Potential Nitrite Scavengers as Inhibitors of the Formation of *N*-Nitrosamines in Solution and Tobacco Matrix Systems

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The ability of 20 compounds, all but one tobacco constituents, to inhibit the formation of tobacco-specific *N*-nitrosamines (TSNA) was investigated in buffer and detergent solution and in tobacco midrib and lamina systems. In solution at pH 5.5, ascorbic acid and the phenolic acids caffeic and ferulic acid were the most potent inhibitors of the reaction between nornicotine and nitrite, with nearly complete inhibition at molar ratios test compound/nitrite > 1:1. Also, cysteine > dihydrocaffeic acid > protocatechuic acid ≈ catechin acted as strong inhibitors with >90% inhibition at a ratio of 3:1. Lower inhibitions were observed with chlorogenic acid > *p*-coumaric acid > sclareol > serine. Rutin showed an inhibition of 34% at a ratio of 0.1:1. Sclareol, alanine, proline, and serine did not significantly affect the *N*-nitrosonornicotine (NNN) formation. α-Tocopherol and glutathione enhanced NNN formation at pH 5.5 but were inhibitors at pH 3. Cinnamic acid, vanillic acid, eugenol, and esculin enhanced NNN formation. Increased NNN formation was also observed for dihydrocaffeic acid, chlorogenic acid, protocatechuic acid, and catechin at a less-than-equimolar ratio of test compound to nitrite. The tobacco matrix experiments were performed with air-cured, ground tobacco midrib and lamina. Caffeic acid, ferulic acid, dihydrocaffeic acid and catechin were potent inhibitors of the formation of TSNA in the midrib as well as in the lamina. Also protocatechuic acid, glutathione, ascorbic acid, *p*-coumaric acid, chlorogenic acid and cysteine were inhibitors, while α-tocopherol and rutin inhibited the reaction in the midrib but not in the lamina. Cinnamic acid, vanillic acid, eugenol, alanine, proline and serine showed small effects only. The molar ratio of secondary alkaloid(s)/nitrite in the test systems were 0.1:1 (solution), ∼0.25:1 (midrib), and ∼1:1 (lamina) and is most likely the major contributor to the observed order of inhibition potency (solution > midrib > lamina) of the test compounds. The vicinal phenolic hydroxyl groups of polyphenols and the simultaneous presence of a phenol group and an olefinic bond in hydroxycinnamic acids were the most characteristic structural elements of the potent inhibitors.

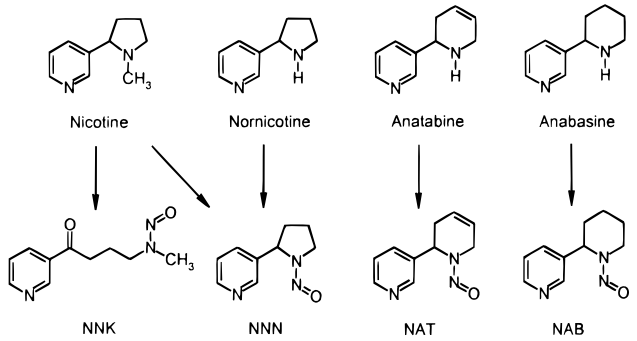
Keywords: *Tobacco-specific N-nitrosamines; nitrosation; nornicotine; secondary alkaloids; nitrite scavengers; ascorbic acid; α-tocopherol; phenolic acids; flavonoids; coumarins, amino acids; tobacco matrix; midrib; lamina*

INTRODUCTION

Nitrosamines have been identified in water, beverages, foodstuffs, cosmetics, and tobacco products (Tricker, 1997). They have received particular attention due to their potential toxicity. *N*-Nitrosamines are formed in the reaction between amines and nitrite-derived species such as NO_2, N_2O_3, and N_2O_4 under acidic conditions. The nitrosation reaction is usually fast with secondary and slow with tertiary amines (Williams, 1988). The reaction with secondary amines is known to be highly pH-dependent, with an optimum at pH 3.0−3.4 (Mirvish et al., 1977).

Tobacco-specific nitrosamines (TSNA) have been the subject of intense research during the past two decades (Hecht, 1998). The group includes as major constituents *N*-nitrosonornicotine (NNN), *N*-nitrosoanatabine (NAT), *N*-nitrosoanabasine (NAB), and 4-methyl-*N*-nitrosamino-1-(3-pyridyl)-1-butanone (NNK). As illustrated in Scheme 1, the first three nitrosamines are formed by *N*-nitrosation of the secondary alkaloids nornicotine, anatabine, and anabasine, respectively. NNN may also derive from

Scheme 1. Tobacco-specific *N*-nitrosamines Formed by Nitrosation of Tobacco Alkaloids



nicotine, which is a tertiary amine. In this case, however, the reaction also involves breakage of a carbon−nitrogen bond, and the reaction rate becomes much slower (Caldwell et al., 1993; Williams, 1988). NNK is derived from nicotine, with nicotine-*N*-oxide and pseudooxynicotine as proposed intermediates (Caldwell et al., 1993; Klimisch and Stadler, 1976; Maeda et al, 1978).

Green tobacco contains virtually no TSNA. These nitrosamines are generated during the post-harvest

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treatment, i.e., curing (drying) and fermentation (Wiernik et al., 1995). Available data suggest that nitrite and TSNA start to accumulate after about two to three weeks of air-curing (Andersen et al., 1987). This is the time when the cells are disrupted due to moisture loss and nutrients are made accessible to bacteria. Some of these bacteria reduce nitrate with accumulation of nitrite. At the existing pH, 5.5, the nitrite may form nitrous anhydride, N_2O_3 (Lewis et al., 1995; Williams, 1988), which reacts with various tobacco constituents, including the tobacco alkaloids. Although substantial amounts of nitrite may be present during the air-curing process (more than 1000 μg/g), the TSNA content in tobacco after a conventional air-curing generally ranges between 2 and 20 μg/g. If the environmental conditions are particularly favorable for microbial growth, the production of nitrite may be enhanced, and higher levels of TSNA are observed (Burton et al., 1989; Wahlberg et al., 1999; Wiernik et al., 1995). Fermentation may, under certain conditions, further increase the levels in smokeless tobacco products. TSNA present in the cured tobacco is partly transferred to tobacco smoke, a minor portion being generated during smoking (Fischer et al., 1990).

Implementation of curing procedures in which the time for microbial growth is reduced would hence be a means to prevent or reduce the formation of TSNA. Another means would be to scavenge the nitrite as it is formed during air-curing by compounds other than the tobacco alkaloids. Support for the viability of the latter alternative comes from model studies which have shown that plant constituents such as ascorbic acid, polyphenols, flavonoids, and cysteine inhibit the formation of nitrosamines in model systems, foodstuffs, and in vivo (Archer, 1984; Bartsch and Frank, 1996; Shenoy and Choughuley, 1992).

