Gas Chromatography–Mass Spectrometry Method for Simultaneous Detection of Nine Alkaloids in Tobacco and Tobacco Products by QuEChERS Sample Preparation

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One method based on QuEChERS sample preparation is presented in this study, which leads to simultaneously detect nine alkaloids in tobacco and tobacco products. Nicotine, nornicotine, myosmine, N-methyl anabasine, β-nicoryrine, anabasine, anatabine, isonicotenine and cotinine can all be found in fresh tobacco leaves, cigars, Virginia-type and blended-type cigarettes. The samples were purified via a certain proportion of adsorbents consisting of anhydrous magnesium sulfate, PSA and carbon after extracting, then centrifuged and filtered before analyzing by GC-MS. The matrix effects were all among 88 - 105%. The limit of detection of all were within the range of 0.0065 – 0.1509 μg/g and limit of quantification were among 0.0217 - 0.5031 μg/g. The recovery rates were higher than 89%. This is the first time that the QuEChERS sample preparation method has been applied for tobacco alkaloids, where more varieties of alkaloids could be quantified regarding sensitivity and reproducibility.

Keywords Gas chromatography–mass spectrometry, nicotine, tobacco alkaloids

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Introduction

Tobacco alkaloids are less, but significant components in tobacco, mainly including nicotine, nornicotine, anabasine, anatabine and nine more alkaloids. The quality of tobacco is also subject to the composition of alkaloids. It was demonstrated that the higher is the proportion of nicotine content, the better is the quality of tobacco leaves and its products.1 In addition, the contents of these alkaloids have a great impact on the safety of tobacco products. Nicotine, the major alkaloid in tobacco leaves, is universally acknowledged regarding its addiction. It turned out that nicotine is able to be absorbed by the human body, followed by stimulating peripheral vasoconstriction and pressing heartbeat, increasing blood pressure and rapid breathing, along with promoting the occurrence of cardiovascular diseases, such as hypertension and stroke.2-3 Another alkaloid, cotinine, is a by-product of nicotine metabolism, as well as the primary biomarker for measuring the amount of cigarette smoke in smokers and passive smokers.10,11 Moreover, it was found that nicotine, nornicotine, anabasine and anatabine are the precursors of volatile N-nitrosamines (TSNAs), which are considered to be the carcinogenic compounds according to previous studies. Therefore, tobacco alkaloids are key substances and ones that require more research. However, nicotine accounts for more than 95% of the total alkaloids in tobacco, which gives hints that several other alkaloids are hardly formed through detection.12-14 The composition of tobacco products is complicated to separate. Consequently, it is a severe problem that less abundant alkaloids can be quantified concurrently with both convenience and quickness.

Over the earlier studies, GC-MS or LC-MS are the most common approaches for the detection of tobacco alkaloids.15-21 It achieved high sensitivity and better reproducibility through the combination of chromatography with mass spectrometry. A selective ion monitoring (SIM) mode in mass spectrometry has been adopted when separating trace alkaloids with similar structures and close retention times, which is able to significantly increase the sensitivity, and well used in analyzing the hugely different contents of ingredients in samples.22 Unfortunately, precise quantification could not be reached in such an analysis process, due to the extremely low content of alkaloids compared with nicotine. Only five or six kinds of alkaloids could be performed by the ways and means in past experiments.23-26 In addition, alkaloids need to be repeatedly obtained in the pretreatment, making the work time mostly over 1 h. Because of the complex operation and long time in the pretreatment, the previous detection ways did not lead to any sensational efficiency. Yet, the application of the QuEChERS method to separate tobacco alkaloids is not only simple and convenient, but also capable of analyzing nine or more tobacco alkaloids. The QuEChERS method is a simplification of solid-phase extraction (SPE), which has been widely applied for the detection of pesticide residues.27 Today, it has gradually become a standard sample processing approach achieving great success in detecting pesticide residues in fruits and vegetables.28-32 The sample was taken out with an organic solvent and subjected to salting and delamination of MgSO₄ or additional salts. Based on the principle of matrix dispersion and separation, different kinds of adsorbents were applied to purify and remove impurities, so that less abundant components in the samples...
were easily measured. PSA, carbon, C18 or something else were suggested in earlier studies.17-19 Compared with the traditional solid-phase extraction, the equipment and operating time are less demanded in the QuEChERS method, which also has a remarkable effect at the same time. In the light of these points, this approach has provided the characteristics of “quick, easy, cheap, effective, rugged and safe”.27 As a result, this article provides a new simple and effective pretreatment method for separating various tobacco alkaloids using modified QuEChERS solid-phase extraction and GC-MS. It is indispensable that the extracted samples are decontaminated by the adsorbent for a period before analyzing in equipment. In the meantime, weak alkaline dissolved, organic solvent, operation time, adsorbent species, and GC-MS conditions were optimized. To our knowledge, this study indicates the first time that the method for determination of nine tobacco alkaloids with uncomplicated and available has been reported, which has been validated in fresh tobacco leaves, cigars, Virginia-type and blended-type cigarettes.

