoking (CORESTA). Method n.85 [12] is carried out with a continuous flow analyzer by reacting the aqueous tobacco extract with sodium citrate and cyanogen chloride. To perform this method, separate spaces are needed within the laboratory because of the formation of cyanogen chloride, which is considered a toxic gas (risk class 2). In addition, the method recommended by CORESTA uses approximately ten different reagents, generating a large amount of waste. Another standardized method (Standard Operating Procedure (SOP) 04, 2014) for extracting nicotine is proposed by the World Health Organization (WHO), which performs extraction through the use of large amounts of highly toxic solvents, such as hexane [13].

One option for nicotine extraction is to use a modified QuEChERS method. The method QuEChERS was introduced in 2003 for the extraction of pesticides in fruits and vegetables, having its name originating from the abbreviation of its main characteristics: **Qu**ick, **E**asy, **Ch**eap, **E**ffective, **R**ugged, and **S**afe when compared to other methods. QuEChERS method features high recoveries, accurate results, reduced analysis time and reagents, and inexpensive equipment for the extraction process [14], obtaining like this widespread application for several matrices and different analytes.

The extraction of the analyte occurs during the agitation and centrifugation processes, and the cleaning of the extract take place in a simple and closed system, minimizing contact with the analyst.

The addition of reagents to change the pH, use of ultrasound in the extraction step, filtration through PTFE membranes (0.45 um), and evaporation of solvent for concentration are examples of modifications employed by other researchers, aiming to improve the extraction of nicotine in samples like black tea, fish, and mushroom tissue, because nicotine is found in trace levels in these matrices. [15–18] However, for nicotine extraction in cigarette tobacco, only a few studies [19] have reportedly employed the QuEChERS method.

Herein we report the results of the modifications performed to optimize the QuEChERS method, such as changes in pH using K_2CO_3 solution that allows the use of a smaller volume to reach the desired pH, without damaging the chromatographic system. In the extraction process, the decrease in the volume of the extraction solvent and the exclusion of the hydration step highlights the importance of these modifications. Consequently, the analysis time is optimized, less amounts of solvent and other reagents are used, and acceptable recoveries were obtained, as determined by gas chromatography using a flame ionization detector (GC-FID).

The aim of this study was to determine the concentration of nicotine in cigarette tobacco smuggled into Brazil, using the modified QuEChERS extraction method.

2. Results and Discussion

In the Doehlert matrix design, the variables at different levels were studied. Greater study intervals were considered for the most important variable, and a second-order model was directly obtained.

Analysis of the response surface shows that better recovery ranges for the total nicotine amount were obtained at basic pH, with the optimum pH being approximately 12. For the solvent quantity factor, the optimum volume was between 7 and 8 mL, shown by Fig. 1 and 2.

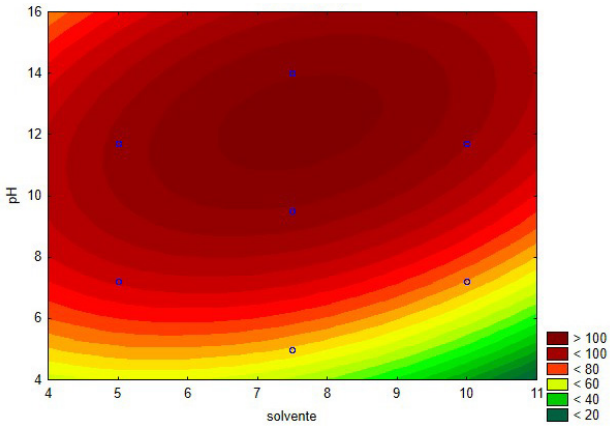

Fig. 1. Contour surface pH × amount of solvent for the extraction of nicotine.

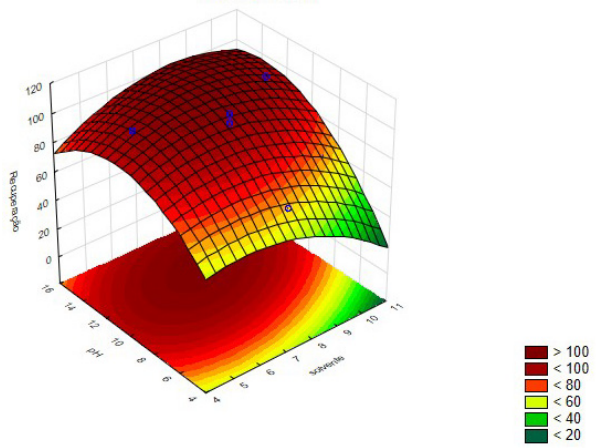

Fig. 2. Response surface pH × amount of solvent for the extraction of nicotine.The model suggests that the optimal pH and solvent volume were 12.4 and 7.6 mL, respectively. Experimentally, it was observed that at pH 12 and volume 7.6 mL, the recoveries obtained ranged from 97.5% to 99.6%.