In the present study, we examined the potential of selected tobacco constituents as inhibitors of the formation of NNN in an aqueous solution system with and without detergent at pH 5.5 and of TSNA in a tobacco matrix system consisting of ground midrib or ground lamina. The test compounds, whose structures are shown in Figure 1, were selected to represent different compound classes covering much of the relevant structural diversity in tobacco.

MATERIALS AND METHODS

Analyses. Nitrosamines were handled according to the directives of the Occupational Safety and Health Administration in Sweden, and the work was carried out in a dedicated and specially designed lab. Analyses of the *N*-nitrosamines formed were performed on an HP-6890 (Hewlett-Packard, Inc.) gas chromatograph, equipped with a Supelco SPB-1 column. Helium was used as carrier gas. A typical temperature program was as follows: 60 °C for 1 min, 40 °C/min to 150 °C, 2 °C/min to 175 °C, 40 °C/min to 240 °C. A TEA 543 (Thermedics Inc.) thermal energy analyzer (TEA) was used as the detector, with the temperatures of the interface and the pyrolyzer kept at 250 and 500 °C, respectively. The ability of each test compound to inhibit NNN (solution) or TSNA (sum of the concentration of NNN, NAT, NAB, and NNK; tobacco matrix) formation with respect to a reference sample, the inhibition, was calculated as $1 - [\text{TSNA}]_{\text{test compound}}/[\text{TSNA}]_{\text{reference}}$, reported in percent, where $[\text{TSNA}]_{\text{test compound}}$ and $[\text{TSNA}]_{\text{reference}}$ correspond to the concentrations of NNN or TSNA formed in the sample containing the test compound and in the reference sample, respectively. The inhibition for the tobacco experiments was calculated after subtraction of the initial TSNA content. A positive value signifies inhibition of TSNA formation; a negative value means that TSNA formation is enhanced. Nitrite was analyzed as $NaNO_2$ by a spectrophotometric procedure using an RFA 300 rapid flow analyzer (OI Analytical). Water activity (a_w) was analyzed at 25 °C using a Novasina a_w-center (Novasina AG).

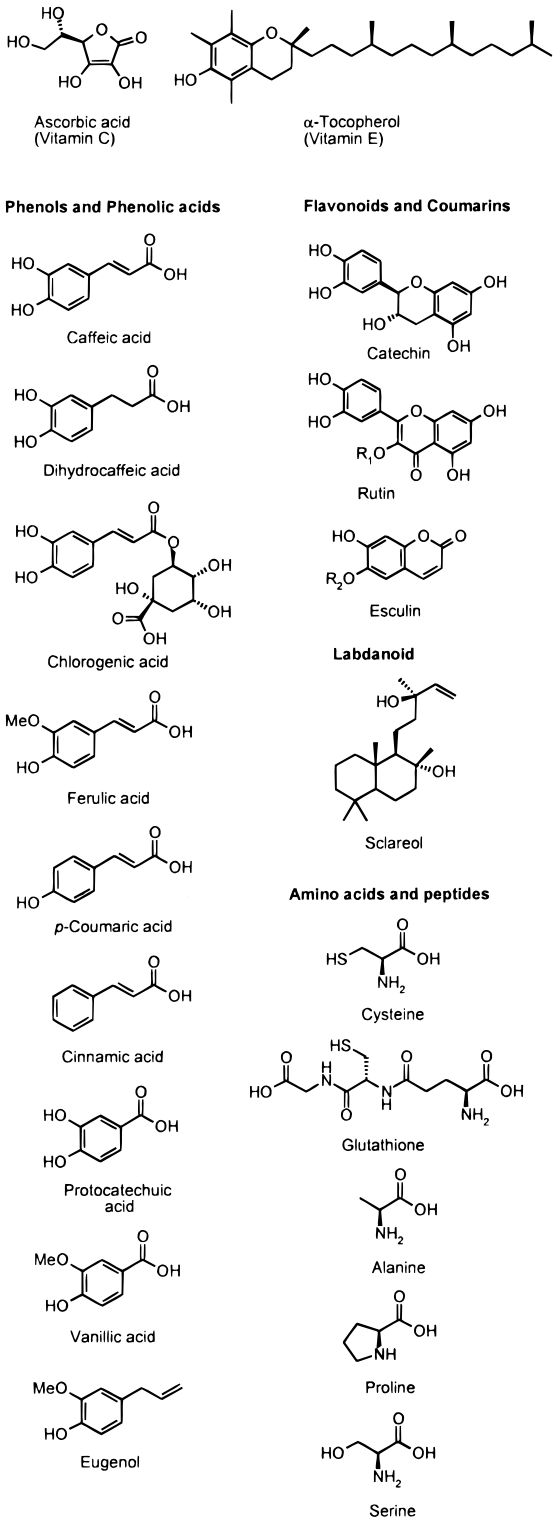


Figure 1. Structures of the test compounds. R_1 = α-L-6-deoxy-mannopyranosyl-(1 → 6)-β-D-glucopyranosyl-(1 → (rutinosyl) and R_2 = β-D-glucopyranosyl-(1 →.

Tobacco. Ground air-cured midrib (Indian tobacco) and lamina (African tobacco) were used for the tobacco matrix systems. The natural content of some chemical constituents in these tobaccos was measured and is listed in Table 1.

Table 1. Chemical Composition of the Tobaccos[a]

	midrib	lamina
nitrite (μg/g)	<10	<10
nicotine (%)	0.9	4.8
nornicotine (%)	0.05	0.2
anatabine (%)	0.02	0.1
anabasine (%)	0.01	0.03
NNN (μg/g)	4.5	0.6
NAT (μg/g)	3.1	1.0
NAB (μg/g)	<0.1	<0.1
NNK (μg/g)	0.9	<0.1
TSNA, total[b] (μg/g)	8.5	1.6
ascorbic acid (%)	<0.001	<0.001
α-tocopherol (%)	0.003	0.013
chlorogenic acid (%)	0.002	0.026
rutin (%)	0.003	0.222
polyphenols, total[c] (%)	0.009	0.327
alanine (%)	0.030	0.106
serine (%)	0.014	0.022

[a] All data are reported on dry-weight basis. [b] Total content of NNN, NAT, NAB, and NNK. [c] Total content of chlorogenic acid, caffeoyl-4- and caffeoyl-5-quinic acid, rutin, kaempferol-3-rutinoside, and scopoletin.

Chemicals and Solvents. All test compounds and other chemicals were commercially available and of high purity. Nornicotine was purchased from Sigma (*p.a.*) or synthesized in our laboratory. All solvents were of the highest available purity. Ultrapure deionized water (Milli-Q, Millipore) was used for all water solutions.

