

Research Article

Nitrate and Nitrite Promote Formation of Tobacco-Specific Nitrosamines via Nitrogen Oxides Intermediates during Postcured Storage under Warm Temperature

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Received 26 December 2016; Revised 7 February 2017; Accepted 7 March 2017; Published 29 March 2017

Academic Editor: Yang Xu

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Tobacco-specific nitrosamines (TSNAs) are carcinogenic and are present in cured tobacco leaves. This study was designed to elucidate the mechanisms of TSNAs formation under warm temperature storage conditions. Results showed that nitrogen oxides (NO_x) were produced from nitrate and nitrite in a short period of time under 45°C and then reacted with alkaloids to form TSNAs. Nitrite was more effective than nitrate in promoting TSNAs formation during 45°C storage which may be due to the fact that nitrite can produce a large amount of NO_x in comparison with nitrate. Presence of activated carbon effectively inhibited the TSNAs formation because of the adsorption of NO_x on the activated carbon. The results indicated that TSNAs are derived from a gas/solid phase nitrosation reaction between NO_x and alkaloids. Nitrate and nitrite are major contributors to the formation of TSNAs during warm temperature storage of tobacco.

1. Introduction

Tobacco-specific nitrosamines (TSNAs) are a group of important and toxic components of tobacco and tobacco smoke [1, 2]. TSNAs mainly consist of N-nitrosornicotine (NNN), N-nitrosoanatabine (NAT), N-nitrosoanabasine (NAB), and 4-(methyl nitrosamino)-1-(3-pyridyl)-1-butanone (NNK). NNN and NNK are strong carcinogens [3, 4]. TSNAs are produced via nitrosation of tobacco alkaloids during the curing and storage of tobacco leaves (Figure 1) [5–8]. In China, both air-cured and flue-cured tobacco leaves are generally stored for approximately 18 months in warehouses before being processed for cigarette production in order to reduce the unfavorable smells [9].

Many studies reported about the factors that influence TSNAs formation during air-curing [7, 10–12]. Cui [10] found that TSNAs levels in the leaf lamina and midrib increased substantially during the fourth to seventh weeks of air-curing

stage. During this stage nitrate is reduced to nitrite via microbial activity, and the resulting nitrite, in turn, is involved in nitrosation reactions with the naturally existing alkaloids during air-curing, then leading to the formation of TSNAs (Figure 2) [11].

However, to our knowledge there are few reports available on the mechanisms of TSNAs formation during storage stage. TSNAs levels may increase several fold in comparison to the levels in freshly air-cured leaves [13, 14], but the mechanisms of TSNAs formation during storage are not clear. It has been reported that, as the storage temperature increased, TSNAs and nitrite contents increased, and the most rapid increase in TSNAs occurred during the warm temperature season [15, 16]. The interactions between temperature and abundant nitrate in cured tobacco leaf could be responsible for TSNAs formation during storage. Treatment of the tobaccos with streptomycin and rifampicin did not inhibit nitrosamine formation during storage indicating that TSNAs formation was

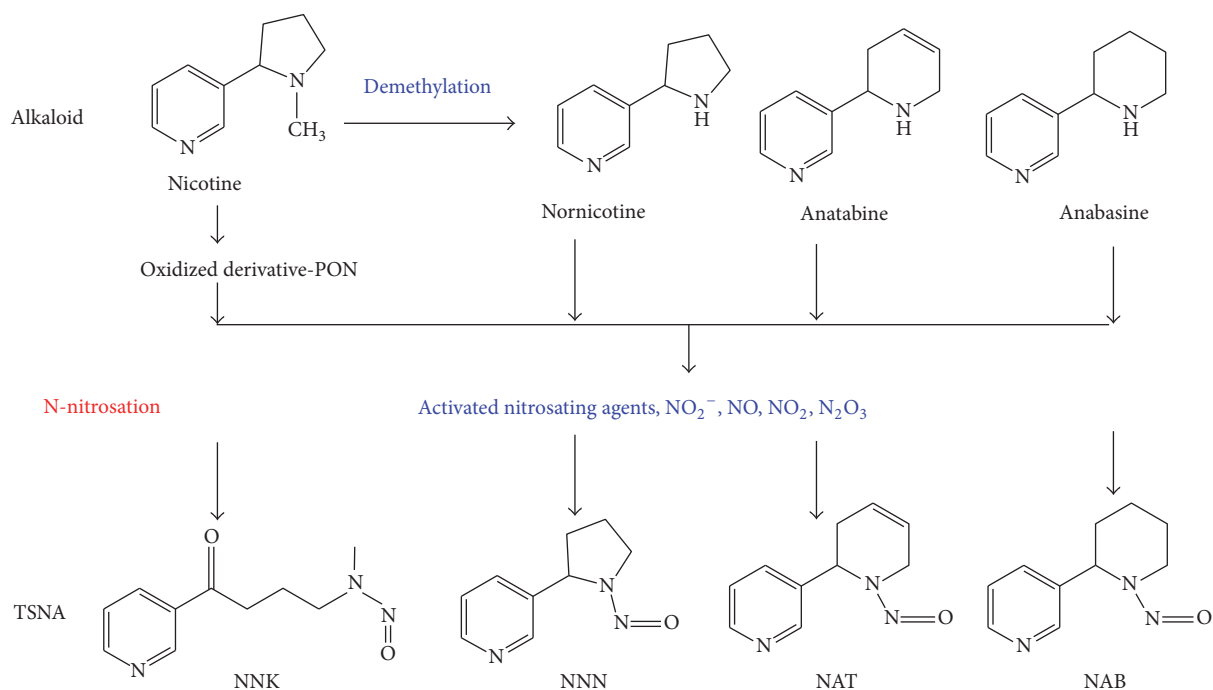


FIGURE 1: Proposed formation pathways of the major TSNA found in cured tobacco leaves. Nitrosating agents can directly interact with nornicotine, anatabine, and anabasine to form NNN, NAT, and NAB, respectively. Nicotine is less susceptible to nitrosation; thus NNK is produced from pseudo-oxynicotine, an oxidized derivative of nicotine.