Materials and Methods

Chemicals and materials

Nine kinds of tobacco alkaloid standards: Nicotine was purchased from Chromadex (USA). (R,S)-Anatabine, S(-)-cotinine, (R,S)-nornicotine, N-methyl anabasine, isonicotinone, (S)-anabasine, myosmine and β-nicotyrine were obtained from Toronto Research Chemicals (Canada). The internal standard quinoline was obtained from Macklin. The purity of (R,S)-anatabine, S(-)-cotinine and β-nicotyrine are 95%, (R,S)-nornicotine is 97%, the remaining five reagents have purity levels of 98%. The analytical purity of ammonia, HPLC grade solutions of nicotine, the ion at 105 was equal to [M–57]+, which indicated that the compounds had nornicotine, anabasine, myosmine and β-nicotyrine were from Fuyu Fine Chemical.

Anhydrous magnesium sulfate (180 – 250 μm), Aluminum Oxide Active Basic (75 – 150 μm) and silicon oxide were purchased from Kermel. Primary secondary amine (PSA, 40 – 60 μm) was obtained from Agela Technologies. Carbon (75 μm) was from Shen Shine.

Sample treatment

The tobacco sample was crushed and passed through a 425-μm sieve and stored in a –10°C freezer. Firstly, 0.5 g tobacco powder (accurate to two decimal places) was placed in a 50-mL polypropylene centrifuge tube; 50 μL of internal standard and 5 mL of 6% of ammonia were added. Secondly, the mixture was rested for 10 min and then diluted with 10 mL of methanol and dichloromethane (v/v = 1:4). Subsequently, the tube was shaken for 20 min with an ultrasonic oscillator (Kunshan Ultrasonic Instrument-KQ 5200 DE, 24 kHz). Afterward, the compound was eluted with 0.5 g of anhydrous magnesium sulfate, 0.125 g of PSA and 0.25 g of carbon, accompanied by shaking for 60 s by hand before the tube was centrifuged at 5000 r/min (Thermo Fisher Scientific) for 5 min. Later, the lower layer was removed to a 1.5-mL injection bottle through a 0.22-μm micron membrane filter. Finally, the mixture was analyzed by GC-MS. For the samples of the optimization conditions, the above steps were performed similarly as the sample treatment procedure mentioned above, except for changing the selected conditions.

Instrument conditions

The samples were analyzed on a gas chromatography (Agilent 7890B) tandem mass spectrometer (Agilent 5977). The GC inlet was operated in the split mode (split ratio = 20:1) and maintained at a temperature of 240°C. High-purity helium was used as a carrier gas with a flow rate of 1.2 mL. An aliquot of 1 μL was injected. Targets were treated separately on an Agilent HP-5 MS (60 μm x 0.25 mm; 0.25 μm film thickness). The oven temperature program was as follows: the initial temperature of the GC was 90°C for 3 min, then raised to 140°C at a rate of 15°C/min and held for 20 min, and next to 280°C at 7°C/min and held for 10 min. The total time of the entire process was 47.14 min. The EI ionization mode was utilized as the mass spectrometric ionization with a voltage 70 eV and a source temperature of 230°C. The solvent delay time was set to 5 min. The target compounds were determined through the retention time in the SIM mode. The quadrupole mass spectrometer detector was operated in the select ion monitoring (SIM) mode during quantitative analysis; the qualifier ion and the quantifier ion together with their abundance ratio were chosen to determine the peak of the target compound. Table 1 lists detailed information.