Experiments Performed in Solution. The molar ratios of test compound to nitrite were between 0.1:1 and 5:1, and the ratio of nornicotine to nitrite was set at 0.1:1. Buffer solutions were made by addition of a 0.1 M citric acid monohydrate solution to a 0.1 M K_2HPO_4 or a 0.2 M Na_2HPO_4 solution. Stock solutions of the test compounds were prepared by dissolving 0.075, 0.225, 0.75, 1.50, 2.25, 3.00, or 3.75 mmol of the compounds in K_2HPO_4−citrate buffer to a final volume of 25 mL. Inhibition experiments were also performed in sodium dodecyl sulfate (SDS) buffer solution to increase the solubility of certain test compounds. The emulsions were obtained by dissolving the test compound, 12.5 mmol of SDS, and 3.75 mL of methanol in Na_2HPO_4−citrate buffer to a final volume of 25 mL. The pH of stock solutions containing acidic test compounds was corrected to 5.5 with NaOH. For α-tocopherol and glutathione, additional experiments were performed at pH 3.0. Samples were prepared by mixing the stock solution of test compound (1 mL) with a 3 mM nornicotine solution in the buffer (1 mL), followed by the addition of a 0.3 M $NaNO_2$ solution in the buffer (0.1 mL). In the reference sample, buffer solution or SDS buffer solution replaced the stock solution. The samples were flushed with N_2 (to avoid oxidation of any NO formed), sealed, shaken, and kept in the dark at 25 °C using a water bath equipped with a thermostat. After 24 h, nitrosation was stopped by addition of 0.2 mL of 2 M NaOH. *N*-Nitrosopentyl-(3-picolyl)-amine (0.215 mL, 0.1 mg/mL in CH_2Cl_2) was added as the internal standard. The internal standard and NNN were extracted into CH_2Cl_2 or ethyl acetate (SDS samples) and analyzed on a GC-TEA. The experiments were performed in triplicate. Test compounds with inhibitory effects showed standard deviations of the calculated inhibitions within each triplicate of less than 10% in the solution systems and less than 15% in the SDS detergent systems.

Experiments Performed in Tobacco Matrix. The model system consisted of ground tobacco midrib (pH of ∼6.3) or lamina (pH ∼5.4). Solutions (10 mL) of the test compounds were added to plastic bags containing 50 g of tobacco to produce test compound/nitrite molar ratios ranging from 0.1:1 to 10:1 after nitrite addition (see below). Caffeic acid, dihydrocaffeic acid, ferulic acid, cinnamic acid, vanillic acid, catechin, and rutin were dissolved in ethanol, *p*-coumaric acid and eugenol were dissolved in methanol, and α-tocopherol was dissolved in hexane. The other test compounds were added as water solutions. The samples were carefully mixed and equilibrated at room temperature overnight. In experiments where the test compound was added as ethanol, methanol, or hexane solutions, the bags were left open overnight to let the solvent evaporate. For experiments performed with ascorbic acid, additional experiments were performed where ascorbic acid was added simultaneously with nitrite at the start of the experiment. NaOH was added to restore the pH when necessary. Reference samples were prepared in the same way, omitting the test compound. After equilibration, a water solution of $NaNO_2$ (1500 μg/g tobacco) was added to each mixture to obtain a final moisture content of 40% in the tobacco samples. The water activity at this moisture level, ∼0.75 in the midrib and ∼0.82 in the lamina samples, was measured to ensure that bacterial activity was prevented. The samples were carefully mixed and incubated at 25 °C. TSNA was analyzed after 1 and 5 days of incubation according to the procedure of Spiegelhalder et al. (1989). The experiments were performed in triplicate. The relative standard deviations of the concentrations of TSNA formed were generally less than 10% and in all cases less than 20%.

RESULTS AND DISCUSSION

Solution. The inhibition experiments were performed for 20 compounds at various test compound/nitrite molar ratios. The results are presented as percent inhibition and are reported in Table 2. The approximate order of inhibition of NNN formation by the most effective inhibitors at the highest test compound/nitrite ratio (3:1, 4:1, or 5:1) was as follows: ascorbic acid ≈ cysteine > caffeic acid > ferulic acid > dihydrocaffeic acid > catechin ≈ protocatechuic acid. These compounds showed inhibitions of 70−100%. Considerably lower inhibitions (less than 40%) were observed with chlorogenic acid > *p*-coumaric acid > sclareol > serine > proline ≈ alanine. For rutin, which showed poor solubility, a value of 34% at a 0.1:1 ratio of rutin/nitrite was observed. α-Tocopherol, cinnamic acid, vanillic acid, eugenol, esculin, and glutathione all promoted the formation of NNN in solution at the highest ratio.

The inhibition observed with ascorbic acid corroborates previous findings that ascorbic acid is a nitrite scavenger and an inhibitor of nitrosamine formation, with an optimum reaction rate at pH 3−4 (Lathia et al., 1988; Mirvish, 1981; Tannenbaum and Mergens, 1980). In particular, Castonguay and van Vunakis (1979) showed that ascorbic acid almost completely inhibits the nitrosation of nornicotine by nitrite at an ascorbic acid/nitrite ratio of 1:1 in strong acid (pH ≈ 1). Caffeic acid and ferulic acid, both potent inhibitors in the present study, have previously been shown to inhibit *N*-nitrosodimethylamine and *N*-nitrosaminopyrine formation in gastric juice at pH 1.7, 3.2, and 6.1 (Dikun et al., 1991). Similarly, the tea substance catechin and other flavonoids have been reported to scavenge nitrite (Choi et al., 1989) and either enhance or inhibit *N*-nitrosoproline formation (Lee and Choi, 1993). Protocatechuic, *p*-coumaric, and chlorogenic acid were all inhibitors in the present study and have previously been reported to inhibit the formation of *N*-nitrosomorpholine (Li et al., 1994). The poor inhibition/catalysis obtained with eugenol is in conflict with the finding that this phenol is a moderate inhibitor of *N*-nitrosamine formation at pH 3.5 (Shenoy and Choughuley, 1989). Limited effects have, in agreement with our results, been reported for cinnamic and vanillic acid (Kikugawa et al., 1983; Li et al., 1994). α-Tocopherol has been reported to scavenge nitrite and to inhibit *N*-nitrosamine formation (Lathia and Blum, 1989; Mergens et al., 1978). In the present study, how-