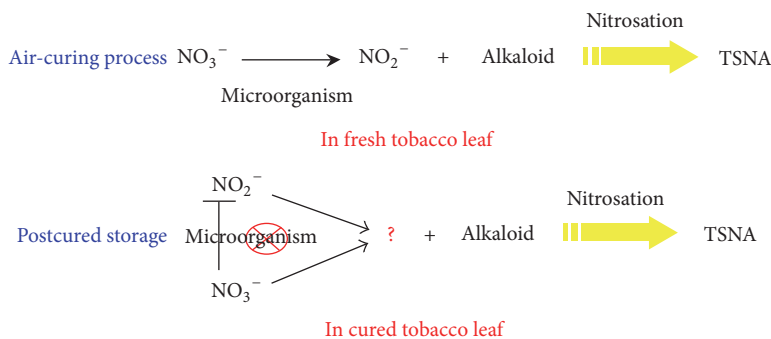


FIGURE 2: The activated nitrosating agents participate in nitrosation of alkaloid to form TSNA during different processes of air-cured tobacco production. Nitrate (NO_3^-) is available for reduction to nitrite (NO_2^-) via microbial activity, and the nitrite, in turn, becomes involved in nitrosation reactions with the alkaloids during air-curing, leading to the formation of TSNA. The nitrite is the most important nitrosating agent during air-curing. TSNA formation was almost not influenced by microorganism during the storage processes.

not influenced by microorganisms during the storage process (Figure 2) [14]. How the nitrate and nitrite trigger the increase in TSNA during warm temperature storage is still not clear (Figure 2). The objectives of this study were to elucidate the mechanisms of TSNA formation during the storage time of cured tobacco leaf and to verify the hypothesis that nitrogen oxides produced from nitrate and nitrite in tobacco are responsible for the formation of TSNA during storage.

2. Experiments

2.1. Plant Materials. Tobacco samples were grown in 2015 and cured locally. Leaves from the middle stalk positions were collected. Flue-cured tobacco (*N. tabacum* cv. "Hongda") was

from Midu county, Yunnan province. Leaf samples were prepared by removing the stems, cut to strips, mixed thoroughly, sealed in plastic bags, and then stored at 4°C for the tests. The moisture content of tobacco was 12%.

2.2. Methods

2.2.1. Direct Addition of Nitrate and Nitrite to Tobacco. The treatments included four levels of NaNO_3 (10, 20, 30, and 40 mg/mL) and NaNO_2 (5, 10, 15, and 20 mg/mL), which were from 10 mL solution each, equal to 73, 146, 219, and 292 mg of NO_3^- and 33, 67, 100, and 133 mg of NO_2^- . The solutions were sprayed onto each of eight 20 g flue-cured tobacco samples. The samples were then placed to an ambient environment for

air-drying. The moisture content of tobacco samples after air-drying was 13%. Then the samples were stored in an airtight vacuum desiccator (15 cm diameter) at 45°C with relative humidity of 70% for 15 d. The sample sprayed with the same volume of deionized water was used as the control. The moisture content of tobacco samples after treatment was 11.8%.

2.2.2. Separating the Tobacco and Nitrate/Nitrite Sources in a Closed System under 45°C Storage Conditions. A vacuum desiccator with a porcelain plate was used to form a closed system in which tobacco cuts and added nitrate and nitrite could be separated during storage. Five mL of each aqueous solution of NH_4NO_3 , KNO_3 , NaNO_3 , and NaNO_2 at 1 mol/L (equal to 310 mg of NO_3^- and 230 mg of NO_2^-) and 5 mL volume of deionized water (control) were sprayed onto the medical gauze pads (5 cm × 5 cm). After air-drying, the sprayed gauzes were placed in the bottom of the vacuum desiccator together with each 20 g sample of flue-cured tobacco. The vacuum desiccators were then tightly closed and sealed with petroleum jelly. The containers were then placed into a chamber at a temperature of 45°C and a relative humidity of 70% and stored for 15 d. The moisture content of tobacco samples after 45°C treatments was 11.1%.

2.2.3. Effect of Indirect Addition of Nitrate and Nitrite on Nitrogen Oxides Formation in a Closed System with Flue-Cured Tobacco. Eight treatments in this experiment were divided to two groups: Group I: (1) tobacco; (2) tobacco and NaNO_3 ; (3) tobacco and NaNO_3 + 2.0 g activated carbon (AC); (4) tobacco and NaNO_3 + 10.0 g AC and Group II: (1) tobacco; (2) tobacco and NaNO_2 ; (3) tobacco and NaNO_2 + 2.0 g AC; (4) tobacco and NaNO_2 + 10.0 g AC. The amount of added NaNO_3 or NaNO_2 was 0.8 g and 0.4 g for treatments 2 to 4 (equal to 583 mg of NO_3^- and 266 mg of NO_2^-) in each group. The weight ratios of tobacco, NO_3^- , and AC were 68 : 1 : 3 and 68 : 1 : 17, respectively. And the weight ratios of tobacco, NO_2^- , and AC were 150 : 1 : 7 and 150 : 1 : 37, respectively. NaNO_3 and NaNO_2 were dissolved in 5 mL deionized water and then sprayed onto the gauze pads. After air-drying, the gauze pads were placed on the bottom of the vacuum desiccator with 40 g sample of flue-cured tobacco leaf in each desiccator. AC sample was made from wood with the particle size being 75 μm , produced by Zhengzhou Tianhe Water Purification Material Co. Ltd. in Henan province. The surface area was 901.7 m^2/g , and the total pore volume was 0.518 cm^3/g with the micropore, mesopore, and macroporous volume being 0.372 cm^3/g , 0.129 cm^3/g , and 0.016 cm^3/g , respectively. AC was activated in 100°C oven for 30 min, then was wrapped in a medical gauze, and suspended under the porcelain plate of the vacuum desiccator for the relevant treatments. All vacuum desiccators were tightly closed, sealed, and placed into a chamber at a temperature of 45°C and a relative humidity of 70% for 24 h.

The AC used in the above experiments were taken out and then were placed in the new airtight desiccator, respectively. Two grams of AC was put into another container as control. All airtight desiccators were placed into a chamber at a

temperature of 60°C and a relative humidity of 70% for 15 min and 90 min.

2.2.4. Effects of Activated Carbon (AC) on TSNA's Formation in Flue-Cured Tobacco in Response to Nitrate and Nitrite Addition. Twelve treatments in this experiment were divided into two groups: Group I: (1) tobacco (4°C); (2) tobacco (warm temperature control 45°C); (3) tobacco and NaNO_3 separately; (4) tobacco and NaNO_3 + 1.0 g AC separately; (5) tobacco and NaNO_3 + 5.0 g AC separately; and (6) tobacco and NaNO_3 + 10.0 g AC separately and Group II: (1) tobacco (4°C); (2) tobacco (warm temperature control 45°C); (3) tobacco and NaNO_2 separately; (4) tobacco and NaNO_2 + 1.0 g AC separately; (5) tobacco and NaNO_2 + 5.0 g AC separately; and (6) tobacco and NaNO_2 + 10.0 g AC separately. The amount of NaNO_3 or NaNO_2 added to each sample of treatments 3 to 6 was 0.3 g (equal to 219 mg of NO_3^- and 200 mg of NO_2^-). The weight ratios of tobacco, NO_3^- , and AC were 91 : 1 : 4, 91 : 1 : 22, and 91 : 1 : 45, respectively. And the weight ratios of tobacco, NO_2^- , and AC were 100 : 1 : 5, 100 : 1 : 25, and 100 : 1 : 50, respectively. $\text{NaNO}_3/\text{NaNO}_2$ were dissolved in 5 mL deionized water and then were sprayed onto the gauze pads. After air-curing, the gauzes were placed on the bottom of the vacuum desiccators with 20 g sample of flue-cured tobacco leaf in each desiccator. All treatments were performed in the vacuum desiccator as described above. The activated AC also was used as described above. The vacuum desiccators were put into a chamber with a constant temperature of 45°C and a relative humidity of 70%, respectively. The moisture content of cured tobacco samples after treatment was 11.6%.