The mass was in the range of 50 – 300 u in the scanning mode. By comparing the ion fragments of the standard with the sample, two characteristic ions were determined for peak-identification, while one was the represented structure and the other was chosen for quantification. The ion fragments of nine tobacco alkaloids in mass spectrometry are displayed in Fig. 1. Most alkaloids can be characterized by their own molecular weight. Consequently, the qualitative ion 133 corresponds to the [M–29] ion formed by the loss of [CH3N]+ for nicotine, the ion at m/z 105 was equal to [M–57]+, which meant the destruction of [CH3CH2CH2NH]+ for anabasine. For nicotine, N-methyl anabasine, anabasine and cotinine, the quantification ions [M–78]+ indicated that the compounds had lost pyridyl. The cleavage of C-N on the pyrrole ring enabled the production of ions [M–29+H]+ of nornicotine, β-nicotyrine and isonicotinone. The peak ion at m/z 105, corresponding to [M–55]+, was caused by the loss of [CH3CH=CHNH]+ for anabasine.

Preparation of matrix standard solution

A 5-g portion of quinoline was weighed and dissolved in a 50-mL brown volumetric flask with methanol to obtain an internal stock solution; it was then diluted 5 times with methanol when involved in samples. The standards of nine alkaloids were weighed (1 mg) and next dissolved in 10 mL brown volumetric flasks with methanol. Ultimately, these batches of the standard stock solution were stored in a refrigerator under an inert atmosphere for further analysis.

| Target compound | Retention time/min | Quantitative ion (m/z) | Qualitative ion (m/z) | Abundance ratio |
|-----------------|-------------------|------------------------|----------------------|-----------------|
| Nicotine        | 15.441            | 84                     | 133                  | 44.1            |
| Nornicotine     | 19.116            | 119                    | 147                  | 37.5            |
| Myosmine        | 19.539            | 118                    | 146                  | 75.9            |
| N-Methyl anabasine | 21.252       | 98                     | 176                  | 15.2            |
| β-Nicotyrine    | 23.696            | 158                    | 130                  | 16.9            |
| Anabasine       | 24.226            | 84                     | 105                  | 69.3            |
| Anatabine       | 26.328            | 105                    | 160                  | 97.9            |
| Isonicotinone   | 27.464            | 156                    | 130                  | 22.7            |
| Cotinine        | 34.422            | 98                     | 176                  | 37.6            |
| Quinoline       | 11.629            | 129                    | 102                  | 22.2            |

Table 1 Retention time, quantitative and qualitative selection of nine tobacco alkaloids and internal standard
atmosphere. Calibration curves were generated by adding a standard mixed working solution of nine alkaloids to a matrix solution of blank samples; they were prepared in the range of 0.1 to 50 mg/g (0.1, 0.2, 0.5, 1, 2, 5, 10, 20, 50) with nicotine, as well as 0.005 to 2 mg/g (0.005, 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1, 2) with the remaining eight alkaloids. The correlation coefficients of all calibration curves were above 0.99.

Results and Discussion

The optimization of ammonia

Alkaloids in tobacco can be liberated by some weak alkaline solution. The strength of the alkaline solution has an effect on the extraction rate of the sample. Since the alkalinity of NaOH is stronger than that of ammonia, the extraction efficiency is lower when using the former. It was proved that five alkaloids were able to be quantified after the sample was treated with 2 mol/L NaOH in a pre-test, while nine alkaloids when diluted ammonia was required. Thus, different concentrations of ammonia (1, 3, 6, 12%) were chosen to verify if they have a better performance on sorts and contents. In the end, 6% of ammonia presented the best results, for the amounts of alkaloids are higher than the other three conditions.

The optimization of extraction time

Ultrasonic extraction provided better responses than extra oscillatory extraction modes. It can shorten the time and increase the rate, and was widely accepted in medicine, chemistry, food and comprehensive fields. Times of 5, 10, 20 and 40 min were selected as the ultrasonic operation time to promote the quantities of alkaloids. As a result, the extraction efficiency obtained great results when the ultrasonic time was 20 min, especially for anabasine, anatabine and isonicotenine.
Here, an ultrasonic time of 20 min was chosen as the best working time for tobacco alkaloids.