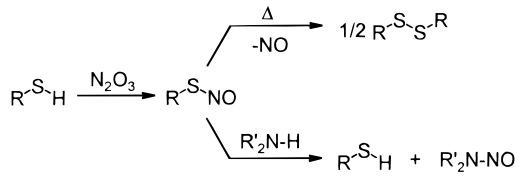
Table 2. Effects of Test Compounds on NNN Formation in Solution[a]

compound	molar equiv.[c]	inhibition[b] (%) solution	detergent
ascorbic acid	0.1	7	37
	1	97	100
	4	97	100
α-tocopherol	0.1		−27, 24[d]
	1		−32, 57[d]
	3		−18, 75[d]
caffeic acid	0.1		36
	1		93
	3		95
dihydrocaffeic acid	0.1	−244	−19
	1	78	75
	3	93	91
chlorogenic acid	0.1	−32	21
	1	−7	54
	3	20	40
ferulic acid	0.1		27
	1		86
	3		94
p-coumaric acid	0.1		5
	1		33
	3		24
cinnamic acid	0.1		−13
	1		−27
	3		−52
protocatechuic acid	0.1	−92	16
	1	53	71
	3	73	88
vanillic acid	0.1		−121
	1		−67
eugenol	0.1		26
	1		−11
	3		−75
catechin	0.1	−1485	
	1	40	
	3	89	
rutin	0.1		34
esculin	0.1	−6	−5
	0.3	−13	−10
	1		−22
sclareol	0.1		−7
	1		−9
	3		21
glutathione	0.1	−95, 6[d]	
	1	−494, 77[d]	
	2	−545, 98[d]	
cysteine	0.1	18	24
	1	84	23
	3	97	96
	5	98	
alanine	0.1	−3	
	1	−2	
	3	0	
proline	0.1	0	
	1	1	
	3	1	
serine	0.1	2	
	1	3	
	3	8	

[a] Studied at pH 5.5 and 25 °C with 1.4 mM nornicotine and 14 mM nitrite. [b] Inhibition with respect to the reference solution. A negative value signifies enhanced NNN formation. [c] Molar equivalent of test compound with respect to nitrite. [d] Measured at pH 3.0.

ever, α-tocopherol either enhanced or inhibited NNN formation (see below). Cysteine and glutathione have been reported to inhibit *N*-nitrosamine formation at pH 3.5 (Shenoy and Choughuley, 1992). The results obtained in this study at pH 5.5 for these thiols are slightly different and are discussed below. Esculin slightly favored the NNN formation at pH 5.5. This is in accord with observations that esculin was not a nitrite scavenger in solution at pH 3 (Choi et al., 1989).

Scheme 2. Reactions of Thiols with Nitrite via N_2O_3 (Williams, 1985)[a]



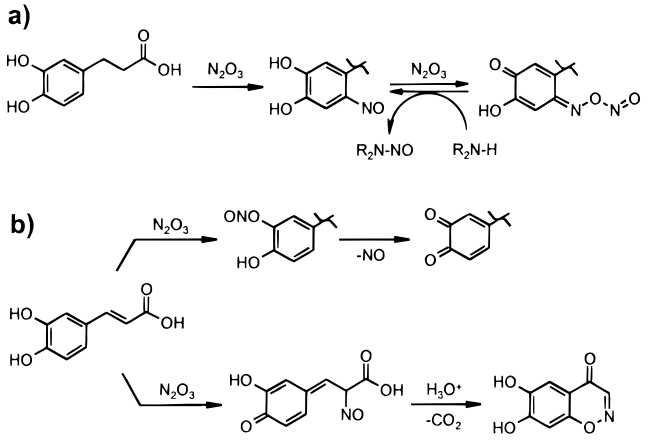
[a] The upper route results in scavenging of nitrite and the lower route results in transnitrosation.

Inhibition versus Catalysis. The actions of cysteine and glutathione may be described by two pathways (Scheme 2) resulting in inhibition and catalysis, respectively (Williams, 1985). The inhibition obtained with cysteine can be explained by the upper route, where the *S*-nitrosothiol formed decomposes to the disulfide. Nitrite is reduced to NO, and the formation of NNN is inhibited. The strong catalysis observed with glutathione at pH 5.5 might be a result of transnitrosation by an *S*-nitrosothiol (depicted by the lower route). Support for the viability of this route comes from reports that the transnitrosation by nitrosocysteine is considerably more rapid at pH 5.5 than at a lower pH (Davies et al., 1978) and that the inhibition of *N*-nitrosamine formation by cysteine and glutathione decreases when the pH increases (Gray and Dugan Jr., 1975). In agreement with this, we observed that glutathione almost completely inhibited the *N*-nitrosation of nornicotine in solution at pH 3.

α-Tocopherol catalyzed NNN formation in detergent solution at pH 5.5 and TSNA formation in the lamina, but inhibited TSNA formation in the midrib (see below). The reason for these conflicting results is not fully understood, but the presence of different reaction pathways should influence the nitrite scavenging. α-Tocopherol scavenges nitrite by producing α-tocopherolquinone and NO (Lathia and Blum, 1989; Tannenbaum and Mergens, 1980). The NO formed may, however, react with the remaining α-tocopherol either directly or after oxidation to NO_2 (Cooney et al., 1993; Janzen et al., 1993). This results in the formation of intermediate nitrite esters that may catalyze *N*-nitrosation. The nitrite scavenging by α-tocopherol has been reported to be pH-dependent, with a much higher rate at lower pH (Kamm et al., 1977). In agreement with this, we observed that at pH 3 the formation of NNN was inhibited.

Catalysis of NNN formation in solution was observed with some of the phenolic test compounds at less than equimolar ratios of test compound/nitrite, particularly for catechin and dihydrocaffeic acid (see Table 2). Analogous results were reported by Pignatelli et al. (1982), who observed a pH dependence with the largest catalytic effect at pH 4 or higher. This effect is most likely explained by the formation of a *C*-nitrosophenol, which reacts with excess N_2O_3 and produces a powerful transnitrosating agent (Archer, 1984; Davies et al., 1978). This route is illustrated for dihydrocaffeic acid in Scheme 3a. At higher than an equimolar test compound/nitrite ratio, however, both catechin and dihydrocaffeic acid were strong inhibitors. Protocatechuic and chlorogenic acid showed a similar behavior. This is in agreement with reports on inhibition as well as catalysis of nitrosamine formation by chlorogenic acid (Challis

Scheme 3. Reactions of Phenolic Acids with Nitrite via N_2O_3[a]



[a] In reaction a, C-nitrosation, exemplified by dihydrocaffeic acid, results in the formation of a nitrosating agent and subsequent catalysis of nitrosamine formation (Davies et al., 1978). In reaction b, inhibition is achieved when nitrite is scavenged by two different routes (Challis and Bartlett, 1975; Rousseau and Rosazza, 1998), exemplified by caffeic acid.