2.3. Chemical Analyses

2.3.1. TSNA's Measurements. In each experiment, tobacco samples were lyophilized, ground to powder, sieved through a 0.25 mm screen, and then measured for the content of NNN, NNK, NAT, and NAB. TSNA's contents were determined at the Beijing Cigarette Factory according to the method of SPE-LC-MS/MS [12, 17, 18].

2.3.2. Nitrate, Nitrite, and Alkaloid Measurements. $\text{NO}_3\text{-N}$ and $\text{NO}_2\text{-N}$ were quantified according to the method of Crutchfield and Grove [19]. The individual alkaloids were analyzed with a gas chromatograph as described by Jack and Bush [20]. Methyl tert-butyl ether was used as the extraction solvent with N-hexadecane as the internal standard.

2.3.3. Nitrogen Oxides Analysis. The first step was diluting the standard gas with a dynamic gas calibrator (Model 146 i, Thermo Scientific, USA EPA) to give a concentration within the operational range of the instrument. The high purity nitric oxide (NO) and nitrogen dioxide (NO_2) standards in N_2 (component content: 69.8 ppm, gas sample number: L120911099, National Institute of Metrology/National Standard Material Research Center, Beijing, China) were configured into nitrogen oxides gas of a low concentration (1 ppm) by 146i calibrator. It was necessary to modify the original

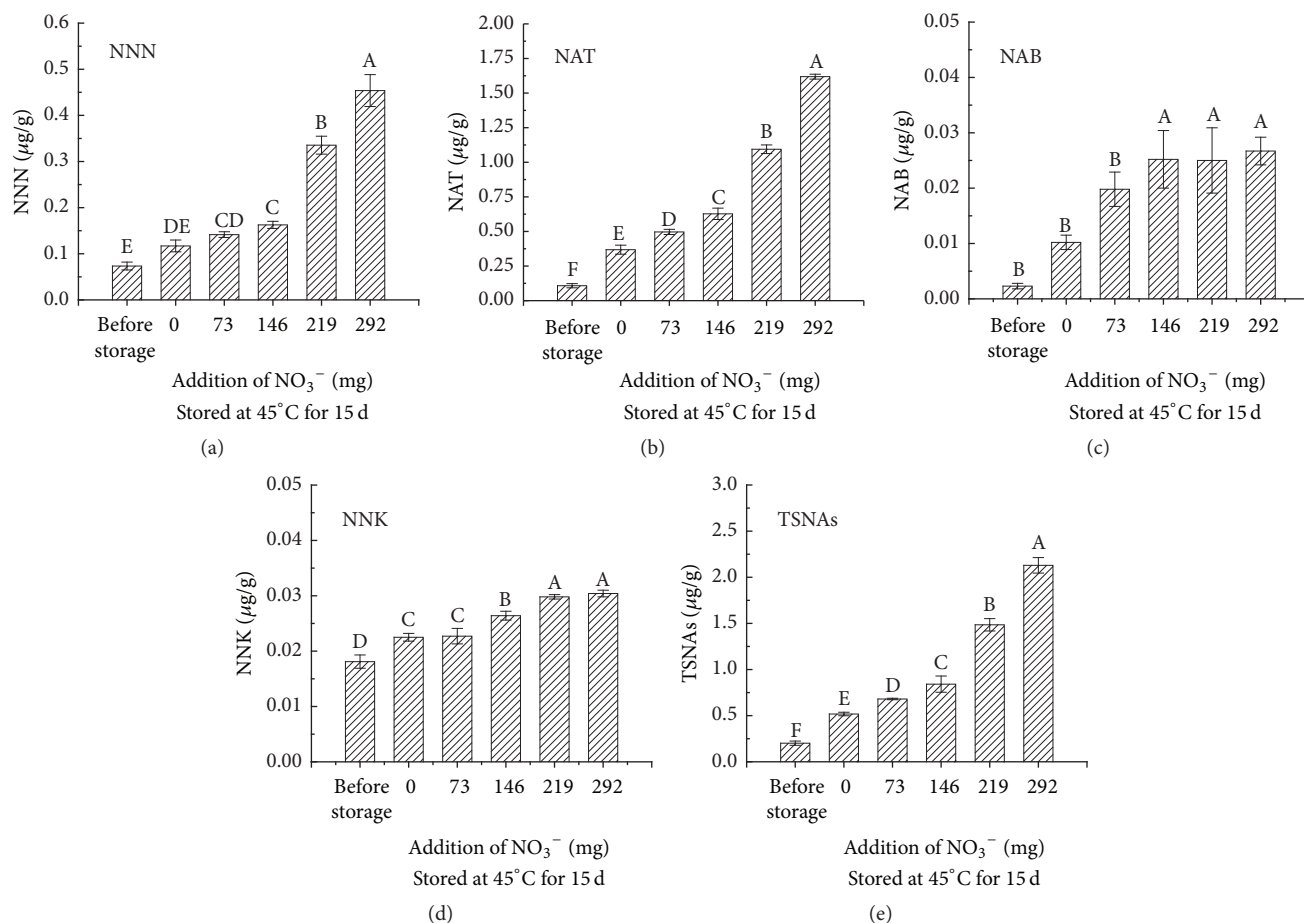


FIGURE 3: Effects of added nitrate on TSNAs formation in flue-cured tobacco stored at 45°C for 15 d. *Note.* For all experiments, each value represents the mean of three independent samples. Uppercase letters indicate significant differences between the treatments at $P < 0.05$.

procedure by configuring the standard gas again if the levels of NO_x exceeded the limit of detection. The NO and NO_x in the air were defaulted to the zero point by the instrument.

After experiment, the airtight vacuum desiccators were taken out and then connected with a vacuum pump (DOA-P504-BN, GAST Manufacturing, A Unit of Idex Corporation, MI, USA) to extract the gas into a gas collecting bag. After 25 s, the gas bag was pulled out and connected with the NO-NO₂-NO_x analyzer (Model 421, Thermo Scientific, USA EPA, Reference method RFNA-1289-074) for NO_x analysis by chemiluminescence detection.

2.4. Statistical Analyses. Analysis of variance (ANOVA) and least significant difference (LSD) of TSNAs and NO_x values were performed at the 0.05 level of significance. Data were statistically analyzed with SPSS 20.0. Figures were drawn with Origin 8.5. All treatments were randomly designed in triplicate.