The decrease in volume is an advantage of this method because the recommended methodologies use up to 40 mL of hexane. In previous studies for nicotine extraction using the QuEChERS methodology, the volumes of organic solvents used were between 9.5 mL and 15 mL [15–18]. The indicated volume is sufficient to involve all the sample material, allowing adequate extraction, and contributing to the reduction of generated waste and reagent savings for analysis of several samples.

Because of the solubility of nicotine in various types of solvents (polar and non-polar), acetonitrile was used as the extraction solvent as well as other studies using the QuEChERS method for nicotine extraction [16–18].

It is noteworthy that water was not used in the partitioning stage, owing to the excessive solubility of the coextractives, compared to extraction without adding water. The water in the extraction process makes sample pores more accessible to the extractor solvent, [14] increasing the efficiency of the extraction of trace analytes. Since nicotine concentration is not in trace levels, acetonitrile was used for extraction without adding water. A previous study with the tobacco matrix [20] showed the need of adding sorbents such as GBC (Graphitized Carbon Black) and C18 as well as low-temperature precipitation in the clean-up step, in order to obtain a clear extract for pesticide determination. In addition, BAO et al. [21] demonstrated that when water was added using the headspace methodology (HS-SPME/GC/MS), a suppression in the chromatographic peak was observed due to the high solubility of nicotine in water.

For nicotine to be absorbed by epithelial tissues, its structure needs to be in a deprotonated form, which occurs in a basic medium because it has values of pKa_1 = 3.12 and pKa_2 = 8.02 [7]. Thus, to change the pH to 12, K_2CO_3 solution was used. Some methods [13,16,18] use sodium hydroxide to raise the pH during the extraction process; however, since the quantification of nicotine is performed by gas chromatography, the frequent use of sodium hydroxide can damage the stationary phase of the capillary column of the chromatographic system [22], compromising the reliability of the results. Other studies used ammonia solution; however, a larger volume is needed to reach pH 12 compared to the K_2CO_3 solution (3.5 mL to 0.5 mL, respectively). Thus, the K_2CO_3 solution was chosen to increase the pH.

Validation extraction method

The calibration curve showed linearity between 2.5 and 1000 mg L^{-1} for GC-FID. The data of the analytical curves were subjected to a variance test, where a lack of adjustment (p-value = 0.993) was not observed, obtaining the coefficient of determination (R^2 = 0.986) and, with the intercept not statistically significant (p-value = 0.189) at the 5% level of significance. Therefore, the model is suitable and the linearity is validated.

The detection limit (LD) and quantification limit (LQ) were calculated to be 0.6 mg L^{-1} and 2.5 mg L^{-1}, respectively. Use of the CG-FID technique to determine nicotine has been reported, e.g., in fermented tobacco leaf extracts with an LQ of 5 mg L^{-1}, [23] since nicotine is not considered a trace-level analyte in tobacco samples and there is no legislation on the limit of nicotine concentration in cigarette tobacco, with only a determined concentration for primary smoke, the values of LQ and LD are considered adequate [24].

Recovery was evaluated in three concentrations: the lowest concentration of 0.06 mg g^{-1}, gave 97.5% recovery with a coefficient of variation (CV) of 2.5%; the intermediate concentration of 7.5 mg g^{-1} gave 99.6% recovery and 2.3% CV; and the highest concentration of 15 mg g^{-1} gave 98.9% recovery and 1.7% CV. Based on these recovery values, the method is considered acceptable according to AOAC [25] which determines the recovery interval for analytes not classified as a trace level of 97–103% recovery.

Thus, the change in pH, the reduction in solvent volume, and the removal of water in the extraction process contributed to the better performance of the method for nicotine extraction in cigarette tobacco. The proposed method is suitable, sensitive, and safe for the determination of nicotine in cigarette tobacco samples.

Application of the optimized method to real samples

Table 1 shows the concentrations of total nicotine presented in nine brands of smuggled cigarettes and two brands of legal cigarettes sold in Brazil.

Table 1. Nicotine concentration in smuggled (A-I) and legal (J and K) cigarettes (n = 3).

