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# A comprehensive methodology for the chiral separation of 40 tobacco alkaloids and their carcinogenic E/Z-(R,S)-tobacco-specific nitrosamine metabolites

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ABSTRACT

The predominant enantiomer of nicotine found in nature is (S)-nicotine and its pharmacology has been widely established. However, pharmacologic information concerning individual enantiomers of nicotine-related compounds is limited. Recently, a modified macrocyclic glycopeptide chiral selector was found to be highly stereoselective for most tobacco alkaloids and metabolites. This study examines the semi-synthetic and native known macrocyclic glycopeptides for chiral recognition, separation, and characterization of the largest group of nicotine-related compounds ever reported (tobacco alkaloids, nicotine metabolites and derivatives, and tobacco-specific nitrosamines). The enantioseparation of nicotine is accomplished in less than 20 s for example. All liquid chromatography separations are mass spectrometry compatible for the tobacco alkaloids, as well as their metabolites. Ring-closed, cyclized structures were identified and separated from their ring-open, straight chain equilibrium structures. Also, E/Z-tobacco-specific nitrosamines and their enantiomers were directly separated. E/Z isomers also are known to have different physical and chemical properties and biological activities. This study provides optimal separation conditions for the analysis of nicotine-related isomers, which in the past have been reported to be ineffectively separated which can result in inaccurate results. The methodology of this study could be applied to cancer studies, and lead to more information about the role of these isomers in other diseases and as treatment for diseases.

## 1. Introduction

Tobacco smoke has been reported to contain at least 60 carcinogens and several have been directly related to cancer [1]. Tobacco and its derived products constitute a leading preventable cause of death in the United States (US) [2]. The Food and Drug Administration (FDA) regulates all commercial tobacco products via the Family Smoking Prevention and Tobacco Control Act and the extension, the Deeming Rule [3,4]. Recently, the FDA also announced a comprehensive plan for lowering the nicotine (NIC) content in cigarettes to make them less or non-addictive [5]. To facilitate dependence, the reduced amount has been estimated to be 0.05 mg NIC compared to the current range of 0.5–1.5 mg NIC yield in one cigarette [1,6]. One challenge might be that smokers turn to other tobacco products for the higher NIC content compared to reduced NIC content cigarettes, such as smokeless tobacco products, which are connected to oral and esophageal cancers [7]. Smokeless tobacco products, like moist snuff, have been determined to contain tobacco-specific nitrosamines (TSNAs), which have been shown to be responsible for oral cavity cancer from smokeless tobacco [7]. The most prevalent and toxic TSNAs have been reported as *N*'-nitrosonornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) [1]. The other main TSNAs, *N*'-nitrosoanatabine (NAT) and *N*'-nitrosoanabasine (NAB), haven't shown as potent carcinogenicity in laboratory animals [7,8]. In one study, 12 rats were treated with racemic NNN and 96 oral cavity tumors and 153 esophageal tumors were observed [8]. Also, the (S)-NNN enantiomer was determined to be more tumorigenic than (R)-NNN indicating that the stereochemistry of this compound is highly important [8].

In 2017, the FDA proposed, "The mean level of *N*'-nitrosonornicotine in any batch of finished smokeless tobacco product not exceed 1 microgram per gram (μg/g) of tobacco (on a dry weight basis) at any time through the product's labelled expiration date as determined by specified product testing." [9]. Current commercial US smokeless tobacco products contain NNN levels ranging from 1 to

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10 μg/g dry weight [10]. NNN is formed by the nitrosation of NIC and nornicotine (NNIC), which is a tobacco alkaloid native to tobacco, as well as a nicotine metabolite [7]. The level of NNIC is dependent on the leaf senescence and curing process [7,11]. Tobacco strains with less (S)-NNIC have been reported to contain less (S)-NNN [11]. Therefore, genetic engineering efforts have been focused on reducing the inherent amount of NNIC [11]. Also, NNN can be formed endogenously, which was shown when NNN was found in saliva after using NIC replacement therapies [12]. Furthermore, NNN metabolizes to another TSNA, *N*'-nitrosonornicotine-1-*N*-oxide (NNNO), which has been shown to be less carcinogenic than NNN in F344 rats and Syrian golden hamsters [13].

The other major carcinogen found in unburnt tobacco and tobacco smoke is NNK, an achiral TSNA, which is formed from NIC during the curing and processing of tobacco [7]. NNK was found to be the only potent lung carcinogen that formed tumors in rats, mice, and hamsters [14]. Metabolites of NNK and other TSNAs are known to bind to DNA once activated, forming adducts that can cause oncogene activation leading to tumor development if they persist [7]. Long-term exposure to these mutation events can lead to cancer and death [7]. NNK is known to metabolize mainly to 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol, NNAL, and its glucuronides [15]. Since NNK and NNAL are only found in tobacco and not from any other source, they can be used as highly specific biomarkers of carcinogen exposure, especially second-hand smoke exposure [16]. Also, the ratio of NNAL-glucuronide to NNAL has been used as a biomarker of susceptibility to lung cancer [16].