3. Results and Discussion

3.1. Effect of Nitrate Concentration on TSNAs Formation in Flue-Cured Tobacco. The mean contents of nicotine, nor-nicotine, anabasine, and anatabine of tobacco used in this

experiment were 19.8, 1.2, 0.3, and 1.5 mg/g, respectively, and the NO₃-N and NO₂-N content were correspondingly 119 µg/g and 10 µg/g. With the increasing amount of NO₃⁻ added, both individual and total TSNAs contents increased significantly (Figure 3). After the addition of 0.4 g NaNO₃ (equal to 292 mg of NO₃⁻), the NAT and total TSNAs contents increased by 340% and 311%, respectively, in comparison with the control.

No significant change of NNN was observed in the tobacco samples when less than 0.2 g of NaNO₃ (146 mg of NO₃⁻) was added. However, when the amount of NO₃⁻ added increased to 219 mg (0.3 g NaNO₃), the NNN content was approximately double that of 0.2 g NaNO₃ addition. Although the NAB content was the lowest of the four individual TSNAs, a significant increase in NAB content occurred as a result of the addition of NaNO₃ reached to 0.2 g. The NNK content also increased significantly as nitrate addition increased.

3.2. Effect of Nitrite Concentration on TSNAs Formation in Flue-Cured Tobacco. Table 1 shows that as the concentration of added NO₂⁻ increased, the individual and total TSNAs contents showed rapid and dramatic increases, and the relative increments were greater for the samples with greater

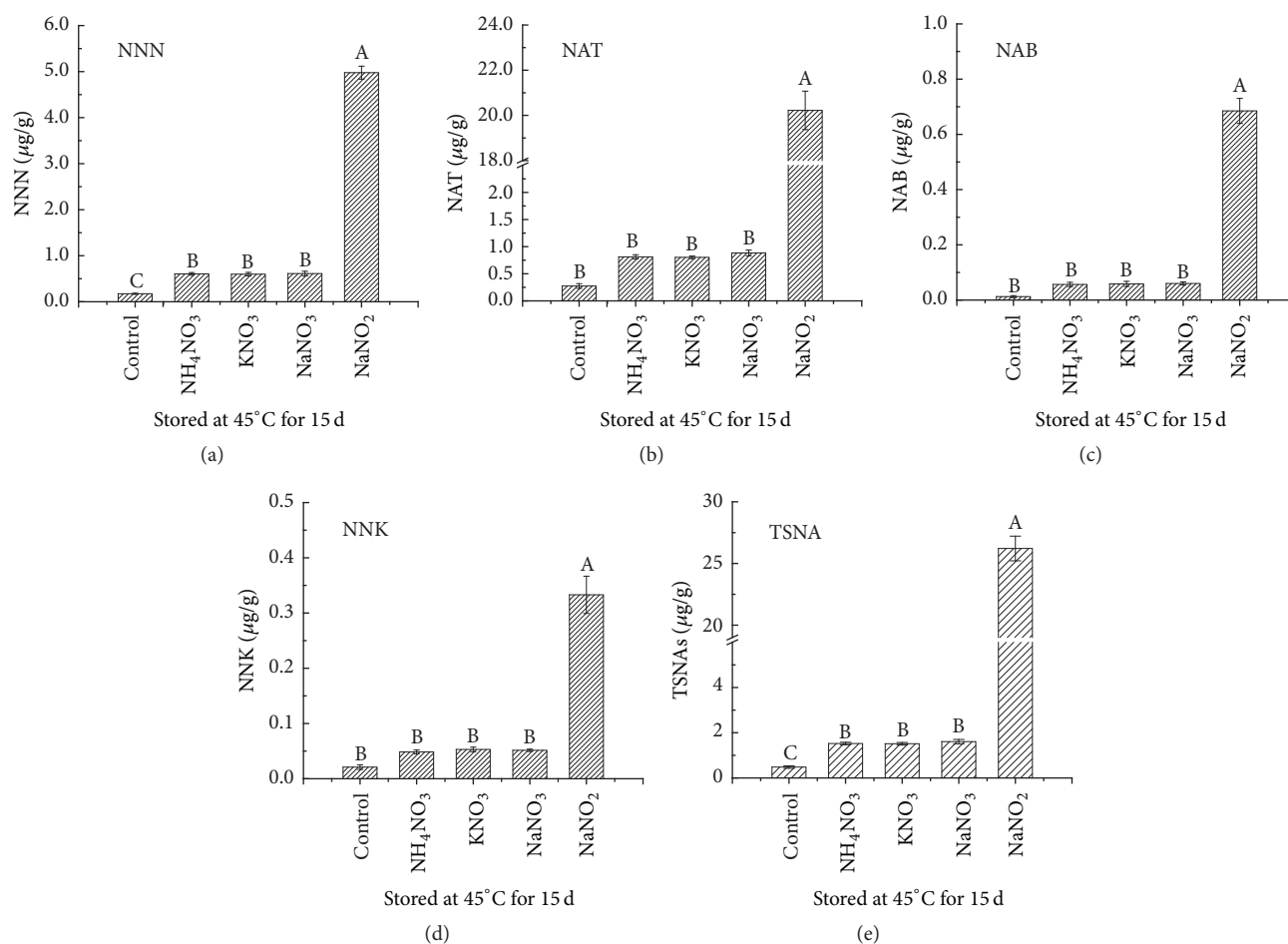


FIGURE 4: Effects of indirect addition of three nitrate compounds and sodium nitrite on TSNA formation in flue-cured tobacco during warm temperature storage. *Note.* The 1 mol/L of nitrate, nitrite compounds (310 mg of NO_3^- and 230 mg of NO_2^- , resp.), and tobacco were added separately in the closed vessel which was then stored at 45°C for 15 d. Uppercase letters indicate significant differences between the treatments at $P < 0.05$.

amounts of added NO_2^- . When tobacco samples were treated with 133 mg of NO_2^- (0.2 g of NaNO_2), total TSNA content was 117 $\mu\text{g/g}$, at a 207% increment compared with the control (0.56 $\mu\text{g/g}$). For the individual TSNA, levels of NNK, NAT, and NAB showed 257-, 38-, 203-, and 110-fold increases, respectively. The results showed that NO_2^- was more effective in promoting TSNA formation in the cured leaf than NO_3^- during warm temperature storage.

The higher TSNA levels in burley tobacco are partly due to the relatively higher levels of TSNA precursors, such as alkaloids and oxide of nitrogen, that are present in the leaf tissue [21–23]. It is reported that nitrite which is derived from the bacterially mediated reduction of nitrate is considered to be the limiting factor in TSNA formation in air-cured tobacco during air-curing [6, 24]. In this research, the addition of nitrate and nitrite in flue-cured tobacco to the levels which are equivalent to those in burley tobacco can increase the TSNA concentration comparable

to burley tobacco especially coupled with warm temperature. Results indicated that the great amount of nitrosating species available is a major contributor to the formation of TSNA observed in cured tobacco during storage.