Brand	Concentration (mg/cigarette)
A	6.7 ± 0.1
B	5.5 ± 0.2
C	6.7 ± 0.4
D	6.0 ± 0.7
E	6.2 ± 0.0
F	5.8 ± 0.1
G	6.3 ± 0.2
H	6.1 ± 0.7
I	6.7 ± 0.2
J	10.7 ± 0.7
K	9.0 ± 0.8

Source: The authors.

The tobacco samples of cigarettes from the brands analyzed by the optimized method exhibited a difference in concentration of nicotine; smuggled cigarettes showed lower concentrations compared to legal cigarettes. However, the nicotine levels presented corroborate the nicotine

concentration of legal cigarettes in Brazil, previously described using other methods, as shown in Table 2.

Table 2. Nicotine concentration in cigarettes legal in other studies.

Reference	Nicotine concentration (mg g^{-1})	Site	Sample (cigarette)
This study	5.5 a 6.7	Brazil	Smuggled
This study	9.0 a 10.7	Brazil	Legal Commercial
INABA et al., 2013	13.7 a 17.2	Japan	Legal Commercial
WU; ASHLEY; WATSON, (2002)	8 a 22	Others countries	Legal Commercial
TAUJENIS; OLŠAUSKAITĖ; PADARAUSKAS, (2015)	14.3 a 16.1	Lithuania	Legal Commercial

Source: The authors.

The amount of nicotine in cigarettes can vary according to the type of mixture used in the manufacturing process [26]. Tang et al. [27] determined the concentration of nicotine in leaves of 51 tobacco samples, which underwent four types of curing and different classifications after curing, concluding that different cultivars and classifications have different concentrations of nicotine. In addition, some brands of contraband tobacco bring the addition of plant extracts to the blend as information on their packaging. A study by Da Silva et al. [5] highlighted the presence of grass in samples of smuggled cigarette tobacco. Thus, nicotine concentration can be directly linked to the quality of tobacco in cigarettes.

Although the smuggled cigarettes have lower nicotine concentration than legal cigarettes, the content is considered sufficient to cause dependence, as studies show that the absolute bioavailability of nicotine can reach 40%. Considering that the daily dose of nicotine must be less than 5 mg to avoid dependency, a cigarette to be less addictive must have an average concentration of 0.4–0.5 mg of nicotine; however, even with a smaller concentration of nicotine, these cigarettes contain carcinogens similar to cigarettes with higher concentration of nicotine, and are not considered safer [28,29]. The lack of quality of smuggled cigarette tobacco has increased the level of alert regarding the health of the population, as there has been an increase in migration from legal to illegal consumer market in recent years.

These consumers tend to consume the same amount of nicotine on a daily basis to achieve desired effects, adjusting consumption to compensate for the difference in nicotine availability when using low-quality cigarettes [28,30]. These data are consolidated with the survey carried out by the Brazilian Institute of Ethics in Competition [2], which points out that smuggled cigarette consumers consume two more units per day compared to legal cigarette consumers.

Thus, a smuggled cigarette consumer is exposed to other physical and chemical contaminants, such as potentially toxic metals and higher levels of carbon monoxide and tar, because the amount of these generated substances does not vary with the amount of nicotine in the cigarette [31].

Based on these data, although there are no established concentration limits for nicotine in tobacco, it is very important to determine the amount of nicotine in cigarettes, because it can imply the quality of tobacco present in products as well as the health of its users.

3. Material and Methods

For optimization of the QuEChERS method, we selected the Doehlert matrix design, an experimental design of second order that requires a reduced number of experiments to achieve the optimal region. The recovery of the analytical nicotine standard (Pestanal®) from Sigma-Aldrich was evaluated. The nicotine stock solution (10 g L^{-1}) was prepared considering the purity of the standard in acetonitrile (HPLC grade) and was maintained at −17 °C. Tomato leaves were used as the representative matrix in all stages of optimization.

Instrumental conditions

The sample extracts were quantified by gas chromatography using a flame ionization detector (Shimadzu 2014). For separation, an RTX-5 capillary column (5% phenyl - 95% methylpolysiloxane), with dimensions of 30 m × 0.25 mm and 0.25 µm film thickness was used. The oven temperature program started at 60 °C and was, maintained for 1 min. Then, the temperature was increased to 180 °C at 15 °C min^{-1}, and was maintained for 1 min. Subsequently, it was increased to 280 °C at 40 °C min^{-1} and was maintained for 2 min. The temperatures of the injector and detector were maintained at 230 °C and 300 °C, respectively. Helium was used as the carrier gas at a flow rate of 1.2 mL min^{-1}. The injection volume was 1 µL in splitless mode [22].