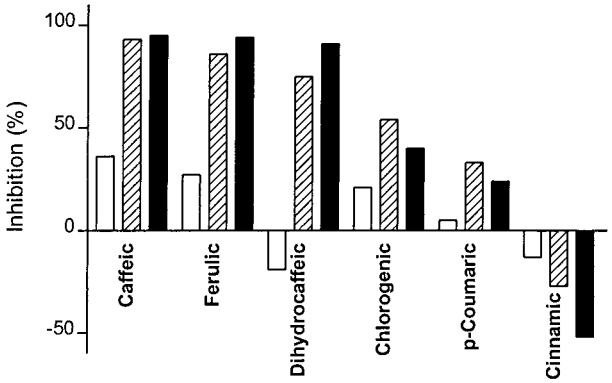


Figure 2. Inhibition of NNN formation in detergent solution by cinnamic acid and various hydroxycinnamic acids at compound/nitrite ratios of 0.1:1 (open bars), 1:1 (dashed), and 3:1 (solid bars).

and Bartlett, 1975; Kikugawa et al., 1983; Kono et al., 1995; Rao et al., 1982).

Structure−Activity of Phenolic Acids. The inhibition potencies of thirteen phenolic acids have been related to their chemical structures by Li et al. (1994), who reported inhibitions at pH 3 for a mixture equimolar in morpholine, nitrite, and the test compound. In their study, the order of activity was caffeic acid > ferulic acid > chlorogenic acid > protocatechuic acid > *p*-coumaric acid > vanillic acid. Effective inhibition was attributed to (i) the number and position(s) of the phenolic hydroxyl group(s) and (ii) the presence of a double bond of the side chain in the cinnamic acid derivatives. A similar relationship between structure and inhibition is found here for the inhibition of the NNN formation in SDS detergent solution at pH 5.5 by the phenolic acids structurally related to cinnamic acid. The inhibitions obtained are illustrated in Figure 2. The presence of the 3,4-dihydroxy groups in caffeic, dihydrocaffeic, and protocatechuic acid resulted in strong inhibition. As shown by the upper route in Scheme 3b, the diphenol is oxidized to an *ortho*-quinone, while the nitrite is reduced to NO (Challis and Bartlett, 1975). Furthermore, the effectiveness of caffeic and ferulic acid as inhibitors can be ascribed to the interplay between the *para*-phenolic hydroxyl group and the conjugated olefinic bond upon C-nitrosation at the side-chain (Kikugawa et al., 1983; Rousseau and Rosazza, 1998), as shown in the lower route of Scheme 3b. Chlorogenic acid (caffeoyl-3-quinic acid) showed limited inhibition of NNN formation in detergent solution despite the strong inhibition by its analogue caffeic acid. Most likely, the ability of C-nitrosation at the conjugated olefinic bond is limited or prevented upon esterification of the carboxy group. Monophenolic compounds, which lack the conjugated olefinic bond, i.e., eugenol and vanillic acid, cannot form a quinone and may therefore scavenge nitrite by C-nitrosation at a carbon ortho to the hydroxyl group (González-Mancebo et al., 1999). Consequently, a nitrosating species is formed which catalyzes NNN formation. Cinnamic acid, which lacks phenolic groups, was a catalyst at all test compound/nitrite ratios studied.

Tobacco Matrix. The tobacco matrixes are considerably more complex than the solutions as model systems, and there are distinct chemical differences between lamina and midrib. It may therefore be worthwhile to discuss briefly the chemical composition of the tobacco systems. Thus, as reported in Table 1, the concentrations of the tobacco alkaloids were 3−6 times higher in the lamina than in the midrib. Similarly, several of the test compounds, e.g., α-tocopherol, alanine, serine, rutin, and chlorogenic acid, the latter the major polyphenol in green leaves of tobacco (Court and Hendel, 1985), showed higher concentrations in the lamina than in the midrib. The polyphenol concentration exceeded 0.3% (dry weight) in the lamina, which corresponds to ∼6 μmol/g tobacco (assuming an average molecular weight of 500). This value can be compared to the ∼22 μmol/g of nitrite added at the start of the experiment. The content of ascorbic acid was below the detection limit in both the midrib and the lamina. Ascorbic acid is, however, present in green leaves of tobacco (Weston, 1968), and the levels are higher in lamina than in midrib (Wahlberg et al., 1995). There are also endogenous compounds other than the alkaloids and test compounds in tobacco that may react with nitrite, e.g., amines, alcohols (carbohydrates), unsaturated fatty acids, polyphenols, thiols, amino acids, and peptides. Several of these occur in higher concentrations in lamina than in midrib (Tso, 1972). Lignin, which has been reported to scavenge nitrite (Møller et al., 1988), is present in substantial amounts both in lamina and midrib (Andersen and Litton, 1975).

TSNA Formation in the Tobacco Reference Samples. The initial concentrations of TSNA were 8.5 μg/g tobacco (dry weight) in the midrib and 1.6 μg/g in the lamina, NNN and NAT being more abundant than NAB and NNK (Table 1). After incubation with 1500 μg/g of sodium nitrite at a moisture level of 40% for 1 and 5 days, the concentration of TSNA increased to 32 and 46 μg/g, respectively, in the midrib and to 52 and 55 μg/g, respectively, in the lamina reference samples. The nitrite content decreased to ∼250 μg/g in the midrib and to ∼10 μg/g in the lamina reference samples after 1 day of incubation. After 5 days, most of the nitrite was consumed in both matrixes.

The substantially higher concentrations of tobacco alkaloids and the slightly lower pH in the lamina samples reasonably account for the more rapid accumulation of TSNA in the lamina than in the midrib reference samples. Lamina is also likely to contain a

Table 3. NNN and NAT Concentrations in the Reference Tobacco Matrix Experiments[a]

	midrib		lamina	
	NNN (μg/g)	NAT (μg/g)	NNN (μg/g)	NAT (μg/g)
tobacco[b]	4.5	3.1	0.6	1.0
day 1	11.1	19.4	11.0	38.8
day 5	16.2	27.7	11.7	40.5

[a] Average values reported on dry-weight basis. [b] Measured in the ground tobaccos.

larger amount of efficient endogenous nitrite scavengers than does the midrib. This may be the reason the final concentrations of TSNA were not much higher in the lamina than in the midrib reference samples, despite the higher alkaloid content in the lamina.

It is of interest to note that more NAT than NNN was formed in the midrib and, even more pronounced, in the lamina after incubation with nitrite (see Table 3), despite the fact that the content of nornicotine in the tobaccos was about twice the content of anatabine. Favored formation of NAT over NNN was also observed in the inhibition experiments (Supporting Information available) and can be observed in studies of air-curing of tobacco (Andersen et al., 1987). Since the alkaloids and the nitrite scavenger(s) compete for the available nitrite, the observed difference in reactivity should be due to individual properties of nornicotine and anatabine.