3.3. Effects of Indirect Addition of Nitrate/Nitrite on TSNA Formation in Flue-Cured Tobacco during Warm Temperature Storage. TSNA contents increased as the storage temperature increased [15, 16], and the abundance of nitrite and nitrate could be a major contributor. To clarify how nitrate or nitrite affects the formation of TSNA under warm temperature, indirect addition of nitrate or nitrite experiments was carried out.

After tobacco leaf treated with 1 mol/L NaNO_2 , total TSNA content increased almost by 54 times compared with that in the control sample (Figure 4). When tobacco leaf is placed separately with gauze pad containing 310 mg nitrate, obvious increases were observed both in individual and

TABLE 1: Effects of added nitrite on TSNA's formation in flue-cured tobacco stored at 45°C for 15 d.

Storage conditions	Addition of NO_2^- (mg)	NNN ($\mu\text{g/g}$)	NAT ($\mu\text{g/g}$)	NAB ($\mu\text{g/g}$)	NNK ($\mu\text{g/g}$)	Total TSNA's ($\mu\text{g/g}$)
Before storage	0	0.08 ± 0.01^E	0.11 ± 0.01^E	0.004 ± 0.00^E	0.03 ± 0.00^D	0.22 ± 0.00^E
	0	0.13 ± 0.01^E	0.39 ± 0.02^E	0.02 ± 0.00^E	0.03 ± 0.01^D	0.56 ± 0.03^E
45°C for 15 d	33	5.85 ± 0.28^D	18.97 ± 0.31^D	0.31 ± 0.03^D	0.09 ± 0.01^{CD}	25.22 ± 0.07^D
	67	12.85 ± 0.88^C	41.73 ± 1.95^C	0.77 ± 0.06^C	0.23 ± 0.03^C	55.57 ± 2.74^C
	100	19.22 ± 1.13^B	51.96 ± 1.54^B	1.15 ± 0.15^B	0.66 ± 0.09^B	72.99 ± 2.44^B
	133	33.66 ± 2.18^A	79.68 ± 0.96^A	2.25 ± 0.23^A	1.21 ± 0.16^A	116.80 ± 3.53^A

Note. Each value represents the mean of three independent samples, and uppercase letters indicate significant differences between the treatments at $P < 0.05$.

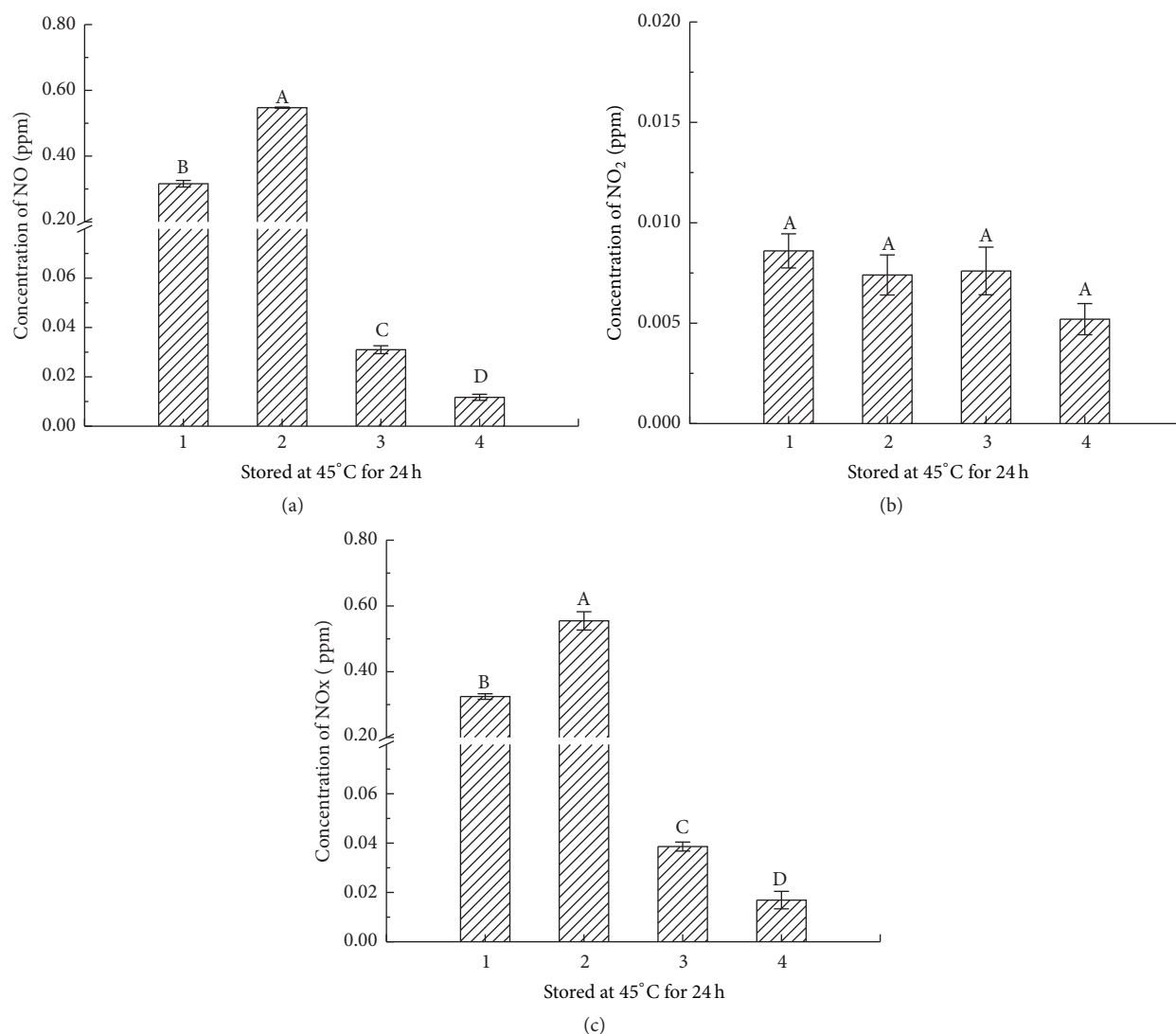


FIGURE 5: Effect of indirect addition of nitrate and activated carbon (AC) on nitrogen oxides formation in a closed system with flue-cured tobacco. Note. (1) tobacco, (2) tobacco + NaNO_3 , (3) tobacco + NaNO_3 + AC 2.0 g, and (4) tobacco + NaNO_3 + AC 10.0 g, 40 g of flue-cured tobacco, and 0.8 g NaNO_3 (583 mg of NO_3^-). The NO_3^- , AC, and tobacco were added separately in the vacuum desiccators stored at 45°C for 24 h. Uppercase letters indicate significant differences between the treatments at $P < 0.05$.

in total TSNA content compared with the control. Data presented here suggested that even though the tobacco sample is placed separately with nitrate or nitrite, TSNA content in tobacco also increased. The formation of TSNA's in storage triggered by nitrate and nitrite is likely a gas phase reaction.