**The optimization of extractant**

Previous studies demonstrated that the organic solvents used for alkaloids detection were dichloromethane, methanol and chloroform. But only four alkaloids could be formed when using chloroform in a pre-test. The most common solvent for tobacco alkaloids is dichloromethane; however, it is not as good as methanol for analyzing certain trace alkaloids. Therefore, a mingled solution of methanol and dichloromethane was required as an extractant to determine whether the extracting function can combine the advantages of both. The same volume of dichloromethane, methanol and the mixed dissolvent of dichloromethane and methanol (v/v = 1:4, 4:1) were practiced in this comparison. The results showed that the combination did best when dichloromethane:methanol = 4:1.

**The optimization of adsorbent**

Carbon, PSA, silica and alkaline alumina were picked as the adsorbent for removing impurities. PSA has been used as an adsorbent many times in other experiments using the QuEChERS pretreatment method. It was investigated that the polar components of fatty acids, certain pigments and sugars in the sample can be removed by PSA without affecting the composition of the samples. When using a single adsorbent, carbon worked best; the rates of the remaining were not much different. In view of the above, carbon and PSA were joined in proportion as an adsorbent to purify the sample. Different ratios of anhydrous magnesium sulfate were set and adjusted to discover the optimum condition. Details were as following, six varieties of the adsorbent mixture were run in the experiment. 0.5 g MgSO₄ + 0.25 g PSA + 0.25 g carbon (2:1:1), 0.5 g MgSO₄ + 0.25 g PSA + 0.125 g carbon (4:2:1), 0.5 g MgSO₄ + 0.125 g PSA + 0.25 g carbon (4:1:2), 0.75 g MgSO₄ + 0.25 g PSA + 0.25 g carbon (3:1:1), 0.75 g MgSO₄ + 0.25 g PSA + 0.125 g carbon (6:2:1), 0.75 g MgSO₄ + 0.125 g PSA + 0.25 g carbon (6:1:2). The ability of the extraction efficiency measured after merging carbon and PSA was 10 - 30% higher than when using one of them alone. As more carbon was used, the efficiency was better. The amount of anhydrous magnesium sulfate had little impact on extraction rate. Finally, the combination (0.5 g MgSO₄ + 0.125 g PSA + 0.25 g carbon) was determined.

**Optimization of chromatographic conditions**

The temperature program is a significant factor that affects component separation. It is suggested that the oven temperature program be roughly divided into two modes in previous analysis of alkaloids. One is to raise the temperature to a maximum temperature at a certain rate from the initial temperature. The other has an intermediate temperature, with two different heating rates before and after it. It turned out that trace alkaloids could not be separated well in the former, whereas the good performance of latter may be attributed to flexible temperature, for myosmine, N-methyl anabasine and β-nicotyrine to be detected. A rapid heating rate was required in the early phase so that nicotine, nornicotine and myosmine could be detected, but the trace alkaloids could be clearly separated by a slow rate between 140 and 180°C. Consequently, 140°C was a good break point in this program. The increasing rate ran faster from the beginning, making the oven reach this temperature in a shorter period so that the alkaloids could be separated. The second proportion was slightly slower, contributing to the separation of anatabine, isonicotenine and cotinine. The split mode was also optimized; after several experiments, 20:1 was determined to be the most suitable split ratio. Figure 2 introduces the total ion chromatogram and Fig. S2 (Supporting Information) is the chromatogram of each alkaloid in the selective ion-monitoring mode.