Inhibition Potencies. The inhibition experiments were performed for 18 compounds at various test compound/nitrite ratios. The results are reported in Table 4. In most cases the inhibitions were of roughly the same magnitude after 1 and 5 days of incubation. Therefore, only the values after 5 days are reported. α-Tocopherol and protocatechuic acid were exceptional and showed up to 30% higher inhibitions after 5 days than after 1 day in the midrib experiments.

When midrib was used as the matrix, the order of inhibition among the highly effective inhibitors (more than 70% inhibition) was caffeic acid > ferulic acid ≈ catechin ≈ dihydrocaffeic acid > protocatechuic acid. The test compounds showing inhibitions of 0−60% were as follows: *p*-coumaric acid > chlorogenic acid > glutathione > ascorbic acid > α-tocopherol > cysteine > rutin > vanillic acid > cinnamic acid > serine > proline > alanine > eugenol. In the experiments performed with lamina, only caffeic acid and catechin showed high inhibitions (70−80%). Ferulic acid > glutathione > dihydrocaffeic acid were also fairly effective inhibitors. The other test compounds just slightly inhibited, or even favored, the formation of TSNA. The inhibitions of caffeic, dihydrocaffeic and ferulic acid, and catechin in the midrib and lamina experiments are compared in Figure 3.

Comparison of the Results in the Various Test Systems. Since chlorogenic acid, ascorbic acid, dihydrocaffeic acid, and cysteine were investigated in all four test systems, the influence by the test system on the inhibition could be evaluated. Chlorogenic acid showed similar inhibition in solution and in the tobacco matrix systems, but also clear differences between buffer and detergent solution. These differences may originate from the propensity of chlorogenic acid to catalyze as well as inhibit *N*-nitrosation (discussed above). Ascorbic acid, dihydrocaffeic acid, and cysteine were strong inhibitors in buffer and detergent solution at higher than equimolar ratios of test compound/nitrite, whereas the

Table 4. Effects of Test Compounds on TSNA Formation in Tobacco Matrix Systems[a]

compound	molar equiv.[c]	inhibition[b] (%) midrib	lamina
ascorbic acid	2	6 (31)[d]	10 (23)[d]
	4	52 (85)[d]	11 (54)[d]
	10	83 (94)[d]	53 (61)[d]
α-tocopherol	1	22	−11
	3.6	48	−13
	6	61	0
caffeic acid	0.1	11	11
	1	80	64
	3	99	80
dihydrocaffeic acid	1	57	18
	3	91	47
chlorogenic acid	0.1	1	12
	1	35	21
	3	58	28
ferulic acid	0.1	11	11
	1	82	53
	3	92	69
p-coumaric acid	1	34	20
	3	60	28
cinnamic acid	1	16	0
	3	19	0
protocatechuic acid	1	52	6
	3	79	18
vanillic acid	1	−8	0
	3	34	−3
eugenol	1	3	−14
	3	1	−10
catechin	1	50	47
	3	94	78
rutin	0.1	11[e]	−11[e]
	1	37[e]	−14[e]
glutathione	1	8	30
	4	56	62
cysteine	1	7	5
	5	40	25
alanine	1	−1	−1
	5	10	0
proline	1	2	7
	5	14	6
serine	1	4	15
	5	24	14

[a] Studied after 5 days of incubation with 1500 μg/g sodium nitrite at 25 °C. [b] Inhibition with respect to the reference sample. A negative value signifies enhanced TSNA formation. [c] Molar equivalent of test compound with respect to nitrite. [d] Ascorbic acid was added at the experimental start. [e] Measured after 6 days.

inhibitions in the tobacco matrix systems, particularly in the lamina, were substantially lower. Since the ability of the test compound to compete with the tobacco alkaloid(s) for the available nitrite reasonably decreases when the alkaloid/nitrite ratio increases, the observed order of inhibition in the test systems, i.e., solution > midrib > lamina, is most likely related to the differences in the ratio of secondary alkaloid(s) to nitrite. This ratio takes values of 0.1:1 in the solution experiments, ∼0.25:1 in the tobacco midrib experiments, and ∼1:1 in the tobacco lamina experiments. Similar trends were observed for the inhibitions of other test compounds, cf. Tables 2 and 4.

Furthermore, the presence of endogenous nitrite scavengers in the tobacco systems is likely to lower the amount of nitrite available for both TSNA formation and nitrite scavenging by the test compound. As a result, the inhibition should be lower in such a system than in a system that lacks "competing" endogenous nitrite scavengers.

Another contributing factor is most likely the presence of oxygen in the tobacco matrixes, which may result in

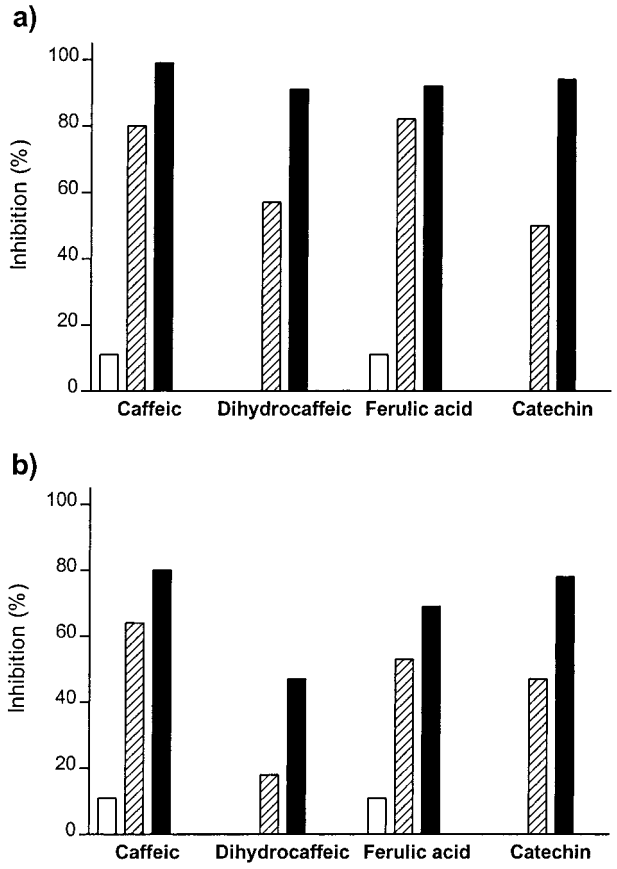


Figure 3. Inhibition of the TSNA formation in (a) midrib and (b) lamina by caffeic acid, dihydrocaffeic acid, ferulic acid, and catechin at test compound/nitrite ratios of 0.1:1 (open bars), 1:1 (dashed), and 3:1 (solid bars).

the regeneration of nitrosating agents by oxidation of the NO formed when nitrite is scavenged by compounds such as ascorbic acid (Licht et al., 1988; Mirvish, 1981). Shortened exposure to air atmosphere obviously resulted in higher inhibition when the easily autooxidized ascorbic acid was added simultaneously with the nitrite at the experimental start (see Table 4). In addition, certain test compounds are oxidized in air atmosphere by enzymes such as ascorbate or polyphenol oxidase, which may still have some activity in the air-cured tobacco (Barrett, 1957).