3.4. Effect of Indirect Addition of Nitrate/Nitrite on NO_x Formation in a Closed System with Flue-Cured Tobacco. Flue-cured tobacco leaves could generate trace concentrations of NO_x under 45°C after 24 h treatment (Figures 5 and 6). As a main component, NO accounted for more than 95% of the NO_x produced from the tobacco sample. As 0.8 g of

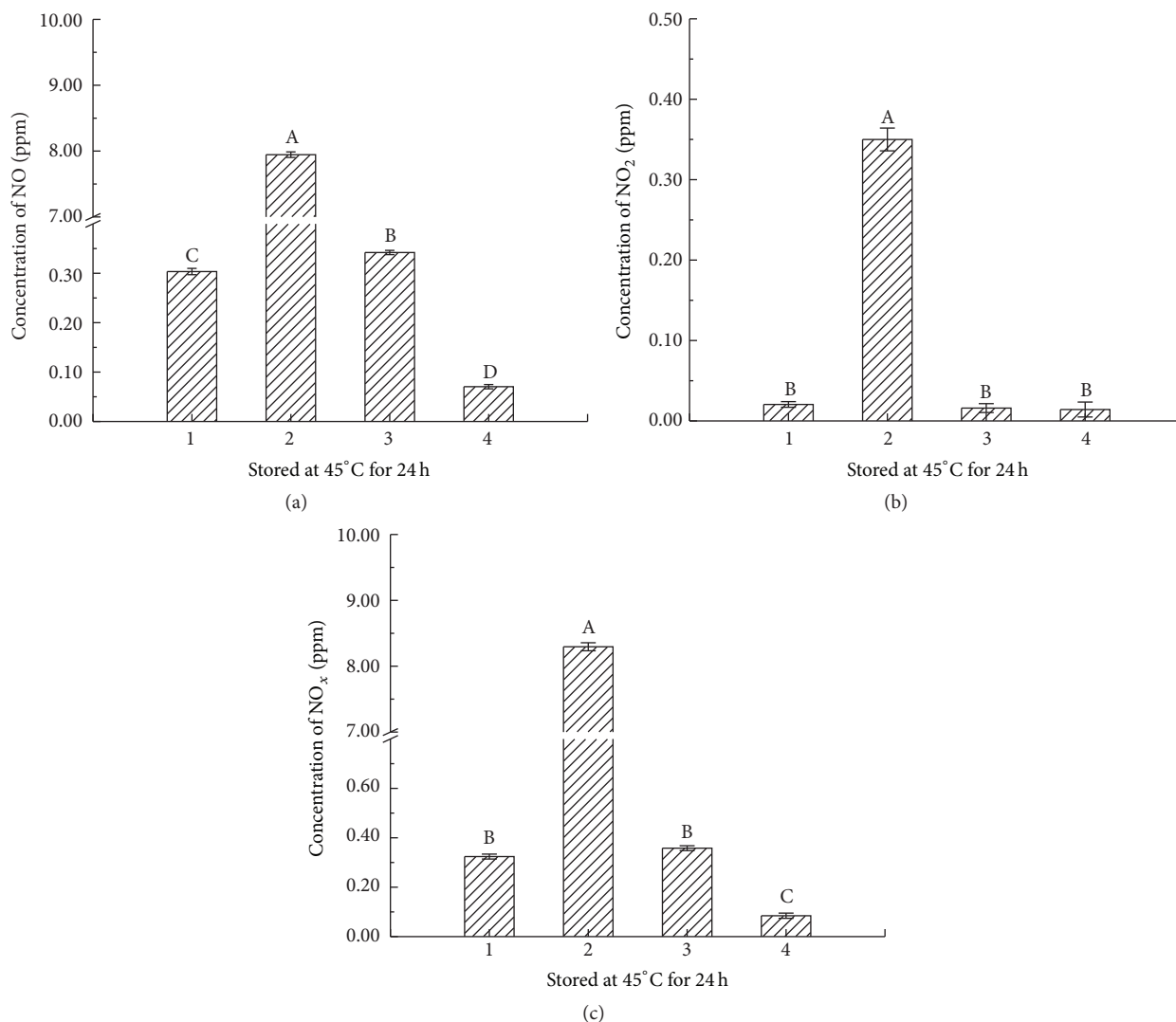


FIGURE 6: Effect of indirect addition of nitrite and activated carbon (AC) on nitrogen oxides formation in a closed system with flue-cured tobacco. Note. (1) tobacco, (2) tobacco + NaNO₂, (3) tobacco + NaNO₂ + AC 2.0 g, and (4) tobacco + NaNO₂ + AC 10.0 g, 40 g of flue-cured tobacco, and 0.4 g NaNO₂ (266 mg of NO₂⁻). The NO₂⁻, AC, and tobacco were added separately in vacuum desiccators stored at 45°C for 24 h. Uppercase letters indicate significant differences between the treatments at $P < 0.05$.

NaNO₃ (equal to 583 mg of nitrate) was added in the vacuum desiccator, the concentration of NO and NO_x increased significantly ($P < 0.05$); NO_x reached 0.6 ppm (Figure 5(c)). When 0.4 g of NaNO₂ (equal to 266 mg of nitrite) was added, NO_x concentrations in the desiccator dramatically increased to 8.3 ppm, which was 24.6-fold greater than the control (Figure 6(c)).

It is interesting that when 2 g of AC was added to the system (the weight ratio of tobacco, NO₃⁻, and AC is 68 : 1 : 3; the weight ratio of tobacco, NO₂⁻, and AC is 150 : 1 : 7), a 97% decrease of NO_x level was observed in comparison to the added nitrate treatment (Figure 5(c)), and almost 7.9 ppm of NO_x were adsorbed by AC (Figure 6(c)). Meanwhile, similar adsorption effects were observed for NO. The concentration of NO and NO_x decreased significantly ($P < 0.05$) as the addition of AC increased, indicating strong adsorption for NO_x generated from nitrate and nitrite by AC.

Table 2 presents the desorption rate of NO_x from the AC samples which were used for the adsorption experiments and were significantly higher than those from control group. The desorption rates of NO_x and NO were obviously increased with the prolonging of treating time and the increase of NO_x adsorption observed in Figures 5 and 6. Results indicated that NO_x were indeed adsorbed by AC. As the weight ratio of tobacco, NO₂⁻, and AC is 150 : 1 : 7, NO_x decreased by 7.9 ppm (Figure 6(c)); at the same time 0.632 ppm of NO_x was desorbed by the AC after treatment at 60°C for 90 min. Since the desorption rate of NO_x in AC has close relationship with the temperature and time [25], further research is needed to determine the effect of desorbing temperature and time on the desorption rate of NO_x adsorbed by AC.