**Matrix calibration, LOD and LOQ**

The standard stock solution of nicotine was diluted with the matrix solution to a concentration of 0.1 to 50 mg/g, and the other eight were from 0.005 to 2 mg/g. An internal standard in each gradient was added to 2 mg/g. The relative concentration of each alkaloid and the internal standard was taken as the ordinate (y), and the relative peak area of quantitative ion was taken as the ordinate (y) when the calibration curve was drawn. The outcomes (Table 2) certificated that the linear relationships of nine alkaloids were good, and the correlation coefficients were all greater than 0.99. The limit of detection (LOD) and the limit of quantitation (LOQ) are, respectively, the value of the
eight samples. In general, the highest is nicotine, followed by Table 5 points out that nine alkaloids can all be found in the Virginia-type cigarettes, blended-type cigarettes, cigars and fresh tobacco leaves (40 days of growth), were extracted by the QuEChERS pretreatment method and then submitted to GC-MS analysis. The main alkaloids contained in the fresh tobacco leaves at this period are nicotine, nornicotine and anatabine. While the total of eight trace alkaloids in anabasine, cotinine and myosmine. From a part view, cigars have the highest contents among these fresh tobacco leaves have the lowest alkaloid contents because they are not fully mature. The main alkaloids contained in the tobacco leaves at this period are nicotine, nornicotine and anatabine.

Conclusions

In this study, the QuEChERS sample preparation method was developed for the simultaneous detection of nine alkaloids in tobacco and tobacco products. Dichloromethane and methanol (v/v = 4:1) were offered as an extracting reagent, anhydrous.
magnesium sulfate, PSA and carbon for removing water and impurities. It was quick, cheap, effective and was capable to accurately detect trace alkaloids. The QuEChERS sample preparation was used for the first time in the determination of tobacco alkaloids.

In recent years, many researchers have explored the extraction and detection of tobacco alkaloids with GC-MS.25,26,36–38 Although the extraction conditions were improved, some trace alkaloids were still not detected in these studies. Yet the method used in this study can detect nine alkaloids presented in most varieties of tobacco, which is quiet more than before. The values of LOD and LOQ were lower than any other methods established for detecting tobacco alkaloids by the predecessors.33,34 Furthermore, the extraction time and efficiency were also greatly improved compared to other studies. This approach was suitable for the rapid detection of nine alkaloids in fresh leaves, cured leaves, cigarettes and more tobacco products. It might also provide technical support for the quality control of cigarette production and tobacco formula experiment.

Supporting Information
This material is available free of charge on the Web at http://www.jsac.or.jp/analsci/.

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Table 5 Analysis results of nine alkaloids in practical samples (mg g–1)

| Target compound | 1#     | 2#     | 3#     | 4#     | 5#     | 6#     | 7#     | 8#     |
|-----------------|--------|--------|--------|--------|--------|--------|--------|--------|
| Nicotine        | 21.06  | 22.11  | 17.10  | 18.07  | 27.07  | 26.71  | 10.62  | 9.92   |
| Nornicotine     | 0.41   | 0.39   | 0.40   | 0.31   | 0.27   | 0.31   | 0.22   | 0.22   |
| Myosmine        | 0.0360 | 0.0370 | 0.0421 | 0.0350 | 0.0863 | 0.0642 | 0.0089 | 0.0028 |
| α-Methyl anabasine | 0.0144 | 0.0143 | 0.0141 | 0.0142 | 0.0127 | 0.0127 | 0.0025 | 0.0012 |
| β-Nicotyrine     | 0.0104 | 0.0104 | 0.0106 | 0.0103 | 0.0169 | 0.0271 | 0.0098 | 0.0103 |
| Anabasine        | 0.1099 | 0.1131 | 0.1067 | 0.1089 | 0.0683 | 0.0682 | 0.0408 | 0.0160 |
| Anatabine        | 1.3966 | 1.3350 | 1.1913 | 1.2323 | 0.3234 | 0.4859 | 0.3524 | 0.2188 |
| Isonicotinone    | 0.0362 | 0.0383 | 0.0460 | 0.0366 | 0.0596 | 0.0943 | 0.0274 | 0.0252 |
| Cotinine         | 0.0431 | 0.0462 | 0.0514 | 0.0368 | 0.0237 | 0.0322 | 0.0057 | 0.0045 |
| Total            | 23.1210| 24.1017| 18.9664| 19.8573| 27.9264| 27.8032| 11.2889| 10.4141|

Note: 1# and 2# are Virginia-type cigarettes from China, 3# and 4# are blended-type cigarettes from America, 5# and 6# are cigars, 7# and 8# are different varieties of fresh tobacco leaves.