Because of the complexity of the tobacco systems, it cannot be excluded that gas-phase reactions via nitric oxide, nitrous anhydride, or dinitrogen tetraoxide (Mergens, 1992) and C-, O-, and S-transnitrosation reactions by tobacco constituents may affect the amount of TSNA formed. It is also unclear whether synergistic effects such as those reported for α-tocopherol and ascorbic acid (Lathia and Blum, 1989), which would result in increased inhibition, are of importance in lamina and midrib.

CONCLUSION

Some highly potential inhibitors of TSNA formation have been identified. The tobacco constituents caffeic acid, dihydrocaffeic acid, and ferulic acid as well as catechin were found to inhibit both the formation of NNN at pH 5.5 in solution and the formation of TSNA in the tobacco matrix systems. Ascorbic acid was an excellent inhibitor of the NNN formation in solution, but high ratios (>4:1) of ascorbic acid/nitrite were required for effective TSNA inhibition in the tobacco matrix systems. The 3,4-dihydroxy groups in phenols and the conjugated olefinic bond together with the free carboxy group of hydroxycinnamic acids were found to be important structural elements for effective blocking of NNN and TSNA formation. The general order of inhibition, solution > midrib > lamina, correlated well with the corresponding order of ratios of secondary tobacco alkaloid(s)/nitrite of 0.1:1, ~0.25:1, and ~1:1 for these systems. The explanation resides most likely in the competition between the alkaloid(s) and the endogenous nitrite scavengers, on one hand, and the test compound, on the other, for the available nitrite.

ABBREVIATIONS USED

TSNA, tobacco-specific *N*-nitrosamines; NNN, *N*-nitrosonornicotine; NAT, *N*-nitrosoanatabine; NAB, *N*-nitrosoanabasine; NNK, 4-methyl-*N*-nitrosamino-1-(3-pyridyl)-1-butanone.

Supporting Information Available: Table with concentrations of NNN, NAT, NAB, and NNK containing data from all inhibition experiments performed in the tobacco matrix systems. This material is available free of charge via the Internet at http://pubs.acs.org.

LITERATURE CITED

Andersen, R. A.; Burton, H. R.; Fleming, P. D.; Hamilton-Kemp, T. R.; Gay, S. L. Effects of air-curing environment on alkaloid-derived nitrosamines in burley tobacco. *IARC Sci. Publ.* **1987**, *84*, 451−455.

Andersen, R. A.; Litton, C. C. Variation of carbohydrate, phenolic, and alkaloid contents in different parts of air-cured tobacco. *Tobacco Science* **1975**, *19*, 64−65.

Archer, M. C. Catalysis and inhibition of *N*-nitrosation reactions. *IARC Sci. Publ.* **1984**, *57*, 263−274.

Barrett, R. E. Enzymes of bright and burley tobaccos. *J. Agric. Food Chem.* **1957**, *5*, 220−224.

Bartsch, H.; Frank, N. Blocking the endogenous formation of *N*-nitroso compounds and related carcinogens. *IARC Sci. Publ.* **1996**, *139*, 189−201.

Burton, H. R.; Childs, G. H., Jr.; Andersen, R. A.; Fleming, P. D. Changes in chemical composition of burley tobacco during senescence and curing. 3. Tobacco-specific nitrosamines. *J. Agric. Food Chem.* **1989**, *37*, 426−430.

Caldwell, W. S.; Greene, J. M.; Dobson, G. P.; deBethizy, J. D. Intragastric nitrosation of nicotine is not a significant contributor to nitrosamine exposure. *Ann. N. Y. Acad. Sci.* **1993**, *686*, 213−228.

Castonguay, A.; van Vunakis, H. Nitrosation and transnitrosation of nornicotine: inhibitory effect of some antioxidants. *Toxicol. Lett.* **1979**, *4*, 475−480.

Challis, B. C.; Bartlett, C. D. Possible cocarcinogenic effects of coffee constituents. *Nature* **1975**, *254*, 532−533.

Choi, J. S.; Park, S. H.; Choi, J.-H. Nitrite scavenging effect by flavonoids and its structure-effect relationship. *Arch. Pharm. Res.* **1989**, *12*, 26−33.

Cooney, R. V.; Franke, A. A.; Harwood, P. J.; Hatch-Pigott, V.; Custer, L. J.; Mordan, L. J. γ-Tocopherol detoxification of nitrogen dioxide: superiority to α-tocopherol. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 1771−1775.

Court, W. A.; Hendel, J. G. Phenolic constituents of flue-cured tobacco at different stages of plant growth. *Tobacco Sci.* **1985**, *29*, 73−76.

Davies, R.; Dennis, M. J.; Massey, R. C.; McWeeny, D. J. Some effects of phenol- and thiol-nitrosation reactions on *N*-nitrosamine formation. *IARC Sci. Publ.* **1978**, *19*, 183−197.

Dikun, P. P.; Ermilov, V. B.; Shendrikova, I. A. Some approaches to prevention of endogenous formation of *N*-nitrosamines in humans. *IARC Sci. Publ.* **1991**, *105*, 552−557.

Fischer, S.; Spiegelhalder, B.; Eisenbarth, J.; Preussman, R. Investigations on the origin of tobacco-specific nitrosamines in mainstream smoke of cigarettes. *Carcinogenesis* **1990**, *11*, 723−730.

González-Mancebo, S.; García-Santos, M. P.; Hernández-Benito, J.; Calle, B.; Casado, J. Nitrosation of phenolic compounds: inhibition and enhancement. *J. Agric. Food Chem.* **1999**, *47*, 2235−2240.

Gray, J. I.; Dugan, L. R., Jr. Inhibition of *N*-nitrosamine formation in model food systems. *J. Food Sci.* **1975**, *40*, 981−984.

Hecht, S. S. Biochemistry, biology, and carcinogenicity of tobacco-specific *N*-nitrosamines. *Chem. Res. Toxicol.* **1998**, *11*, 559−603.

Janzen, E. G.; Wilcox, A. L.; Manoharan, V. Reactions of nitric oxide with phenolic antioxidants and phenoxyl radicals. *J. Org. Chem.* **1993**, *58*, 3597−3599.

Kamm, J. J.; Dashman, T.; Newmark, H.; Mergens, W. J. Inhibition of amine-nitrite hepatotoxicity by α-tocopherol. *Toxicol. Appl. Pharmacol.* **1977**, *41*, 575−583.