NNAL has been reported to have similar toxicity as NNK, with a higher tumorigenicity of the (R)-NNAL enantiomer than (S)-NNAL, due to preferential metabolic activation [17]. NNK, NNAL, and NNN were reported to form E/Z isomers [18–20]. The relative level of E isomers was higher than Z isomers [18–20]. Some previous reports have shown the separation of a few TSNAs, but most do not report the separation of both their enantiomers and E/Z isomers [20–23]. Thus, some researchers have expressed confusion because the tops of their TSNA chromatographic peaks show splitting [24]. However, TSNAs are known to interconvert between E/Z isomers [18–20]. Chiral capillary electrophoresis has been used to separate E/Z-NNK and (R,S)-(E/Z)-NNAL [20]. Also, achiral nitrosamines, other than TSNAs, have been separated by LC into their E/Z isomers. For example, fish toxicants like 6′,7′-acetylenic nitrosamines were efficiently resolved with an achiral LC method [25]. Using a similar LC method, but with the addition of chiral derivatizing agents, the indirect separation of (R)-(E/Z) and (S)-(E/Z)-TSNA isomers was performed [18,19]. The approach described in this work provides a direct and efficient separation of both E/Z isomers and their enantiomers as well as indicating if isomeric interconversions occur under "ordinary" conditions. In jaundice phototherapy, toxic, unconjugated bilirubin is isomerized to several E/Z configurations [26]. This isomerization makes bilirubin become more soluble in plasma so it can be excreted by the liver [26]. Therefore, E/Z isomers have different physical and biological properties and should be further studied with TSNAs.

TSNAs are nitrosated metabolites of chiral tobacco alkaloids, which have similar structures as NIC [7]. NIC is predominantly found as the (S)-(-) enantiomer in tobacco plants [27]. The percent (R)-(+)-NIC in tobacco, and medicinal products derived from tobacco was reported to be in the 0.1–1.2% range [27]. The pharmacology of (R)-(+)-NIC has not been an area of great concern, most likely because human exposure and intake of (R)-(+)-NIC is minimal. However, the individual enantiomers have been examined for their use as therapies for neurodegenerative diseases. These studies have reported that NIC enantiomers have different pharmacological effects, such as oxidative stress, weight loss, and binding mechanisms [28–30]. (R)-NIC has been reported as eighty times less cytotoxic than (S)-NIC, when considering their metabolites [30]. A recent study determined new smoking products (e-liquids), which have synthetic NIC (tobacco-free nicotine, TFN), contained 50% of (R)-(+)-NIC [31]. Since new products contain higher (R)-NIC levels than in tobacco-derived products, it was suggested that the pharmacology of (R)-NIC should be more extensively studied [31]. The binding affinity of (R)-NIC to nicotine acetylcholine receptors was estimated to be 10 times lower than (S)-NIC, which might result with a less stimulating dopaminergic response [30]. New TFN products with higher (R)-NIC might be analogous to commercial products with less addictive NIC levels.

While (S)-NIC is the main alkaloid in tobacco products, minor chiral alkaloids also are present including NNIC, anatabine (AT), and anabasine (AB) [32]. The R-enantiomers of minor tobacco alkaloids have been reported to be present at higher relative levels than (R)-(+)-NIC [32]. Most biomarker strategies utilize tobacco alkaloids or their metabolites, such as the major chiral metabolite, cotinine (COT). COT is used to measure NIC uptake, due to its long half-life, such as in smoking cessation trials and tobacco exposure tests [33]. However, tobacco alkaloids are useful to differentiate the use of tobacco while using NIC replacement therapies [34]. Also, chiral alkaloids have been reported to be useful as therapies for neurodegenerative diseases by mimicking NIC's neuropharmacological and neuroprotective effects [30,35]. Enantiomers are well known to have different pharmacological effects, e.g. (R)-AB was reported to be more toxic and cause more birth defects than (S)-AB [36]. So, if these alkaloids were developed into medicinal products, the FDA would require, in their words, "the pharmacology and toxicology of the enantiomer should be characterized for the principal effects and any other pharmacological effect, with respect to potency, specificity, maximum effect, etc." [37]. However, most analytical methods do not have the capability to analyze the individual enantiomers of these alkaloids and metabolites, so new more effective methods are needed. To quantitate and perform biological studies, it would be useful if such "chiral methods" were compatible with mass spectrometry (MS).

Some separation approaches for chiral nicotine-related compounds, more importantly the carcinogenic compounds, have been reported, but most have disadvantages that limit the analysis. Most analyses are similar to those of achiral nitrosamine analysis and do not have the capability of separating enantiomers, such as a study which determined the amount of TSNAs in replacement liquids for electronic cigarettes [38]. One chiral approach reported the separation of NIC and several alkaloids using a packed liquid chromatography (LC) microcolumn with a β-cyclodextrin mobile phase, but required three hours [39]. Other previous approaches mainly utilized chiral gas chromatography (GC) or chiral derivatization LC [18,23,32]. GC isn't best suited for the biological analysis of these compounds due to the thermal liability of the sample. Chiral derivatization LC methods increase cost and analysis times and rely on the purity of the chiral derivatization agent. The best approach for chiral separations of nicotine-related compounds is using LC chiral stationary phases (CSPs). Enantioseparations of three tobacco alkaloids using LC CSPs have been reported, but they used normal phase solvents, which are not compatible with MS [36,40]. These alkaloids might be possible targets for neurodegenerative therapies, but these methods won't be compatible for biological analysis [30].

Recently, a fast, high efficiency, mass spectrometry compatible, chiral LC approach was developed to analyze NIC in TFN commercial e-liquids [31]. Herein, we examine this approach for applicability for the sensitive identification and enantiomeric quantification of most nicotine-related compounds and metabolites in commercial tobacco products and biological samples. Focus is paid to the LC separation of carcinogenic compounds, like NNN or NNK, and other complex isomeric mixtures that have not been reported to separate previously. This study examines the effectiveness of new and known macrocyclic glycopeptide chiral selectors in resolving the most comprehensive set of chiral nicotine-related compounds yet investigated, including minor tobacco alkaloids, metabolites, synthetic related compounds, and E/Z-TSNAs [31,41–44]. Further, only LC-MS compatible formats were considered.