Sample preparation

In the absence of a sample blank matrix, it is advisable to use a representative matrix, which presents similarities with the sample matrix [25]. For the study of nicotine recovery, tomato leaves have been used as representative matrix [32–34]. All the stages of the optimization process were conducted using tomato leaves collected from an organic crop. Briefly, 0.6 g of tomato leaves were dried in an oven at 80 °C, crushed in a processor, spiked with 100 µL of 10 g L^{-1} nicotine standard, and left to stand for 24 h at 10 °C. Based on the Doehlert matrix design, five different pH levels and three different extraction solvent volumes were studied, as shown in Table 3 including coded levels and actual values used.

Extraction procedure

First, 0.6 g of the sample was weighed in a 15 mL polypropylene tube. Then, 7.6 mL of acetonitrile was added, and the mixture was stirred. The pH was adjusted with 500 µL of 1 mol L^{-1} K_2CO_3 solution. The mixture was stirred again, and 1.4 g of magnesium sulfate and 0.3 g of sodium chloride were added to the same tube. The tube was vortexed vigorously for 1 min followed by centrifugation (4000 rpm) for 5 min. Next, 2 mL of the supernatant was removed and placed in another tube containing 0.3 g of $MgSO_4$ and 0.1 g of PSA. The extract was vortexed for 1 min and centrifuged (4000 rpm) for 5 min. After that, the supernatant was filtered through a nylon filter

(0.22 µm), and the extract was transferred to a vial for chromatographic determination.

Table 3. Doehlert matrix of the optimization QuEChERS method.

FACTORS	LEVELS	
	Codified	Real (mL)
Solvent Volume	-0.866	5
	0	7.5
	+0.866	10
pH	-1.0	5
	-0.5	7.2
	0	9.5
	+0.5	11.7
	+1.0	14

Source: The authors.

Method validation

To guarantee the validity of the optimized method, the performance parameters established by the AOAC [25], IUPAC [35], ANVISA [36], and INMETRO [37] were used. The validation of the study was performed in terms of linearity, accuracy (recovery), precision, detection limit, and quantification limit. Linearity was assessed by the significance of the linear regression equation coefficients through variance analysis of the calibration curve, ranging from 2.5 to 1000 mg L^{-1} in authentic replicates. Accuracy studies (recovery) were performed by recovery at concentrations of 4, 500, and 1000 mg L^{-1}. Precision was expressed in relation to the coefficient of variation (CV) at concentrations of 4, 500, and 1000 mg L^{-1}. Both in seven replicates. The limits of detection and quantification were calculated using signal-to-noise ratios of 3 and 10, respectively.

Real samples

After studying the influence of pH and solvent volume, the optimized method was applied to real samples of smuggled and legal cigarettes. Nine cigarette brands seized by the Federal Revenue Service 9th region of the state of Paraná, Brazil. Two brands of legal cigarettes, which were most sold in local shops in the city of Ponta Grossa, PR, were analyzed. An arbitrary sampling was carried out according to UNODC, [38] considered a widely accepted approach, according to the formula n = √N, where n is the sample size and N is the population size.

Tobacco was separated from the wrapper and filter. In this study, 22 packs from each box (brand) were used, with four cigarettes from each pack. The tobacco from each brand was mixed, and the sample was quartered [39] to remove a homogeneous aliquot. Nicotine determination was performed in triplicate.

4. Conclusions

The modified QuEChERS method for extracting nicotine using a lower volume of extraction solvent, at pH 12, and without hydration, proved to be effective for cigarette tobacco sample analysis. These modifications contributed to the development of the method, reducing extraction time, generating less waste, and obtaining good recoveries.

With increasing consumption of contraband cigarettes, studies on their quality are extremely important, and nicotine concentration is a crucial factor to be analyzed; thus, this study is relevant to investigate the quality of contraband cigarettes.

Based on the results, it is concluded that the proposed method is a useful tool for the determination of nicotine concentration in cigarette tobacco, and as demonstrated by the application in real samples, it can be used in laboratories as a routine method.

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Author Contributions

C.E.D., J.K. and C.R.P. method modification, chromatographic analyzes, formal analysis and validation; T.R.O.S., R.Z. and P.L.W. data curation and validation; C.M.S.V. and S.X.C. conceptualization, funding acquisition, project administration, resources and supervision. All authors contributed for the written, review and editing of the manuscript.

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