Kikugawa, K.; Hakamada, T.; Hasunuma, M.; Kurechi, T. Reaction of *p*-hydroxycinnamic acid derivatives with nitrite and its relevance to nitrosamine formation. *J. Agric. Food Chem.* **1983**, *31*, 780−785.

Klimisch, H. J.; Stadler, L. Untersuchungen zur bildung von *N*-nitroso-nornikotin aus nikotin-*N*′-oxid. *Talanta* **1976**, *23*, 614−616.

Kono, Y.; Shibata, H.; Kodama, Y.; Sawa, Y. The suppression of the *N*-nitrosating reaction by chlorogenic acid. *Biochem. J.* **1995**, *312*, 947−953.

Lathia, D.; Blum, A. Role of vitamin E as nitrite scavenger and *N*-nitrosamine inhibitor: a review. *Int. J. Vitam. Nutr. Res.* **1989**, *59*, 430−438.

Lathia, D.; Braasch, A.; Theissen, U. Inhibitory effects of vitamin C and E on *in-vitro* formation of *N*-nitrosamine under physiological conditions. *Front. Gastrointest. Res.* **1988**, *14*, 151−156.

Lee, J.-H.; Choi, J.-S. Influence of some flavonoids on *N*-nitrosoproline formation in vitro and in vivo. *J. Korean Soc. Food Nutr.* **1993**, *22*, 266−272.

Lewis, R. S.; Tannenbaum, S. R.; Deen, W. M. Kinetics of *N*-nitrosation in oxygenated nitric oxide solutions at physiological pH: role of nitrous anhydride and effects of phosphate and chloride. *J. Am. Chem. Soc.* **1995**, *117*, 3933−3939.

Li, P.; Wang, H.-Z.; Wang, X.-Q.; Wu, Y.-N. The blocking effect of phenolic acid on *N*-nitrosomorpholine formation in vitro. *Biomed. Environ. Sci.* **1994**, *7*, 68−78.

Licht, W. R.; Tannenbaum, S. R.; Deen, W. M. Use of ascorbic acid to inhibit nitrosation: kinetic and mass transfer considerations for an in vitro system. *Carcinogenesis* **1988**, *9*, 365−372.

Maeda, S.; Uchida, S.; Kisaki, T. Microbial degradation of nicotine-*N*′-oxide degradation products. *Agric. Biol. Chem.* **1978**, *42*, 1455−1460.

Mergens, W. J.; Kamm, J. J.; Newmark, H. L.; Fiddler, W.; Pensabene, J. α-Tocopherol: uses in preventing nitrosamine formation. *IARC Sci. Publ.* **1978**, *19*, 199−212.

Mergens, W. J. Tocopherol: natural phenolic inhibitor of nitrosation. *ACS Symp. Ser.* **1992**, *507*, 350−366.

Mirvish, S. S.; Sams, J.; Hecht, S. S. Kinetics of nornicotine and anabasine nitrosation in relation to *N*′-nitrosonornicotine occurrence in tobacco and to tobacco-induced cancer. *J. Natl. Cancer Inst.* **1977**, *59*, 1211−1213.

Mirvish, S. S. Inhibition of the formation of carcinogenic *N*-nitroso compounds by ascorbic acid and other compounds. In *Cancer: Achiv., Challenges, Prospects 1980s, Proc. Int. Symp. Cancer*; Burchenal, J. H., Oettgen, H. F., Eds.; Grune & Stratton: New York, 1981; pp 557−587.

Møller, M. E.; Dahl, R.; Bøckman, O. C. A possible role of the dietary fibre product, wheat bran, as a nitrite scavenger. *Food Chem. Toxicol.* **1988**, *26*, 841−845.

Pignatelli, B.; Bereziat, J.-C.; Descotes, G.; Bartsch, H. Catalysis of nitrosation in vitro and in vivo in rats by catechin and resorcinol and inhibition by chlorogenic acid. *Carcinogenesis* **1982**, *3*, 1045−1049.

Rao, G. S.; Osborn, J. C.; Adatia, M. R. Drug-nitrite interactions in human saliva: effects of food constituents on carcinogenic *N*-nitrosamine formation. *J. Dent. Res.* **1982**, *61*, 768−771.

Rousseau, B.; Rosazza, J. P. N. Reaction of ferulic acid with nitrite: formation of 7-hydroxy-6-methoxy-1,2(*4H*)-benzoxazin-4-one. *J. Agric. Food Chem.* **1998**, *46*, 3314−3317.

Shenoy, N. R.; Choughuley, A. S. U. Effect of certain plant phenolics on nitrosamine formation. *J. Agric. Food Chem.* **1989**, *37*, 721−725.

Shenoy, N. R.; Choughuley, A. S. U. Inhibitory effect of diet related sulphydryl compounds on the formation of carcinogenic nitrosamines. *Cancer Lett.* **1992**, *65*, 227−232.

Spiegelhalder, B.; Kubacki, S. J.; Fischer, S. A method for the determination of tobacco-specific nitrosamines (TSNA), nitrate and nitrite in tobacco leaves and processed tobacco. *Beitr. Tabakforsch. Int.* **1989**, *14*, 135−144.

Tannenbaum, S. R.; Mergens, W. Reaction of nitrite with vitamins C and E. *Ann. N. Y. Acad. Sci.* **1980**, *355*, 267−279.

Tricker, A. R. *N*-Nitroso compounds and man: sources of exposure, endogenous formation and occurrence in body fluids. *Eur. J. Cancer Prev.* **1997**, *6*, 226−268.

Tso, T. C. *Physiology and Biochemistry of Tobacco Plants*; Dowden, Hutchinson & Ross: Stroudsburg, PA, 1972.

Wahlberg, I.; Long, R. C.; Brandt, P. Unpublished results, 1995.

Wahlberg, I.; Wiernik, A.; Christakopoulos, A.; Johansson, L. Tobacco-specific nitrosamines. A multidisciplinary research area. *Agro-Food-Ind. Hi-Tech* **1999**, *10* (4), 23−28.

Weston, T. J. Biochemical characteristics of tobacco leaves during flue-curing. *Phytochemistry* **1968**, *7*, 921−930.

Wiernik, A.; Christakopoulos, A.; Johansson, L.; Wahlberg, I. Effect of air-curing on the chemical composition of tobacco. In *Recent Advances in Tobacco Science*; Burton, H. R., Ed.; The Tobacco Chemists' Research Conference: Lexington, 1995; pp 39−80.

Williams, D. L. H. *S*-Nitrosation and the reactions of *S*-nitroso compounds. *Chem. Soc. Rev.* **1985**, *14*, 171−196.

Williams, D. L. H. *Nitrosation*. Cambridge University press: Cambridge, U.K., 1988.

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