During flue-curing process, direct-fired systems allow combustion products, specifically NO_x, to mix with the air and expose the green tobacco leaves to these gases [26]. The

TABLE 2: The desorption rate of NO_x at 60 °C for different time.

Treatment		NO (ppm)		NO _x (ppm)	
		15 min	90 min	15 min	90 min
AC (Control)		0.0094 ^C	0.0249 ^C	0.0203 ^B	0.0486 ^B
AC used in Figure 5	Treatment 3 (2 g AC)	0.0200 ^B	0.0466 ^B	0.0220 ^B	0.0505 ^B
	Treatment 4 (10 g AC)	0.0216 ^B	0.0471 ^B	0.0244 ^B	0.0511 ^B
AC used in Figure 6	Treatment 3 (2 g AC)	0.0732 ^A	0.6200 ^A	0.0864 ^A	0.6320 ^A
	Treatment 4 (10 g AC)	0.0740 ^A	0.6302 ^A	0.0887 ^A	0.6435 ^A

Note. Each value represents the mean of three independent samples, and uppercase letters indicate significant differences between the treatments at $P < 0.05$.

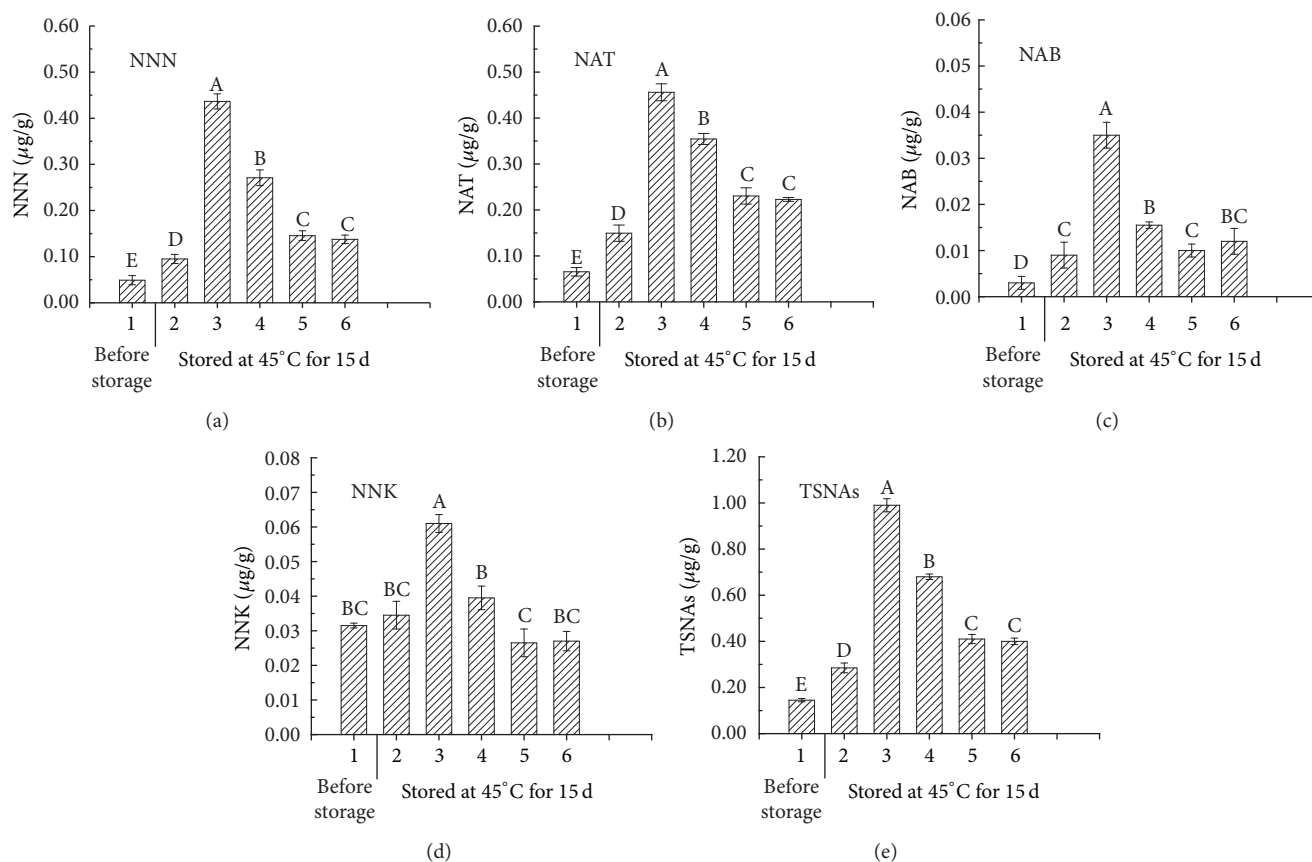


FIGURE 7: Effect of activated carbon (AC) on TSNAs formation in flue-cured tobacco in response to nitrate addition. Note. (1) tobacco (before storage, 4 °C), (2) tobacco, (3) tobacco + NaNO₃, (4) tobacco + NaNO₃ + AC 1.0 g, (5) tobacco + NaNO₃ + AC 5.0 g, and (6) tobacco + NaNO₃ + AC 10.0 g, 20 g of flue-cured tobacco, and 0.3 g NaNO₃ (219 mg of NO₃⁻). The NO₃⁻, AC, and tobacco were added separately in the vacuum desiccators stored at 45 °C for 15 d. Uppercase letters indicate significant differences between the treatments at $P < 0.05$.

previous research showed that TSNAs may be formed by nitrosation via the elevated levels of NO_x in the air surrounding the leaves during the curing process [27]. However, there were few reports about the relationship between NO_x and TSNAs during storage process. Data in Figure 4 suggested that TSNAs in storage is likely a gas phase reaction. This result showed that NO_x can be generated from NO₂-N and NO₃-N under 45 °C after 24 h. Nitrite was more effective than nitrate in the production of NO_x which in turn would probably promote the formation of TSNAs.

3.5. Effect of AC on TSNAs Formation in Flue-Cured Tobacco in Response to Nitrate and Nitrite Added. Having a very porous structure and special surface properties, AC has been used to trap TSNAs in tobacco solution [28, 29]. Lin et al. [30] reported that AC made from coconut shells exhibited a high affinity for TSNAs. The impregnated sorbent ZnAC can remove 73% of the TSNAs in solution, offering a cost-effective candidate for industrial applications [29].

As shown in Figures 7(e) and 8(e), total TSNAs content in tobacco incubated separately with NO₂⁻ (treatment 3) at

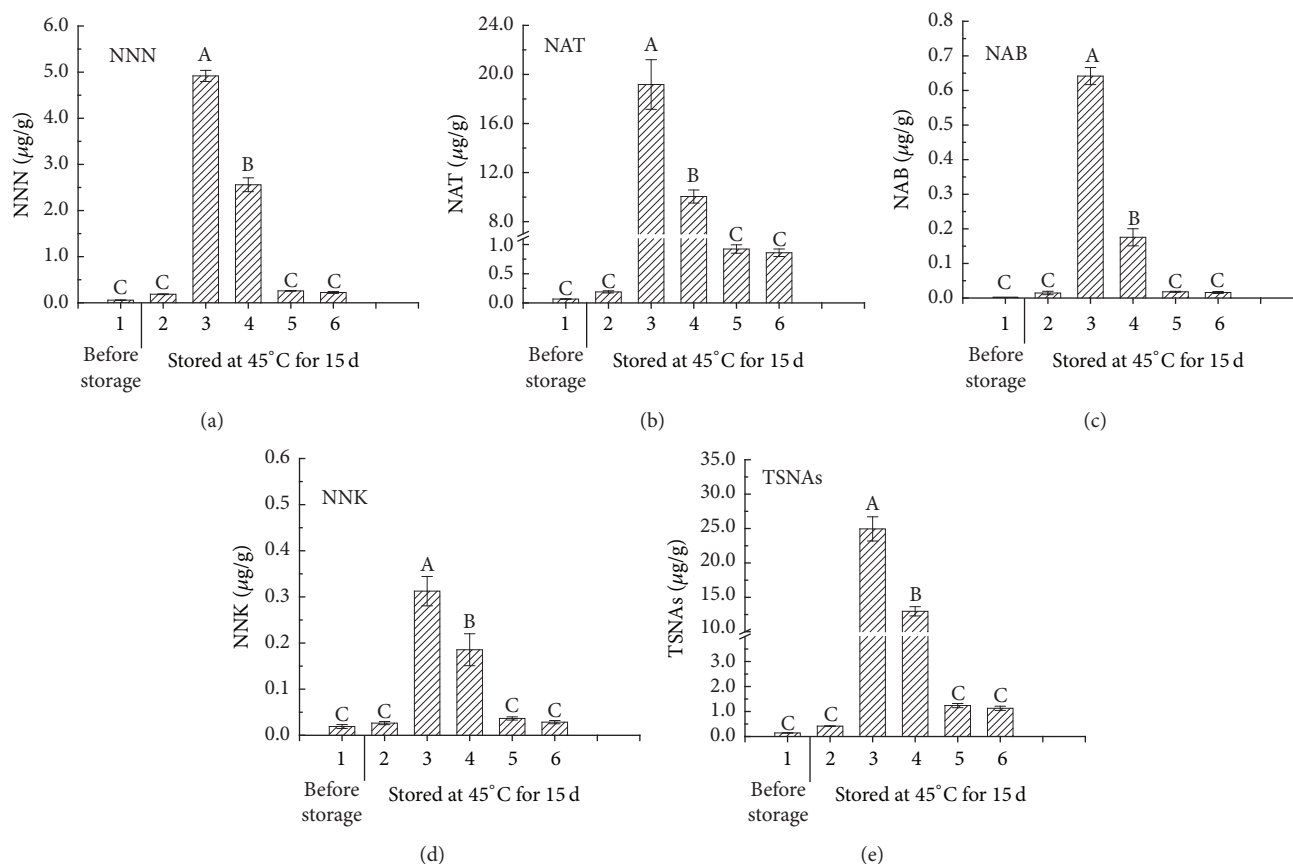


FIGURE 8: Effect of activated carbon (AC) on TSNAs formation in flue-cured tobacco in response to nitrite addition. *Note.* (1) tobacco (before storage, 4°C), (2) tobacco, (3) tobacco + NaNO_2 , (4) tobacco + NaNO_2 + AC 1.0 g, (5) tobacco + NaNO_2 + AC 5.0 g, and (6) tobacco + NaNO_2 + AC 10.0 g, 20 g of flue-cured tobacco, and 0.3 g NaNO_2 (200 mg of NO_2^-). The NO_2^- , AC, and tobacco were added separately in the vacuum desiccators stored at 45°C for 15 d. Uppercase letters indicate significant differences between the treatments at $P < 0.05$.

45°C increased markedly to 25 $\mu\text{g/g}$, a 59-fold increase over the control (treatment 2). By treating tobacco with NO_3^- , however, total TSNAs increased to 1 $\mu\text{g/g}$, also significantly higher than the control. The huge difference of TSNAs content between tobacco treated with NO_2^- and NO_3^- could be attributed to the high concentration of NOx which were emitted from NO_2^- under the same condition.

However, after adding 1 g of AC to the separating system of tobacco and NO_2^- , the weight ratio of tobacco, NO_2^- , and AC was 100:1:5, both individual and total TSNAs contents of tobacco decreased markedly, with total TSNAs content decreasing to 13 $\mu\text{g/g}$, 48% lower than in treatment 3, and NNN, NAT, NAB, and NNK decreasing by 47.9%, 47.6%, 67.3% and 41%, respectively. When the addition of AC increased to 5 g, the weight ratio of tobacco, NO_2^- and AC reached to 100:1:25, total TSNAs levels further decreased to 1.2 $\mu\text{g/g}$, indicating a crucial role of AC in inhibiting TSNAs formation by adsorbing the NOx generated from NO_2^- -N and NO_3^- -N. The results also showed no significant differences in reducing TSNAs levels by increasing AC usage greater than 5 g (Figures 7 and 8). The maximum inhibitory effect of AC on TSNAs formation occurred at an AC/ NO_2^- ratio of 25/1. The above results indicated that AC may have the potential to be

used as adsorbent agent to reduce TSNAs formation during tobacco leaf storage. Our results strongly support the theory that TSNAs formation in storage is a gas phase reaction, and the NOx which is produced from nitrate and nitrite could intermediate the TSNAs formation during warm temperature postcured storage of tobacco. Since NOx can be produced from nitrate and nitrite under warm temperature in a short period, it can readily react with alkaloids to form TSNAs. This result also explained why the most rapid increase in TSNAs occurred during the warm temperature season [16].

Decreasing NOx level by AC adsorption significantly reduced TSNAs formation of tobacco which indicated that the removal of NOx from storage environment could be an effective way to inhibit TSNAs formation in storing tobacco leaf. Therefore, controlling the storage environment and scavenging gaseous nitrosation agents would be crucial to reduce or inhibit TSNAs formation during leaf storage.

4. Conclusion

The results proved that TSNAs are derived from a gas/solid phase nitrosation reaction between NOx and alkaloids during

storage. Nitrogen oxides produced from nitrate and nitrite are responsible for the formation of TSNAs during storage under warm temperature. Presence of activated carbon in the tobacco storage containers effectively inhibited the TSNAs formation due to the adsorption of NO_x on the activated carbon.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Authors' Contributions

Jun Wang and Huijuan Yang contributed equally to this work and should be considered co-first authors.

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